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Tricistronic Lentivirus Vector Construction using Scar-less DNA Assembly Methods and Web-based Software j5 to Help Study GRK4

A Project Presented to
the Faculty of the Undergraduate
College of Integrated Science and Technology
James Madison University

in Partial Fulfillment of the Requirements
for the Degree of Bachelor of Science

by Christophe Jacques-Yves Langouët-Astrie

May 2015

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Abstract

About one third of the world's population is affected by hypertension, or high blood pressure, which increases an individual's risk for cardiovascular disease. A major contributor to hypertension is dietary sodium intake. To assess an individual's risk for hypertension, patients are put on a low sodium diet. However, research has shown that low salt intake can also have different and potentially harmful effects. Because of this, a genetic screen for salt sensitivity is needed to assess an individual's salt sensitivity classification before testing and these results matched to a recommended dietary change. The kidney regulates the body's fluid volume, so genetic factor within the renal system could be used for such a genetic screen. The dopaminergic system within renal proximal tubule cells is responsible for the majority of excretion and is highly influenced by a G-protein kinase, GRK4. The expression of GRK4, its association with the dopaminergic system, and the activity in varying salt sensitivity cell types can be indicators for salt sensitivity and provide a definitive diagnosis of the type of salt-related hypertension. Thus, a finely tuned treatment plan would be possible. To help study GRK4 expression, tricistronic lentivirus delivery systems were constructed to measure protein expression *in vivo*. To construct the vectors, scar-less DNA assembly methods allow for simpler and multipart construction, while web-based software j5 was developed to optimize cost, time efficiency, and reduce construction difficulty. Developing tricistronic vectors allows for introduction of a protein with two modes of measurement separated by 2A self-cleaving peptide sequences that impart protein autonomy. Bicistronic vectors, pLVX-GZ and pLVX-GC were constructed for utility and to establish a protocol for constructing tricistronic vectors. A tricistronic vector pLVX-GZC2 was been constructed containing GRK4, Zeocin resistance, and a fluorescence protein with P2A and T2A. Construction of such vectors will serve as a fast and cost-effective platform to fine-tune the differential diagnosis of salt-related hypertension.

Introduction

Approximately, one third of the world's population is affected by hypertension, a clinical syndrome defined as an increase in heart contraction force due to rising blood pressure (1). The additional force can lead to problems in the cardiovascular system leading to heart failure and is a major contributor to coronary heart disease (1,2). Dietary salt has been shown to be an important factor in increasing blood pressure (2). Illustrating this effect, research has found a correlation between left ventricle mass and cardiovascular mortality and morbidity, independent of the blood pressure (2). This is because as the mass of the left ventricle increase, the amount of diastolic filling that occurs during a heart contraction decreases; summing to the increased contraction force needed to circulate the same volume of blood through the heart (2). In addition to this relationship, research has shown that left ventricular mass and diastolic filling have been found to be positively correlated with urinary sodium excretion in normotensive subjects, that is, those having systolic blood pressure greater than 121 mmHg (Figure 1) (2). Also, normotensive subjects were followed up for 3 to 8 years revealing that the initial left ventricular mass and wall thickness were significantly related to the subsequent development of hypertension (2). Reducing salt intake can decrease the left ventricular mass associated with essential hypertension, as shown by The Treatment of Mild Hypertension Study Research Group (2). Dietary salt intake is an important factor for patient's risk for hypertension and cardiovascular diseases.

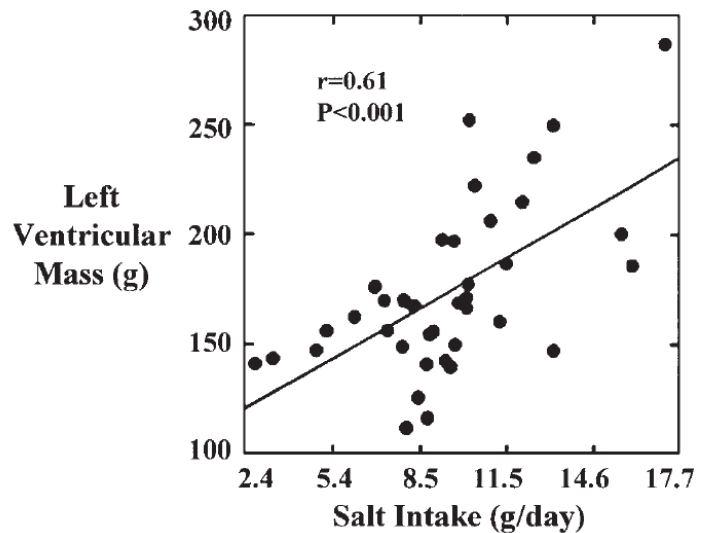


Figure 1: Correlation between Left Ventricular Mass and Salt intake in normotensive subjects (adapted from 2).

Renal Dopaminergic System as a Genetic Indicator for Hypertension:

The kidney is responsible for long-term regulation of blood pressure by controlling the body's fluid volume (3). Under moderate sodium intake, over 50% of renal excretion is accomplished by the dopaminergic system in the proximal tubule (1,3-4). The importance of this system is that dopamine aids in the kidney's antihypertensive function by being both a vasodilator and a natriuretic (2).

Dopamine, secreted by the renal proximal tubule cell, stimulates dopamine receptors (D₁, D₂, D₃, D₄, D₅) where stimulation of the D₁ and D₅ receptors leads to natriuresis (urine excretion of sodium) through inhibition of the sodium/hydrogen exchanger 3 (NHE3) and the sodium-potassium pump, NaKATPase (3,4).

The ability of dopamine to decrease renal proximal tubular sodium reabsorption was found to be impaired in genetic rodent hypertension and human essential hypertension (3).

G protein-coupled receptor kinase 4 (GRK4), one of the 7-member GRK family, phosphorylates the receptor causing D₁ internalization resulting in the reabsorption of sodium (Figure 2) (1,3-4). Regulating expression of GRK4 is important due to its implications in hypertension, because high renal GRK4 expression inhibits D₁ receptor's ability to excrete sodium (3,4). An experiment with spontaneously hypertensive rats found that the basal levels of GRK4 is 90% higher in the kidney compared to their normotensive control (4). Similar results in humans found basal phosphorylation of the D₁ receptor was greater in hypertensive (HT) subjects than normotensive (NT) subjects

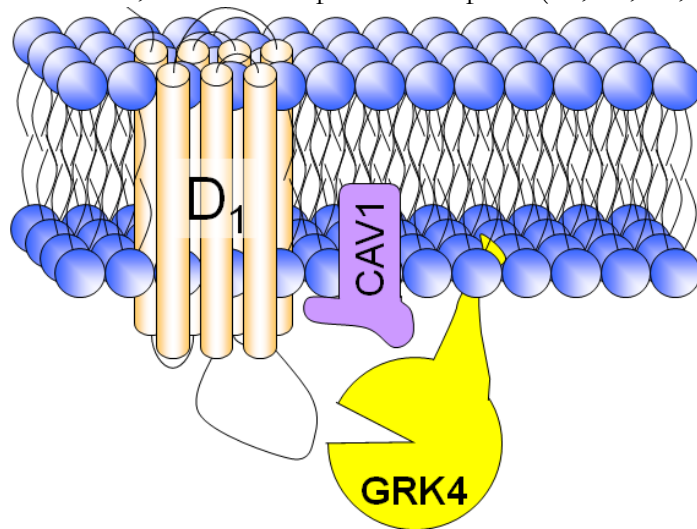


Figure 2: Visualization of Dopamine receptor complex in human proximal tubule cells. Dopamine receptor 1 (D₁) regulates renal excretion which controls the body's fluid levels. G protein-coupled receptor kinase 4 (GRK4) phosphorylates the D₁ receptor causing internalization of the receptor complex. Caviolin 1 (Cav1) inhibits GRK4 activity and is associated with the D₁ receptor (Provided by Dr. John Gildea).

(Figure 3) (3). Fenoldopam, a D₁-like agonist used clinically as an antihypertensive, increased the phosphorylation of the D₁ receptor in cells from NT individuals but not in cells from HT individuals (Figure 3). Fenoldopam is used as a high salt scenario control for NT cells while for HT cells, GRK4 over activity prevents fenoldopam from binding to the D₁ receptor (Figure 3) (1,3-4). Antisense GRK4 oligonucleotides inhibited the phosphorylation of the D₁ receptor in both HT and NT subjects with a greater difference in HT subjects (Figure 3) (1). As a control for antisense GRK4 oligonucleotides, sense or scrambled GRK4 oligonucleotides had no significant effect on the quantity of phosphorylated D₁ receptor in HT or NT subjects (Figure 3) (1). Based on these results, the model of D₁ receptor activation and inactivation is controlled by GRK4 activity. GRK4 phosphorylation of the receptor leads to a net internalization of the receptor complex which has also

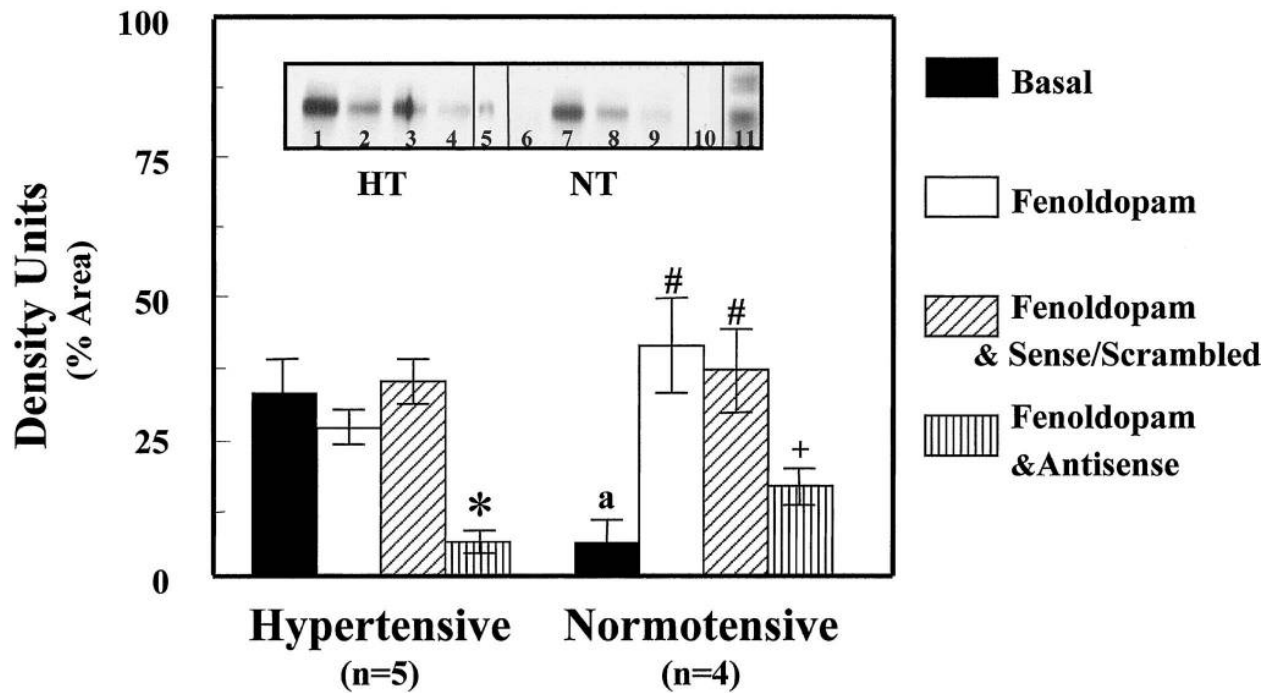


Figure 3: Renal proximal tubule cells phosphorylation of the D₁ receptor in normotensive (NT) and hypertensive (HT) subjects (3). Lysates of renal proximal tubule cells were immunoprecipitated with a D₁ receptor antibody and immunoblotted with an anti-phosphoserine antibody. (Inset) Lanes 1–4 are HT while lanes 6–9 are NT with lane 5 being the 80 kDa molecular size marker. Lanes 1 and 6 are basal phosphorylation, lanes 2 and 7 are the effect of fenoldopam, lanes 3 and 8 are the effect of GRK4 sense/scrambled oligonucleotides, lanes 4 and 9 are the effect of GRK4 antisense oligonucleotides, lane 10 is the western blot of human proximal tubule cells with D₁ antibody reabsorbed with the immunizing peptide, and lane 11 is the western blot of human proximal tubule cells with D₁ antibody. The graph depicts the composite studies from five hypertensive and four normotensive subjects. Homozygous GRK4 gene variants were found in four of the five hypertensive subjects. The % area was normalized to 100% for either hypertensive or normotensive subjects. a, P > 0.05 normotensive vs. hypertensive, t test; *, P > 0.05 vs. other hypertensive groups, ANOVA, Scheffe' s test; #, P > 0.05 vs. normotensive basal, ANOVA, Scheffe' s test; and +, P > 0.05 vs. normotensive fenoldopam alone, t test. Data are mean ± SE (Adapted from 3).

been previously proven by a GRK4 triple mutant that permanently turns on the kinase and removes inhibition activity from Cav1 (Figure 4) (3,4). Activation of the D₁ receptor complex and integration to the plasma membrane requires GRK4 inhibition, which occurs in low salt conditions (Figure 4) (3,4). Studying the phosphorylation of the D₁ receptor and GRK4 activity can be used to determine individual's salt sensitivity and their risk/predisposition to hypertension.

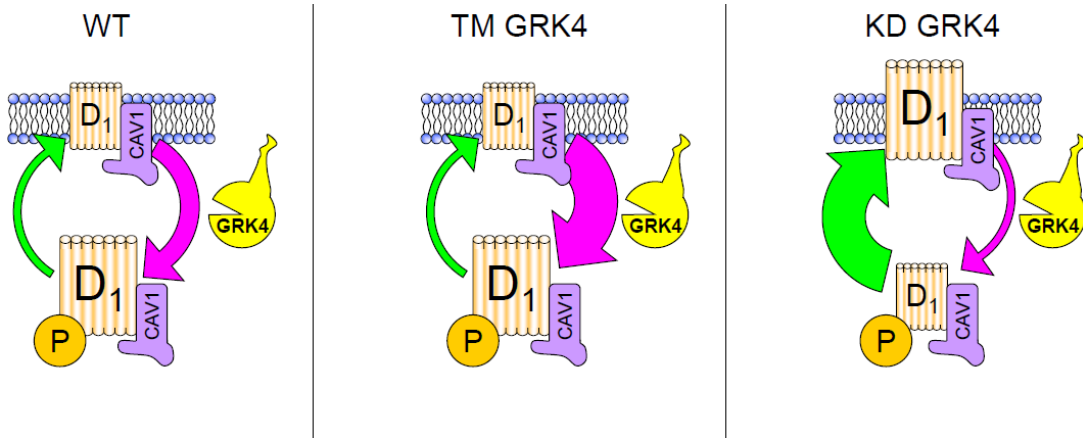
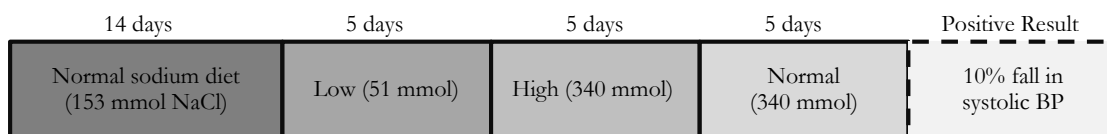


Figure 4: Visualization of G protein-coupled receptor kinase 4 (GRK4) activity with Dopamine receptor 1 (D₁) and GRK4's inhibitor Caviolin 1 (Cav1) in normal salt concentrations provided by Dr. John Gildea. First column is with the wild type of GRK4, Second column is with the triple mutant of GRK4, and the third column is with GRK4 knockdown. Purple arrows indicated internalization of the D₁, Cav1 complex and green arrows indicate reintegration of the D₁ complex to the plasma membrane. Internalization of the D₁ complex is catalyzed with GRK4 phosphorylating D₁ with arrow size indicating net direction (Provided by Dr. John Gildea).

Hypertension and Salt Sensitivity Diagnosis:

Hypertension is currently diagnosed using an arm cuff or ideally a mercury sphygmomanometer (JNCVII) though no genomic screens for hypertension are conducted (1,3). Variability in blood pressure should be monitored for it is indicative of an increased risk for cardiovascular disease (1). Genomic screens would distinguish salt-sensitivity from hypertension, which is clinically important as a risk factor for cardiovascular morbidity and mortality is salt-sensitivity (1,2). Salt-sensitivity is clinically defined by two different methods: (i) a change in blood pressure of 5–10% or at least 5 mmHg, in response to changes in salt intake and (ii) an increase in mean arterial blood pressure (MAP) of at least 4 mmHg with an increase in salt intake (1). The most reliable method of diagnosis

29 Day Protocol for Determining Salt-Sensitivity



Abbreviated 5 Day Protocol for Determining Salt-Sensitivity

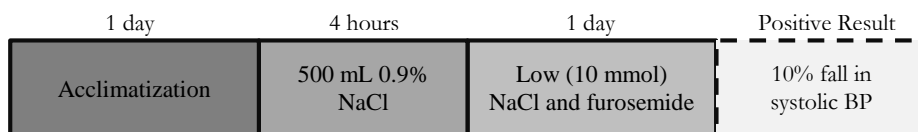
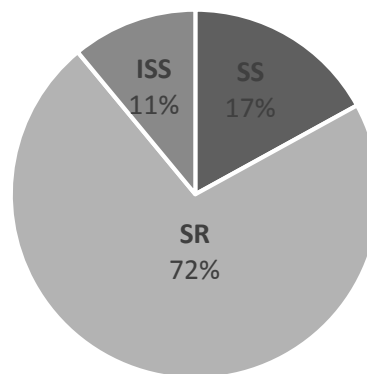


Figure 5: Diagram representation of two protocols for diagnosing salt sensitivity (1). The top method requires 29 days for determination while the other requires only 5 days for determination of salt sensitivity. A positive diagnosis of salt sensitivity is made if the individual has a 5–10% increase or decrease in blood pressure following an increase or decrease in salt intake, relative to blood pressure on a normal salt intake (Adapted from 1).

for salt sensitivity is by measuring blood pressure responses to changes in dietary salt intake, which can be performed using either a 29 day or 5 day restrictive diet examination (Figure 5) (1). Even with these practices in place, the restricted diet experiments are expensive, patients have low compliance for enrollment, and many do not complete the test, all of which contribute to the lack of diagnosing salt sensitivity (1).

The Institute for Medicine current dietary sodium recommendation is a ‘one size fits all’ model, although research has suggested that a spectrum of salt sensitivity and sodium dietary changes should be tailored to the individual based on their personal genetics (1). This spectrum of salt sensitivity can be divided into three categories: salt-sensitive (SS), salt resistant (SR), and inverse salt-sensitive (ISS), each having varying responses to dietary salt changes (Figure 6) (1).



SALT INTAKE	ISS	SR	SS
HIGH SALT	Normal MAP	Normal MAP	High MAP
LOW SALT	High MAP	Normal MAP	Normal MAP

Figure 6: Distribution of salt-sensitive (SS), salt-resistant (SR), and inverse salt-sensitive (ISS) individuals in a population of 183 individuals tested for salt sensitivity (1). Trends which occur when administering low/high salt is listed below each group. Mean arterial pressure (MAP) increases over 7 mmHg on a high-sodium diet were classified as salt-sensitive, whereas blood pressure decreases over 7mmHg were considered as inverse-salt-sensitive (1).

Administering high sodium diet to a SS individual will elevate MAP by over 7 mmHg while SR will have no relative changes in their MAP regardless of sodium diet (Figure 6) (1). ISS individuals have an inverse increase in MAP of at least 7 mmHg on a low sodium diet (Figure 6) (1). With the variability of blood pressure responses, the ‘one-size-fits all’ can be potentially harmful to individuals along with being ineffective (1).

Because of the importance of establishing an effective, personalized genetic screen for diagnosing salt-sensitivity, GRK4 and its association with the dopaminergic system in proximal tubules is a potential candidate for an accurate diagnosis (1,3). Determining GRK4 activity in SS, SR, and ISS individuals could be developed into a diagnostic tool leading to appropriate dietary restrictions with minimal adverse effect from inappropriate dietary restrictions (1). Being able to analyze an individual’s genetics and their risk for hypertension and cardiovascular disease would encourage dietary salt restriction in individuals with increased likelihood of the disease.

Development of a Tricistronic Viral Expression Systems:

In order to study protein expression and regulation, standard cloning techniques require that the gene of interest is isolated, cloned into an expression vector, and transformed or transfected into competent cells or cell lines respectively. Biotechnology companies have developed bicistronic expression vectors, which allows for a protein of interest and a selectable marker to be transcribed together. One of the earliest detections of a bicistronic system was of α -crystallin synthesis of A₂ and B₂ polypeptide chains for calf lenses (5). Commercialized bicistronic vectors contain a multiple cloning site for cloning the insert, an internal ribosomal entry site (IRES), and a selectable marker, most commonly a fluorescence protein. Bicistronic vectors allow studying proteins through indirectly methods, an alternative to direct measurement requiring cell lysing.

Tricistronic vectors can be an improvement to the bicistronic vector system by allowing researchers to study the interaction of two proteins with one antibiotic selectable marker or study one protein with two selectable markers for precise measurement. Few tricistronic vectors have been constructed with the purpose for monoclonal antibody production (7-9). Traditional methods of monoclonal antibody production include using either a co-transfection with each vector containing a light chain (LC) or a heavy chain (HC) along with a selection marker on each, or one vector that uses multiple promoters and contains both LC and HC and a selection marker (7,8). Both designs have disadvantages, as the co-infections produce false positive clones which have the antibiotic resistance but contain only one of the vectors (8). When both vectors were present in a cell, either the LC or the HC were produced in greater quantities, which reduced the antibody yield (8). The multiple-promoter, single vector guarantees introduction and expression of LC and HC, but the antibody production was reduced due to transcriptional interference by having multiple promoters in close proximity (7). The variation in LC:HC ratio expression could affect antibody expression as a whole because LC facilitates the folding of the antibody and minimizes aggregation (7,8). The development of a tricistronic vector for monoclonal antibody production allows a greater control of relative expression of LC over HC (7). The increased control was due to the vector expressing LC, HC, and a selection marker in one mRNA transcript which reduces any variation in LC:HC ratios (8). If the transcript was fragmented or transfected improperly, the clone would not survive drug selection, eliminating false positive clones (8).

The utilization of a tricistronic vector allows for approximately equal expression of LC and HC along with a control where transformed antibiotic resistant colonies have an intact mRNA strand containing both the LC and HC. This application can be broadened from production of two proteins to measuring protein expression by two different modes. Another tricistronic vector was constructed with three proteins, all of which were fluorescent proteins, with 2A peptide sequences

and cloned into a lentivirus (see below) (9). This system was used to develop transgenic sheep in order to investigate methylation in transgene expression (9). Within this research, the 2A peptide sequence was found to efficiently mediate the co-expression of all three fluorescent proteins (9). Construction of a tricistronic vector with one gene of interest and two selectable markers is the first of its kind at this time.

The project was to design a tricistronic vector which contains the protein of interest, GRK4, a Zeocin resistance, and a fluorescence marker, CherryPicker (Clontech), in order to select for clones using Zeocin that have been properly transfected and measuring GRK4 activity indirectly using CherryPicker *in vivo*. The benefit of constructing this type of vector would be that an indirect relationship could be made between the expression of GRK4 and the fluorescent protein, eliminating any lysing. Expression over time using confocal microscopy could be measured in different salt conditions and in SS, ISS, and SR proximal tubule cells. Changes between high sodium to low sodium conditions and vice versa could also be investigated and measured *in vivo*. Built into this vector is an automatic control for if mutations within the tricistronic transcript or improper transfection, result in corrupted mRNA and the inability for producing a resistance to Zeocin, then the cell will lyse leaving the properly infected cells with the intact mRNA to survive and propagate.

Utility of 2A Peptide Sequences in Cloning:

One disadvantage to tricistronic viral vectors is that increases in vector size leads to low infection rates which can be circumvented through the use of 2A peptide sequences. The 2A peptide sequences are small, viral peptide sequences which use an unusual process called ribosomal skipping to “self-cleave” leading for the two proteins on either end to function autonomous from each other (Figure 7) (6, 8-11). Compared to the commercially available IRES sequence, both ensure co-expression of genes upstream and downstream of the sequence (6-11). However, a tricistronic

vector with IRESs includes an addition of 500 base pairs per IRES, increasing the size of the vector along with decreasing the infection rate (6). In addition, research has shown that the expression of the two genes before and after the IRES have unequal expression, with the second gene downstream of the IRES has a reduced expression (6, 10).

To reduce the size of the final vector in order to maintain a high infection rate, 2A peptide sequences range from 18 – 22 residues (54 – 66 bp), perform similarly to IRESs, and have a high cleavage affinity leading to

almost 100% autonomy between the two proteins being expressed at approximately a 1:1 ratio.

This sequence was first discovered in foot-and-mouth disease viruses (FMDV) and was used to express two proteins at relatively equal rates on a

smaller cistron (6, 8-11). The most commonly investigated 2A peptide sequences are E2A, T2A, P2A, and F2A (6, 8-11). This ribosomal skipping occurs during translation in the ribosome near the C-terminal region of the 2A peptide sequence at a conserved PGP residue sequence (Figure 7). The peptide bond between the second to last residue, glycine, and the last residue, proline, is unable to be attached by the ribosome, allowing the N-terminal portion to be released with the glycine at the C-terminus (Figure 7). The ribosome then reengages allowing continued translation of the remaining mRNA with the addition of a proline at the N-terminus (Figure 7) (6,10). Cleaving efficiency is

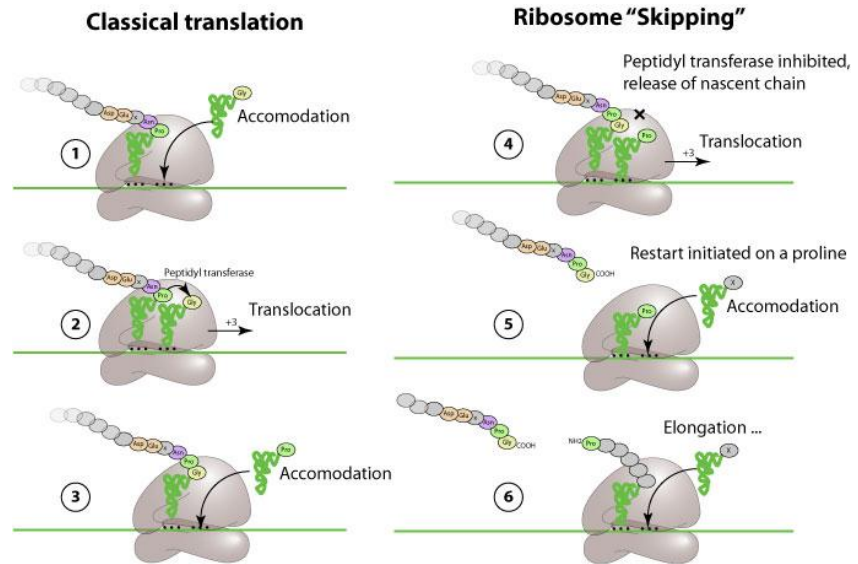


Figure 7: Visualization of 2A peptide cleavage by peptide skipping (ExPASy). Skipping occurs between a conserved PGP residue sequence at the C-terminus of the 2A peptide. Translation occurs normally (steps 1-3) until reaching the PGP residue where the peptide bond is unable to form between the Glycine and Proline allowing the N-terminus portion of the amino acid sequence to disassociate (step 4). The Ribosome reengages restarting translations to finish the transcript (step 5 and 6). This allows to proteins to be translated at the same rate with autonomy (ExPASy).

variable on model organism, cell line, and other environmental conditions although its efficiency is greater than 90% (6,10). P2A has been shown to have the highest cleaving efficiency in three human cell lines, zebrafish embryos, and mouse liver cells (6) while T2A had the highest efficiency in *Drosophila* models both *in vitro* and *in vivo* (10). With the reduce sequence length, 2A self-cleaving peptide sequences P2A and T2A will be used in the constructed vectors.

Integration of j5 Software Into Cloning Projects:

Current methods of creating recombinant vectors require the utilization of restriction enzymes, ligases, polymerases, and homologous ends on the 5' and 3' ends of the insert and backbone for annealing. A limitation with traditional cloning is the low probability of multiple fragments being ligated together which lead to the necessity of multiple single digests and ligations (12). These intermediary steps, finding complementary restriction sites, confirming digests via agarose gels along with additional PCR steps, and creating intermediary vectors can be a time consuming and costly process. To help reduce the time, cost, and difficulty of cloning, biological computerized aided design (BioCAD) programs have been developed to aid the researcher (12).

Dr. Hillson et al. have developed a web-based software in conjunction with a BioCAD program and a visualization program to help, establish, visualize, and develop protocols for cloning projects (13).

These programs include j5, Device Editor, and Vector Editor which can be accessed by visiting:

http://j5.jbei.org/index.php/Main_Page (12-14). j5 was developed to optimize construction while assuring cloning project are cost efficient by the program's ability to minimize the cost of the project, allow and enforce user design specifications, and develop hierarchical assembly strategies to prevent undesired assembly products (12-13). Device Editor allows users to label all pieces required in construction along with hierarchical assembly requirements for orientation and order of desired pieces being assembled (12). Once everything has been labeled, the user can have a mock assembly

of the vector with a visualization illustrated by Vector Editor (14) to show where all open reading frames and restriction sites are within the vector. If all requirements are met, then Device Editor can design the vector using scar-less multi-part DNA assembly protocols which have been proven to be effective in cloning more than two DNA fragments at the same time (13, 15-16). For cloning projects, j5 aid in designing and virtually constructing the desired vectors based on desired specifications while also provided instructions for physically constructing the vectors.

Isothermic scar-less multipart DNA assembly protocol, Gibson, was used for ligating all the pieces together (Figure 8) (15). The theory behind the Gibson protocol is a T5 polymerase with 5' exonuclease activity digests DNA fragments approximately 25 nucleotides producing single stranded homologous ends on all fragments (Figure 8) (15).

Similar to a restriction enzyme digest, fragments with similar overhangs will anneal to each other. A phusion polymerase ligates the segments together while Taq ligase fills any single stranded gaps (15). After the incubation

period, the mixture containing the newly synthesized vector is transformed (15). This protocol was chosen because of its ease and isothermic reaction unlike SLIC, CPEC, and SLiCE protocols which have more difficult procedures (12-16). Other techniques require special reaction mixtures and temperatures to separate the double stranded DNA and anneal (CPEC) while others requires lysing

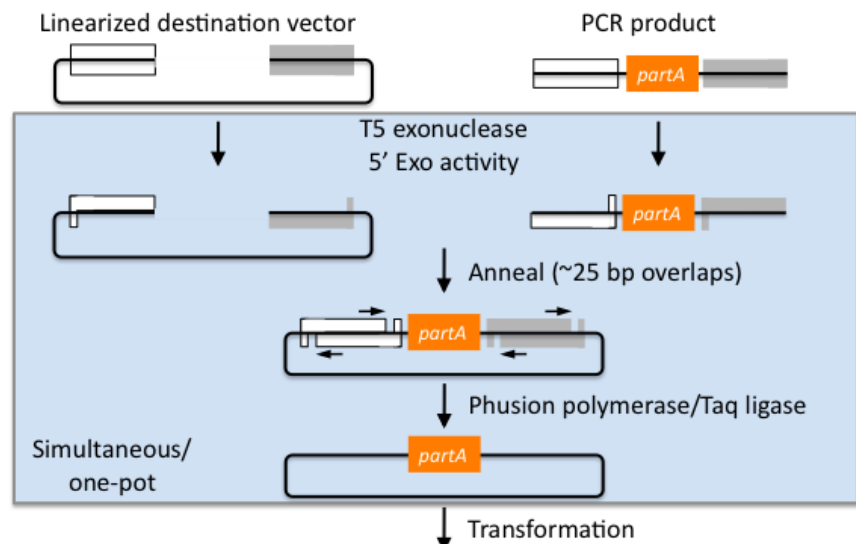


Figure 8: Visualization of Gibson protocol (15) illustrated by j5 (13). The diagram illustrates a two part ligation between a linearized vector and partA. Grey and white boxes indicate regions of homology of about 25 bp which are generated through PCR. The blue region indicates the reaction that occur in the Gibson protocol which are the T5 exonuclease at the region of homology generating overhangs, the overhangs anneal, and phusion polymerase polymerizes the region of homology and the taq ligase fills in the ssDNA gaps (13).

and extracting an engineered bacterial lysate with variable temperatures for DNA annealing (SLiCE) (13,15-16). For the construction of the lentivirus vectors, learning how to use j5, Device Editor, and Vector Editor was done along with developing a brief manual in how to use this software.

Lenti-viral vectors containing GRK4 were created to help study the expression of GRK4 in high and low sodium concentrations *in vivo*. These vectors were designed using j5 and the Gibson protocol was used for ligating the DNA pieces together. A total of three viral vectors were constructed; two bicistronic vectors and one tricistronic vector (Figure 9). The two bicistronic vectors pLVX-GZ and pLVX-GC both contain GRK4 while one has Zeocin resistance and the other has red fluorescence protein CherryPicker (Clontech) respectively. pLVX-GZ has a T2A sequence between GRK4 and Zeocin while pLVX-GC has an IRES between GRK4 and CherryPicker. These vectors serve as controls and measurements of T2A cleaving efficiency compared to IRES efficiency along with establishing a protocol and finding potential problem areas when constructing the tricistronic lentivirus vector. The tricistronic vector was also constructed, pLVX-GZC, which contains GRK4, Zeocin resistance, and CherryPicker. Being able to quantify GRK4 expression *in vivo* could allow possible insight in body response to changes in salt conditions over time in various salt-sensitivity cell types. Discovering trends and analyzing other genetic variants associated with hypertension could be used as a diagnostic tool for salt-sensitivity and help prescribe dietary restriction and changes. The addition of a genetic screen would be an effective method of reducing individual's risk for hypertension potentially leading to cardiovascular disease.

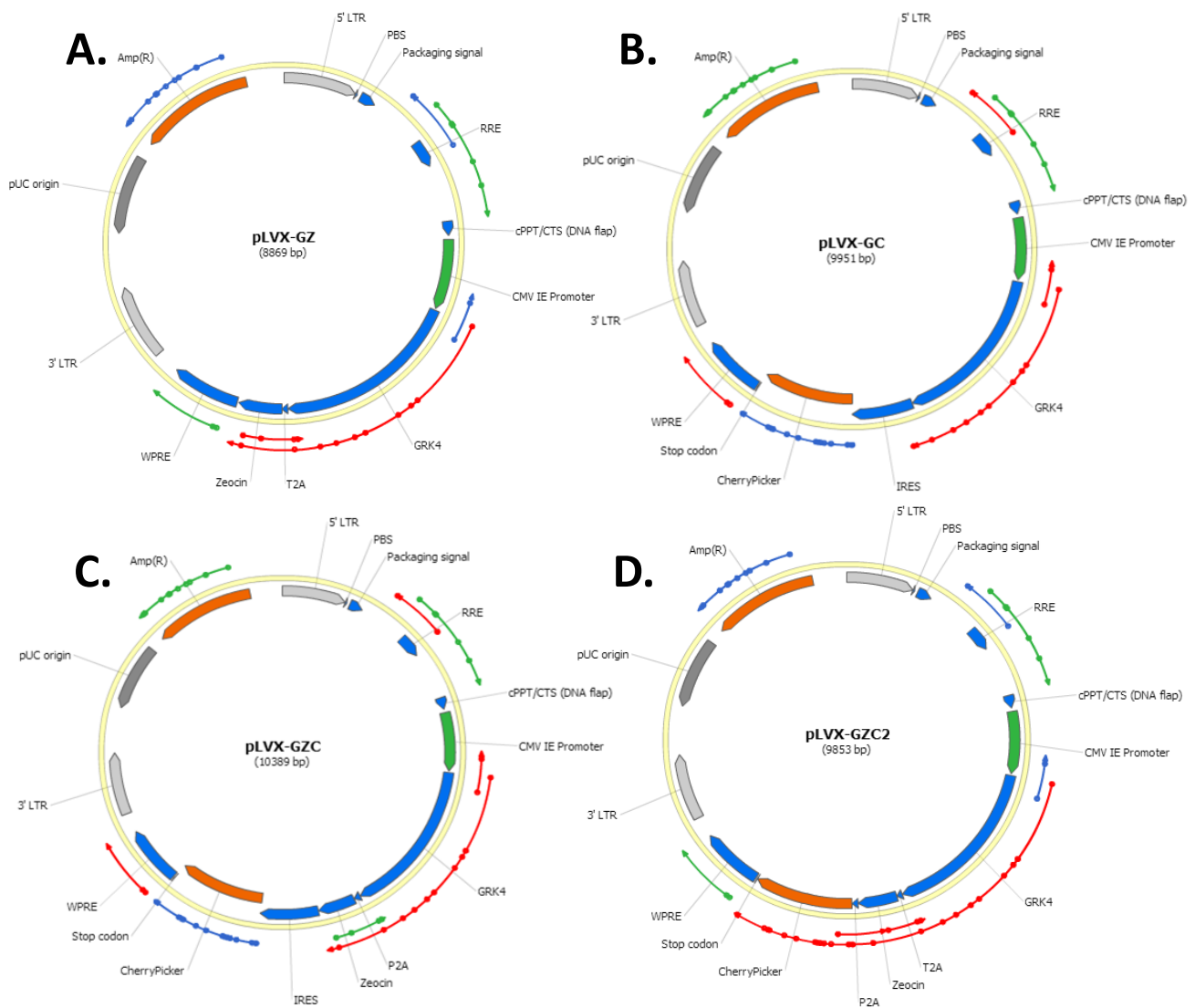


Figure 9: Visualization of vectors pLVX-GZ (A), pLVX-GC (B), and pLVX-GZC (C.) illustrated by Vector Editor (14). Base pair size of each vector is indicated by the number in parenthesis. Bulk arrows inside the vector indicate gene products and thin arrows on the outside indicate ORFs of the vector. pLVX-GZ is comprised of GRK4, 2A self-cleaving peptide sequence T2A, Zeocin resistance, and pLVX vector backbone pLVX-Puro (Clontech). pLVX-GC is comprised of GRK4, IRES and CherryPicker (Clontech), and pLVX vector backbone pLVX-CherryPicker2 (Clontech). pLVX-GZC is comprised of GRK4, 2A peptide sequence P2A, Zeocin resistance, IRES and CherryPicker (Clontech) and pLVX vector backbone pLVX-CherryPicker2 (Clontech). Later on pLVX-GZC was modified, pLVX-GZC2, based on research and experimental results which is explained in Figure 6 (D.). pLVX-GZ and pLVX-GC are both used as controls to ensure that the 2A sequence is functional and IRES and CherryPicker respectively are functional with GRK4 independently (14).

Methods

Cell Cultures and Media:

293T cells were used for viral propagation of pLVX-GZ and pLVX-GZC and were plated on 150 mm dishes. Human Embryonic Kidney (HEK) cells were plated on 16 mm dishes and were used to study GRK4 expression, cell's resistance to a Zeocin challenge, and presence of CherryPicker, a red fluorescence protein (Clontech). HEK cells were specifically used because of their low production of GRK4 and any transfection would result in a significant difference. Cells were grown in warmed IPTG media and cells were stored at 37°C with 5.0% CO₂.

Inserts Used:

Pieces were obtained through PCR from other vectors. The vector backbone originated from pLVX-CherryPicker2 (Clontech) along with IRES and the CherryPicker insert which is identified as IRES_CherryPicker. The IRES_CherryPicker fragment size is 1512 bp while the size of the backbone varies depending on the vector being constructed. GRK4 variation γ and Zeocin resistance gene came from prior constructed vectors with lengths 1684 and 398 bp respectively. The 2A sequences that were used in this research were T2A and P2A due to their high cleavage probability (6, 10). These sequences were obtained from prior research (6, 10) and were added to forward or reverse primers depending on the vector being constructed and j5 algorithms (13).

PCR of Pre-Assembly Pieces:

After j5 output had been generated, primers were ordered (IDT) and were used in PCR while longer primers were synthesized using PAGE purification (IDT) (Table 1). MyTaq Red Mix (Bioline) was used for PCR master mix. Annealing temperatures used were indicated in the T_m (3' only) column found in the Oligo Synthesis section of the j5 Output (13) with an addition of two degrees. For

polymerization of pLVX backbone (pLVX_bb), a PCR gradient was done to determine optimal annealing temperature due to the backbone's large sequence (data not shown). A PCR template on pLVX_bb was designed based on these PCR gradients. The touchdown method of PCR was performed for isolation of GRK4, Zeocin, and IRES_Cherry. DNA fragments were recovered using a Gel Extraction protocol (Zyppy).

Table 1: Primers used for the different vector formation which were generated by j5 (5). GRK4_REV and Zeocin_FWD are longer in length due to having a 2A peptide sequence (T2A and P2A respectively) in the primer.

Vector	Primer Name	Sequence
pLVX-GZ	pLVX_bb_FWD	GGAGCAGGACTGAACGCGTCTGGAACAATCAACCTCTGG
	pLVX_bb_REV	CACGATGTTCTCGAGCTCCATACCGGAAATAGATCCTCTAGTAGAGTCGG
	GRK4_FWD	GAGGATCTATTTCCGGTATGGAGCTCGAGAACATCGTGGCCA
	GRK4_REV	GCACTGGTCAACTTGGCAGGGCCGGGATTCTCCTCCACGTCACCGCATGT TAGAAGACTTCCCTCGCCCTCGCATTGCCTTGGGTTCACCTTCCCTTCTCAC
	Zeocin_FWD	GAATCCCGGCCCTGCCAAGTTGACCAGTGCCGT
	Zeocin_REV	GTTCCAGACGCGTTCAGTCTGCTCCTCGGCCA
pLVX-GC	pLVX_bb_FWD	CGAGCTGTACAAATGAACGCGTCTGGAACAATCAACCTCTGG
	pLVX_bb_REV	ACGATGTTCTCGAGCTCCATACCGGAAATAGATCCTCTAGTAGAGTCGG
	GRK4_FWD	GGATCTATTTCCGGTATGGAGCTCGAGAACATCGTGGCCA
	GRK4_REV	GAGGGAGAGGGGGCGCATTGCCTTGGGTTCACCTTCCCTTCTCAC
	IRES_CherryPicker_FWD	ACCCAAGCAATGCGCCCTCTCCCTCCCCC
	IRES_CherryPicker_REV	GTTCCAGACGCGTTCATTTGTACAGCTCGTCCATGCC
pLVX-GZC	pLVX_bb_FWD	GCTGTACAAATGAACGCGTCTGGAACAATCAACCTCTGG
	pLVX_bb_REV	CACGATGTTCTCGAGCTCCATACCGGAAATAGATCCTCTAGTAGAGTCGG
	GRK4_FWD	GGATCTATTTCCGGTATGGAGCTCGAGAACATCGTGGCCA
	GRK4_REV	GAGGTGAGTTTGGCAGGGCCGGGATTCTCCTCCACGTCACCGCATGTTAG AAGACTTCCCTCGCCCTCGCATTGCCTTGGGTTCACCTTCCCTTCTCAC
	Zeocin_FWD	GAATCCCGGCCCTGCCAACTCACCTCCGCTGTGC
	Zeocin_REV	CCACCATTCCCATAGGTCCAGGGTTCTCCTCCACGTCCTCCAGCCTGCTTCA GCAGGCTGAAGTTAGTAGCGTCTGCTCCTCGGGAC
	IRES_CherryPicker_FWD	AGAACCCTGGACCTATGGGAATGGTGGACGGCG
	IRES_CherryPicker_REV	GTTCCAGACGCGTTCATTTGTACAGCTCGTCCATGCC

DNA Assembly Techniques and Transformation:

The Gibson Protocol (15) was obtained from j5 manual of scar-less DNA assembly protocols (13). Preparation of Gibson Assembly Master Mix and ISO Buffer were followed as instructed by j5 (13). For the Gibson reaction mix, 100 ng of linearized vector backbone and equimolar of additional DNA pieces were added to 15 μ L of Gibson Assembly Master Mix and deionized water was added for a final volume of 20 μ L. The Gibson reaction mix was incubated in a Thermomixer R (Eppendorf) at 50°C for one hour with 300 rpm of shaking. To desalinate the Gibson reaction mixture, a 10 cm dish with 25 mL of 1% agarose was made and let solidify. Wells were made using an agarose gel well mold and the reaction mixture was added to the well and let sit for 2-5 minutes. Because the newly synthesized vector would have not migrated far within the agarose, the well was resuspended in a desired volume of diH₂O and transferred into a new tube on ice. Electro-competent cells, ElectroMAX DH10B™ (Invitrogen), were transferred to the new tube, incubated on ice for 5 minutes, and transferred to a chilled electroporation cuvette (BioRad) sized 0.1 cm. The competent cells were electroporated using GenePulser® II (BioRad) with recommended settings from competent cell manual (Invitrogen), 2.0 kV, 25 μ F, and 200 Ω . The cuvette was suspended in 1 mL of SOC media where the media was then transferred to a new tube placed on a heat block set at 37°C for one hour. Competent cells were plated on LB agar plates with Amp¹⁰⁰ and were incubated at 30°C overnight.

Large scale plasmid preparation:

Pure Yield™ Plasmid Maxiprep System (Promega)™ was used to extract all lentivirusviral vectors for future transduction. Bacteria were grown in volumes of 100-300 mL of LB which were incubated at 30°C for 16 to 24 hours. Cells were then spun in an ultracentrifuge at 7000 x g for 20 min at 4°C after which the supernatant was decanted and the pellet was resuspended in 12 mL of Cell

Resuspension Solution and transferred to a 50 mL conical tube (Corning). Cell Lysis Solution (12 mL) was added, mixed by inversion, and incubated for 3 minutes at room temperature. Following incubation, 12 mL of Neutralization Solution was added and the conical tube was mixed by inversion gently 10-15 times. The lysate was centrifuged at 14000 x g for 20 minutes at room temperature using a fixed angle rotor. The supernatant was poured into the assembled blue PureYield Clearing Column (Promega) and white PureYield Maxi Binding Column (Promega) stack connected to a vacuum inlet (Promega). Once the supernatant had passed through both columns, the vacuum was turned off and the blue PureYield Clearing Column was discarded. To the PureYield Maxi Binding Column, 5 mL of Endotoxin Removal Wash was added and the vacuum was applied to pull the solution through the column. Next, 20 mL of Column Wash was added to the column with the vacuum applied. The column was left to dry for 5 minutes with vacuum suction. Once dry, the binding column was removed from the vacuum and the tip was wiped for excess ethanol. A 1.5 mL centrifuge tube was placed into the base of the Eluator Vacuum Elution Device (Promega) and the binding column was placed on top with the tip inside a 1.5 mL centrifuge tube. The device was added to the vacuum manifold and 1 mL of heated nuclease-free water was added to the binding column. The vacuum was applied until all the liquid had passed through the column. The DNA was quantified using a Nanodrop 2000 (Thermo Scientific).

Transfection:

A triple transfection was performed on 293T cells using two helper viruses, pAx2 and pM2DG. These helper viruses are used to help integration of the viral vector of interest into the cells. Viruses subsequently produced in the supernatant are the vector of interest. 293T cells were grown on High Glucose DMEM media with 10% FBS on 100mm dishes. Viruses were added when cells reached a confluence of 30-40%. Transfection was performed by adding 3 mL of Opti-MEM media into two

15 mL conical tubes (Corning). A 15 μ L aliquot of Lipofectamine2000 (Invitrogen) was added into one of the conical tubes in a drop-wise fashion with intermittent shaking to prevent micelle formation. This solution was allowed to sit for 20 minutes. A DNA mix of the helper viruses, 10 μ g of pAx2 and 5 μ g of pM2DG for one 100 mm dish transfection, was prepared in the conical tube not containing Lipofectamine and was mixed by briefly vortexing. Of the helper virus DNA mix, 1.5 mL was transferred to another 15 mL conical tube. Of the constructed experimental vectors, 15 μ g of the vector was added to one of the DNA-mix conical tubes along with 1.5 mL of Lipofectamine in a drop-wise fashion with intermittent shaking after which the tube was allowed to incubate at room temperature for 20-30 minutes. The two DNA-mixes were added to two 100 mm dishes containing 293T cells in a drop-wise fashion with intermittent shaking and were let to incubate for 48-72 hours at 37°C with 5.0% CO₂. To harvest the virus, the supernatant was filtered through a 0.45 μ M filter and was stored at 4°C. Aliquots were frozen down and placed in a -80°C freezer for long-term storage. Detection of viruses was done using Lenti-X GoStix (Clontech) by adding 20 μ L of the supernatant to the sample well and adding four drops of Chase buffer, waiting until a band appeared. Viral titers ranged from 4.6×10^4 to 4.6×10^5 IFU/mL.

HEK Cells Infection and Antibiotic Challenge:

HEK cells were grown in 6 well dishes to a confluence of 30-40% in DMEM with 10% P/S. The supernatant was aspirated, except for the control well, where empty wells were resuspended in 2 mL of viral fractions and incubated for 18-20 hours. The supernatant was again aspirated and replaced with 2 mL of new media and was left to incubate for a minimum of 24 hours. When the cells had reached a confluence of at least 50%, the media was aspirated and replaced with 2 mL of DMEM containing Zeocin and incubated overnight. Cells infected with pLVX-GZ or pLVX-GZC were observed for Zeocin resistance. Cells infected with pLVX-GC and pLVX-GZC followed the same

procedure except the final resuspension was media without Zeocin. These cells were observed using a confocal microscope to detect fluorescent from the CherryPicker (Clontech). pLVX-GZ and pLVX-GC serve as a positive control for cells obtaining Zeocin resistance and fluorescence respectively.

Using j5 and Related Programs:

For designing vectors, j5 was used to help design, discover potential construction difficulties, and visualize the vector before beginning the wet lab work. The program's premise is an open source software that can be used by researchers starting cloning projects to help them save laboratory time and money (13). Assembly protocols available in j5 are SLIC, Gibson, CPEC, and Golden Gate with the addition of combinatorial library construction using these methods (13). Users can sign up for a free account to have full access to j5 and Device Editor where cloning projects can be saved to their computers (Figure S1). Manuals on the usage of each program and how-to videos are located on the homepage (Figure S1).

The majority of construction was done using Device Editor where all projects can be saved as an .xml file (Figure S2). A basic procedure of using this program will be described below while more information about each program's utility can be found on the j5 manual (13). DNA fragments can be added to Device Editor by adding another column and copying the sequence to the column. This can be done by right clicking the column and selecting either "Copying from Clipboard" or "From File." The sequences can originate from a Genbank file (.gb) which allows for detailed information to be viewed in Vector Editor's virtual construction of the vector or can be copied from a FASTA file with less information. Using a Genbank file is useful when uploading the backbone vector which helps identify specific sequences, including multiple cloning sites, promoters, reporters, and resistance genes. For each piece, an image can be depicted to visually signify the function of each

DNA segment. An example of how Device Editor looks when all desired pieces are listed is illustrated in Figure S3 using pLVX-GZC2. Assembly requirements, including embedding short sequences in a primer, direct synthesis, digest, etc. can be specified in “Forced Assembly Strategy” under the “Part Info” tab. Once all pieces, settings, and assembly requirements are to the user’s specifications, under the collection info tab, a green “True” should appear next to j5 ready when the program is able to construct the vector (Figure S3). Clicking on the j5 button on the top right of the Device Editor page will open a new page inquiring for what assembly method the user prefers (Figure S4). “Mock Assembly” is used for strictly visualizing the vector ensuring all pieces are in the right order, orientation, and meet all the user’s requirements while SLIC/Gibson/CPEC generates the visualization of the vector along with generating a file for construction of the vector. With both options, a link will be generated transferring to the Vector Editor Webpage which will have an image of the construct (Figure S5).

The downloadable output file is a zip file containing 11 files with the file containing the master protocol listed in pj5_000XX.csv (Figure S6). The two XX’s indicate variable numbers depending on the number of runs the user has done on Device Editor. The CSV files are subsections of the master protocol file while the other files can be used on the j5 program. For the master protocol file, pLVX-GZC2 is used as an illustrative example of the output file while further information can be obtained from the j5 and Device Editor Manual on the j5 homepage. The file is divided into sections listing all the DNA pieces along with other information, and at the end of each section, there are warning statements if the program has encountered any assembly issues. For when assembling with SLIC/Gibson/CPEC, warnings may be related to another protocol than the one the user has decided in using. On the first few lines, the type of procedure is indicated along with the citation of the document. Assembly parameter is the first section where j5 specifications, including primer length, homology length, etc. will be shown (Figure S7). If none of the parameters have been

changed, they are the default values set by the program. These settings may be altered in Device Editor before submitting the vector for j5 construction by pressing, “Edit j5 Parameters” (Figure S4). The next section is headed as “Non-degenerate Part IDs and Sources” which list all pieces being assembled, original source of nucleotides, orientation, start and end base pair, and the nucleotide sequence (Figure S7). Warnings in this sequence will be general on the assembly including issues of primer design, hairpins, and T_m discrepancies. The next section is “Target Part Ordering/Selection/Strategy” which indicates what procedure is needed to isolate the DNA fragment mostly indicated with PCR (Figure S8). Warnings in this section are mostly related to CPEC primer design due to the process of the DNA assembly technique. Incompatibilities between Assembly Pieces section indicates any issues that could hinder vector assembly. The numbers indicate the DNA piece listed in the “Non-degenerate Part IDs and Sources” section. A way to resolve assembly issues is to compile some of the pieces prior to performing the DNA assembly technique (Figure S8). Suggested Assembly Piece Contigs for Hierarchical Assembly section indicates the order of how the pieces should be assembled and if prior assembly is necessary before the final vector assembly (Figure S9). The numbers originate from the Incompatibilities between Assembly Pieces section which can identify which pieces should be added together first to avoid problems in vector assembly (Figure S9). The final section is labelled Oligo Synthesis section which generates the primers utilized in isolating the DNA pieces along with the addition of the overlapping regions on each of the pieces for annealing (Figure S9). All primer sequences are in 5' to 3' orientation which allows for direct sequence transferring to a company which synthesizes primers (Figure S9). Prices for the primers can be made but additional information is required in Device Editor to generate an accurate prediction of cost. At the very end of the CSV file, a total DNA sequence of the entire plasmid can be copied and pasted for future reference.

Results

pLVX-GZ:

The constructed vector pLVX-GZ includes GRK4, T2A, Zeocin resistance gene, and pLVX_bb which serves as a control for the activity of the 2A peptide, T2A, and Zeocin resistance. Each piece was isolated by PCR using the j5 generated primers to add approximately 20-25 base pairs of homology to each piece (13). To reduce the number of pieces for the Gibson protocol, the T2A sequence was imbedded in the reverse primer of GRK4. The predicted lengths of each piece including the homology portions are 6881 bp for pLVX_bb, 1684 bp for GRK4, and 398 bp for Zeocin which were confirmed by gel electrophoresis and the DNA was isolated by gel extraction (Figure 10A). The excised DNA pieces were ligated together using the Gibson protocol (15) after which the reaction mixture was transformed into competent cells. Transformed colonies did grow on ampicillin plates, indicating the gain of ampicillin resistance which also occurred with the positive control, pUC19 (Invitrogen). Colony PCR was conducted to detect the presence of GRK4 with T2A

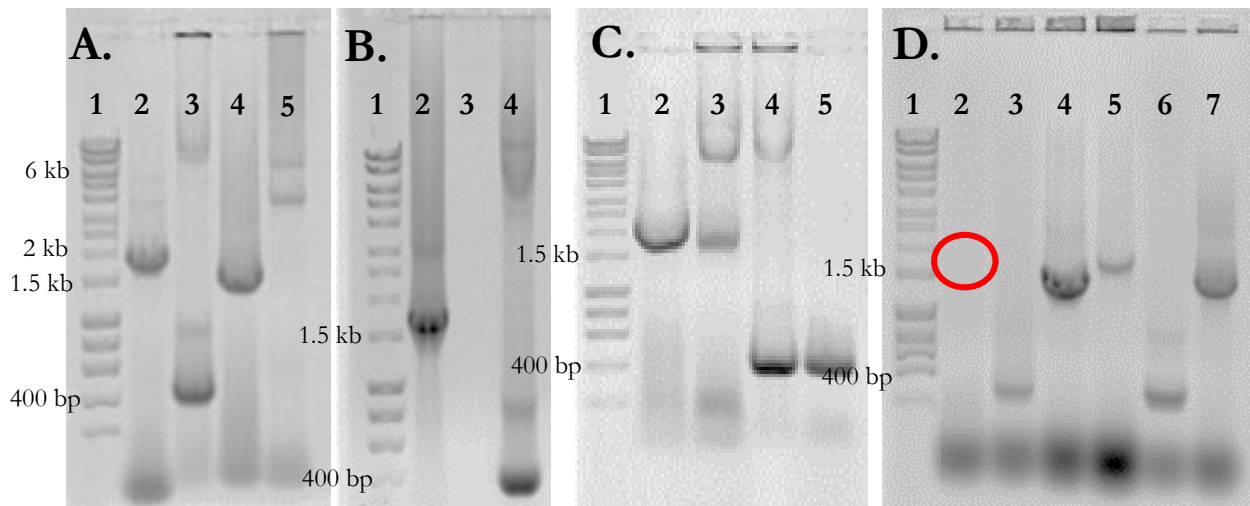


Figure 10: DNA gel electrophoresis of DNA segments of PCR products for each DNA feature (A) pLVX-GZ (B) and from either Maxiprep or colony PCR for pLVX-GZ (C) and pLVX-GZC (D). All pieces were visualized using touchdown PCR. For panel A, lane 1 contains 1 Kb ladder, lane 2 contains GRK4 with P2A, lane 2 contains Zeocin, lane 3 contains CherryPicker with IRES, and lane 4 contains pLVX-CherryPicker backbone with predicted length of about 7 Kb. For panel B, lane 1 contains 1 Kb ladder, lane 2 contains GRK4, predicted length of 1.5 kb, and lane 4 contains Zeocin with T2A, predicted length of 400 bp. Panel C is confirmation of pLVX-GZ in bacterial colonies with ampicillin resistance. Lane 1 contains 1 Kb ladder, lane 2 contains GRK4 DNA control, lane 3 contains GRK4 from colony PCR, lane 4 contains Zeocin and T2A DNA control, and lane 5 contains Zeocin and T2A from Colony PCR. Panel D is confirmation of pLVX-GZC. Lane 1 contains 1 Kb ladder, lane 2 contains Variation 1 GRK4, lane 3 contains Variation 1 Zeocin and P2A, lane 4 contains Variation 1 CherryPicker, lane 5 Variation 2 GRK4, lane 6 contains Variation 2 Zeocin and P2A from colony PCR, and lane 7 contains Variation 2 CherryPicker. For panel D, the red circle indicates missing GRK4 piece.

and Zeocin to determine which colony should be used for plasmid extraction, one of which is shown (Figure 10B). The quantity of vector obtained from maxiprep (Figure 10C) was used in a triple transfection in 293T cells. Once the cells are infected and are producing viruses, the supernatant contains only the virus of interest which in this case is pLVX-GZ. Virus was shown to be in the supernatant using the Lenti-X GoStix (Invitrogen) which was harvested and used to infect HEK cells to determine if pLVX-GZ was a functional virus, if the 2A peptides cleaved in HEK cells, and if GRK4 was functional. HEK cells were challenged with Zeocin and were able to propagate indicating 2A cleavage efficiency allowing the cells to acquire a Zeocin resistance in the cells. Cells were frozen down and stored in liquid nitrogen.

pLVX-GC:

The formation of pLVX-GC required GRK4 and IRES_Cherry, providing a control when looking at the IRES activity. pLVX-GC will be used to compare production of GRK4 and CherryPicker compared to the tricistronic vector along with confirming the result from previous research that the IRES is not as efficient in its function as the 2A peptides (6, 10). Primers were constructed using j5 (13) allowing for homology between the pieces for a three part ligation to occur. The predicted lengths of each piece are 6755 bp for pLVX_bb, 1684 bp for GRK4, and 1512 bp for IRES_CherryPicker for a total size of 9951 bp (Figure 9B). After Gibson Assembly and transformation, colonies developed an ampicillin resistance potentially indicating the presence of pLVX-GC and

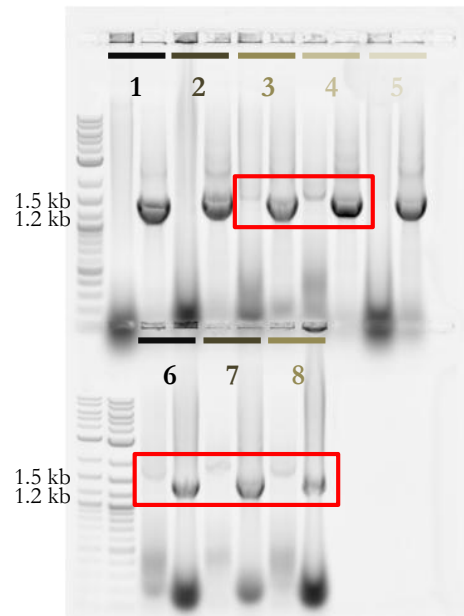


Figure 11: DNA gel electrophoresis of colony PCR for pLVX-GC. Eight colonies were screened with GRK4 in the first lanes and IRES_CherryPicker in the second lane. Red rectangles indicate colony samples that are positive for both pieces. Quick-Load 2-log DNA ladder was used (NEB).

transformed colonies were screened for GRK4 and IRES_Cherry using Colony PCR (Figure 11). From colonies screened, 5 out of the 8 colonies screened had the two inserts, indicating that the full vector was within the colonies (Figure 11). These colonies were grown in large quantities to obtain enough of the vector for a triple transfection in 293T cells and the virus pLVX-GC was detected in the supernatant using the Lenti-X GoStix (Invitrogen).

pLVX-GZC and pLVX-GZC2:

The formation of pLVX-GZC required GRK4, P2A, Zeocin, IRES_CherryPicker (Clontech), and pLVX_bb for a total length of 10839 bp (Figure 9C). A similar process for primer design was followed where P2A was imbedded in the forward primer of Zeocin, and IRES and CherryPicker were isolated together from pLVX-CherryPicker2 (Clontech). In the four part formation, colonies would form on ampicillin plates which indicated that the cells had the vector; however, colony PCR would detect Zeocin with P2A and IRES_CherryPicker but not GRK4 (Figure 10D). Due to the rare event of a four part formation of pLVX-GZC, twenty colonies from the resulting transformation were tested and all had the GRK4 piece missing (data not shown).

To reduce the number of pieces in the transformation, PCR by overlapping extension, SOEing, was used to combine two or more of the pieces together by PCR. Two variations of SOEing were made to combine GRK4, P2A, Zeocin, and IRES_CherryPicker (Clontech) together in order for a two part ligation. The first variation was to polymerize GRK4, Zeocin with P2A, and IRES_CherryPicker individually with their respective j5 primers then SOE the pieces together with the forward primer of GRK4 and the reverse primer of CherryPicker. The second was a two-step SOE with the first step combining GRK4 and Zeocin with P2A and combining Zeocin with P2A and IRES_CherryPicker and the second step was SOEing the two pieces together having the advantage of a two piece SOE and having increased region of homology with both pieces having

P2A and Zeocin as their homology region. Both resulted in a single piece of insert DNA containing all four pieces and both were able to be transformed into *E. coli* resulting in growth on ampicillin plates. The second variation of SOEing was the one that resulted in all pieces to be detected in pLVX-GZC using colony PCR (Figure 10D).

Colonies from the second variation of SOEing that was successful in contain all DNA features in pLVX-GZC were used for viral propagation in 293T cells and harvested from the supernatant with a titer of approximately 4.6×10^5 IFU/mL. Determining to see whether the viral vector was functional, confluent HEK cells were infected with pLVX-GZC and subsequently challenged with Zeocin. Challenged cells were scanned using a confocal microscope to investigate whether pLVX-GZC had properly infected and GRK4, Zeocin^R, and CherryPicker were functional. Only 3 to 5 cells per well were found to have Zeocin resistance and fluorescence above background, indicating that the both the resistance protein, Zeocin^R, and the fluorescence protein, CherryPicker, were active and stable. Attempting to propagate the cells to a higher confluence for storage and GRK4 analysis, the cells were grown for 72 hours checking every 24 hours. Unfortunately, none of the cells survived past 72 hours. Testing whether leaving the media constant with washing the plate, or adding new media to remove particulates from dead cells was performed and neither stimulated HEK cells with pLVX-GZC growth.

Two possible contributing factors in the lack of growth and survival are the large size of the vector and the usage of an IRES. With pLVX-GZC being approximately 10.4 Kb, the infectivity rate of the lentivirus will be lowered because of its greater increase in size than the original vector, pLVX-CherryPicker2 (Clontech), which was approximately 8.4 Kb. Also, with recent research finding that IRESs do not promote equal translation of the upstream and downstream sequences (6,8-11), this can explain the low production of Zeocin resistance and fluorescent protein. To resolve both issues,

The IRES was eliminated and replaced with a P2A peptide sequence and a T2A peptide sequence was placed between GRK4 and Zeocin instead of the P2A peptide sequence (Figure 12). This allows a single ORF to encompass all the proteins of interest with 2A peptides in between. In choosing whether to use two similar 2A sequences or two different 2A sequences, Tian et al. (9) used two similar 2A sequences in their tricistronic vector. Although with using the Gibson

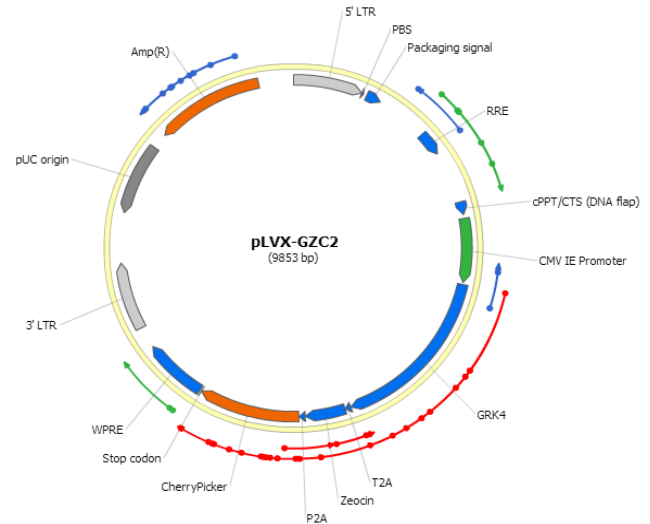


Figure 12: Visualization of the corrected pLVX-GZC2 illustrated by Vector Editor (14). Base pair size of each vector is indicated by the number in parenthesis. Bulk arrows inside the vector indicate gene products and thin arrows on the outside indicate ORFs of the vector. pLVX-GZC is comprised of GRK4, 2A peptide sequence T2A, Zeocin resistance, P2A peptide sequence, CherryPicker (Clontech) and pLVX vector backbone pLVX-CherryPicker2 (Clontech) (14).

Protocol which anneals sequences based on homologous regions, it was decided to use separate 2A sequences to reduce any homologous regions not generated by j5. These modifications have reduced the size of the vector by approximately 500 bps. To help in the construction of pLVX-GZC2, a new oligo-synthesizer product from IDT called gBlock (IDT) synthesized approximately 1389 bps containing T2A, Zeocin, P2A, CherryPicker, and some of the backbone in that order allowing for a three part ligation. Codons were optimized using IDT's codon optimizer with the specifications to HEK cells.

Discussion

Genetic screening for diagnosis of salt-sensitivity would be an effective, relatively inexpensive, and less time consuming compared to traditional methods of 29 day or 5 day restrictive diet tests (1). Determining an individual's salt-sensitivity classification (SS, SR, ISS) based on his/her genetics can allow personal dietary changes that can have a greater impact on reducing their risk for hypertension and cardiovascular diseases (1-4). Having found that the renal dopaminergic system, especially the D₁ receptor, regulates the antihypertensive function of the kidney, factors impacting the expression of the receptor can be used as the genetic screen for salt-sensitivity diagnosis (3,4). One of these factors, GRK4, directly regulates D₁ receptor through phosphorylation and internalization of the receptor complex. Investigating GRK4's activity in different salt-sensitivity groups can be the genetic factor used for diagnosing salt-sensitivity, reducing harmful dietary changes from the "one-size-fits all" model as well as reducing the risk of hypertension and cardiovascular disease.

The development of the tricistronic vectors will allow indirect *in vivo* measurements of GRK4 expression through fluorescence in various cell types and salt conditions. GRK4 expression changes in changing salt conditions, high to low sodium or vice versa, can also be measured live in time-lapse confocal microscopy. Future investigation on the relationship between fluorescence measured and GRK4 expression and efficiency of 2A peptide are needed before testing other salt-sensitivity cell types. Having developed a cost and time effective method in constructing tricistronic viruses using j5 can be used in other systems to help measure protein expression indirectly and *in vivo*.

Utility of j5:

The web-based DNA design automation software, j5, along with Device Editor and Vector Editor are useful and powerful tools for any cloning project (12-14). Manuals and tutorial videos allow for easier transition into using j5 along with the ability to send questions to other j5 users and to the

developer. This program is unique in its ability to optimize cost, design hierarchical assembly strategies to consolidate DNA fragment products, and allow users to enter design specification rules (13). With all of these functions being performed with a simple interface for input data and a consolidated CSV output file, cloning projects can be done at the same time frame or faster along with being cheaper (Table 2). The largest benefit in time and cost for cloning projects can be seen in combinatorial DNA libraries of around 200 plasmids (13). The time saved in combinatorial DNA library construction can be around 3 to 8 fold compared to traditional approaches and the savings can range from 10 to 20 fold over DNA synthesis services (13). Though total duration is equivalent to traditional cloning, designing time, materials cost, and total cost are much lower using SLIC/Gibson/CPEC with j5 (Table 2). Whether to use SLIC, Gibson, or CPEC methods for DNA assembly is not important, as for they are all time efficient DNA assembling protocols. These tools allow researchers to have a control on almost every single nucleotide, whether adding or subtracting a sequence while at the same time optimizing costs (13).

Table 2: Table of time and money efficiency of j5 and scar-less DNA assembly techniques compared to traditional cloning and direct synthesis of two cloning projects. Costs for the various cloning techniques and hands-on time predictions for hypothetical cloning projects were obtained from Hillson et al. (13). Time and cost savings with combinatorial DNA libraries projects can be referred to Supplementary Table 1 from Hillson et al. (13).

Hypothetical Project	Task	Traditional Cloning and Robotics ¹	DNA Synthesis ²	SLIC/Gibson /CPEC ³ with j5 and Robotics ⁴
Metabolic Pathway Construction Vector backbone 4kb + 2 promoters (200 bp each) + 5 ORFs (with RBS, 1000 bp each) + 1 terminator (100 bp)	Hands-on Time⁵	5.7 hr design ⁶ 6.7 hr lab	0.1 hr design 0.1 hr order	1.2 hr design 4.4 hr lab
	Materials cost	\$212	\$2345	\$174
	Total cost	\$1452	\$2365	\$734
	Total duration	2.5 weeks	2 months	2 weeks
Protein Chimera Construction Vector backbone 4kb + 3 domains (500 bp each)	Hands-on Time⁵	3.1 hr design 4.1 hr lab	0.1 hr design 0.1 hr order	0.5 hr design 4.2 hr order
	Materials cost	\$73	\$785	\$64
	Total cost	\$793	\$805	\$534
	Total duration	2 weeks	3 weeks	2 weeks

¹ Best-case scenario (viable restriction enzyme choices). Binary ligations with maximum intermediate re-use.

² Assumes perfect parallel process scaling, \$0.39/bp, \$50/construct for custom destination vector.

³ SLIC/Gibson/CPEC can be used interchangeably to assemble the same j5-designed fragments.

⁴ No automation of transformation/clonal isolation process

⁵ Fully burdened-labor cost estimate: \$100/hr. Design time includes sequence validation.

⁶ Design time refers here to DNA sequence file manipulation (i.e., pasting each insert into the vector backbone)

Device Editor helps organize and lay out all the pieces of the desired vector. Any project can be saved and continued to be worked on as an .xml file. For this project, many files and many runs were performed in order to design each vector with changes being made easily and swiftly. Within minutes, projects can be assessed visually using Vector Editor to make sure all features are present, in frame, and meet all user requirements. The project can also be assessed using j5 to see if there could be potential construction problems and any adjustments need to be made. With Device Editor being the main input method, the utility of the program is intuitive and easy to assess if any changes are needed for a project, unlike other programs where files need to be transferred from one program to another. In constructing the viral vectors, having the Genbank files, especially for backbone pieces, allows the researcher to have additional pertinent information about the vector. Having the Genbank file for pLVX_bb which originated from pLVX-CherryPicker2 (Clontech) identified all features within the vector in Vector Editor allowing the ability of removing unnecessary nucleotides in the backbone to reduce the length of the final vector. Indicating the nucleotide sequence range in the backbone using Device Editor simplified inputting the backbone portion of the vector allowing the program to know where the DNA parts were going to be added. By default, Device Editor assembles the pieces based on column order within the program unless there are problems in construction but “Forced Assembly Strategies” can and was used to indicate the order.

In regards to the 2A peptide sequences, j5 indicated that embedding the sequences within the primers would be easier and cheaper by eliminating more pieces needed to be assembled using Gibson protocol. Recently with companies producing Gibson kits for DNA assembly, there is less need to purchase the materials to mix up the Gibson master mix, for creating the buffer can be cumbersome. With all of these programs, j5 has allowed for a streamlined process for cloning and developing vectors while assessing potential problems and cost assessments. The intuitiveness of the program can be potentially used in a classroom setting to demonstrate to students how vector

cloning works and what the final product should be. Otherwise, this tool can be useful in the laboratory setting where multiple projects can be saved and be recalled, verifying what preferences were set in prior constructions.

Vector Designing:

The three vectors, pLVX-GZ, pLVX-GC, and pLVX-GZC, have been constructed and two out of three have been made into viral titers and have been transfected into HEK cells which were frozen down for storage for later experimentation. pLVX-GZ and pLVX-GC have been relatively straightforward in construction due to the simplicity in design. These projects have provided a template for PCR temperatures, areas of potential construction difficulties, and relative homology lengths needed for a cloning project to be completed swiftly. pLVX-GZC was initially constructed, sequenced to confirm all parts were ligated into the vector, and transfected into HEK cells.

However, once challenged with Zeocin, very few cells survived and were not sustainable and the measured fluorescence was barely above background. With this, pLVX-GZC had to be reconstructed to reduce the size issue of the vector, decreasing the size from 10389 bp to 9853 bp.

This largest contributing factor was removing the IRES sequence and replacing it with a P2A sequence. Two different 2A sequences were used due to gBlock (IDT) construction requirements and reducing the number of additional homologous regions not specified by j5 for improper ligations during the Gibson protocol.

The future direction of this research is to test the expression level of the tricistronic vector, pLVX-GZC2 to ensure the ratio between GRK4 expression, Zeocin antibiotic resistance, and fluorescence along with testing the stability of the protein of interest. Testing the smaller vectors have shown the GRK4 is stable within the HEK cells and are not precipitating within the cells. To determine if similar levels of proteins and antibiotic resistance are being produced, pLVX-GZC2 would be

compared to pLVX-GZ which has GRK4 and Zeocin resistance. Different cell lines will be infected with one of the viruses allowing for GRK4 extraction to be conducted and see whether the relative amounts of GRK4 is equal between pLVX-GZ and pLVX-GZC2 along with being at a greater level than the negative control cells. Antibiotic resistance would simply be tested by serial dilution of the antibiotic and counting the number of viable cells indicating the highest concentration of antibiotic the cell can survive. To determine fluorescence, pLVX-GZC2 and pLVX-GC will be compared to each other to ensure that the fluorescent protein is being translated at relatively similar rates. This can be performed using confocal and measure light intensity after different time points. With ribosomal skipping efficiency variable on cell type, protein production within the vector would need to be tested. At this point, no studies have been conducted or investigated for superinfection or difference in cleaving efficiencies if using two identical 2A sequences or two different 2A sequences. For construction of pLVX-GZC2, two different 2A sequences were used to reduce the number of additional homology regions that were not generated by j5 for Gibson assembly. This would prevent the 2A sequences from annealing from each other leading to improper ligations. Also at this point of time, development of a tricistronic vector with one gene of interest with two modes of selection to infect HEK cells has not been developed. Once the tricistronic vector is constructed and tested, it will be a helpful tool in studying GRK4 expression in varying salt conditions along with transitions between low and high salt conditions. This type of vector can be used in other fields to study other proteins *in vivo* or can be used to produce two proteins. Along with the web-based software j5, producing these vectors can be done in a timely and cost efficient manner.

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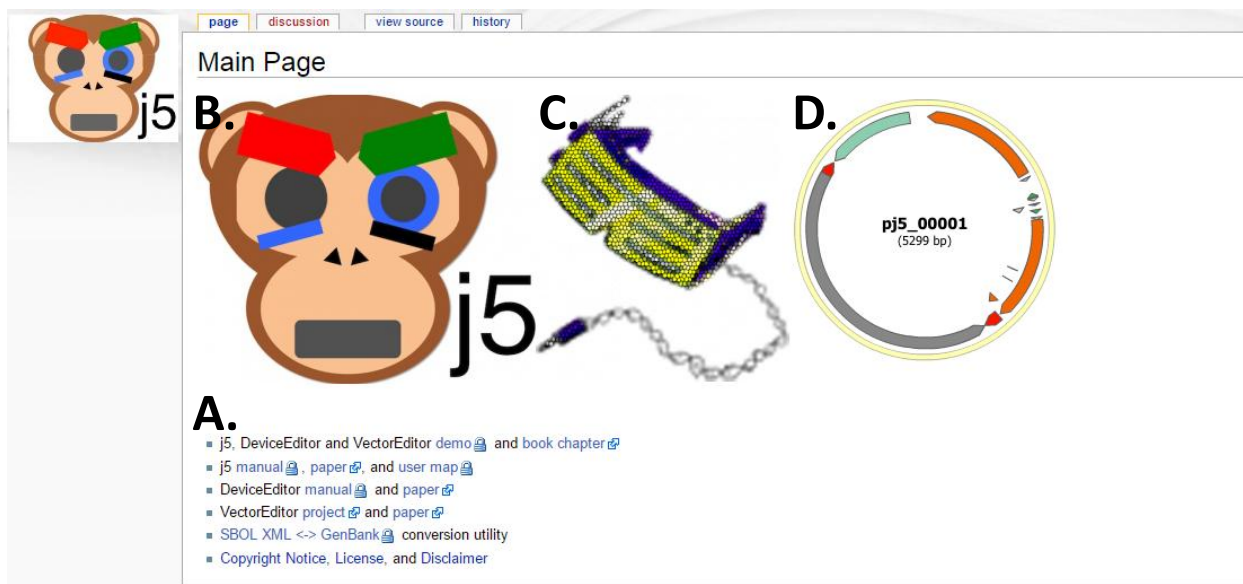


Figure S1: Screenshot of the home page of j5 located at: http://j5.jbei.org/index.php/Main_Page. Manuals of each of the programs, j5, Device Editor, and Vector Editor are listed in the area marked with an A. To use these programs users need to apply for an account to be able to save files. To access j5 algorithms directly, the icon labelled with a B will lead to the webpage of j5. The bioCAD program, Device Editor, allows users to assemble pieces and specify assembly methods and can be accessed by clicking on the icon located by the C. Also Device Editor allows users to assemble the pieces and generate a file with the protocol. To visualize vectors and see where the ORF, restriction cut sites, and generate a picture of the file, the icon located with a D will lead to the program Vector Editor (13).



Figure S2: Screenshot of Device Editor when using the program. Users can add columns by clicking on the “Add Column” button on the right pane which allows for additional DNA fragments to be added for final assembly. Sequences can be copied into Device Editor by right clicking under the column in the grey boxes and clicking either “Map from Clipboard” or “Map from File.” Assembly rules like embed sequence in forward/reverse primer, direct synthesis, or digest can be found in Forced Assembly Strategies. Once all pieces and assembly strategies are met, users can mock assemble the pieces by clicking on the j5 button on the tip left of the page (12).

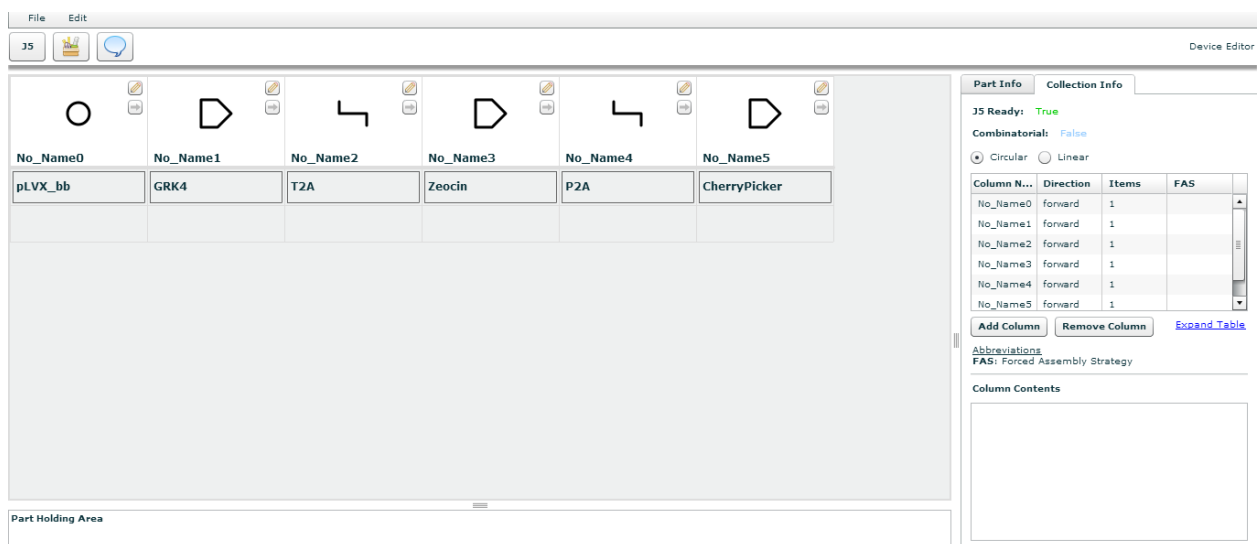


Figure S3: Screenshot of Device Editor when all pieces of pLVX-GZC2 are added to the program and ready to be assembled. The green “True” in the upper right side of the window on the line stating j5 Ready indicates that the pieces are added in a manner that the program can assemble them into a vector (12).

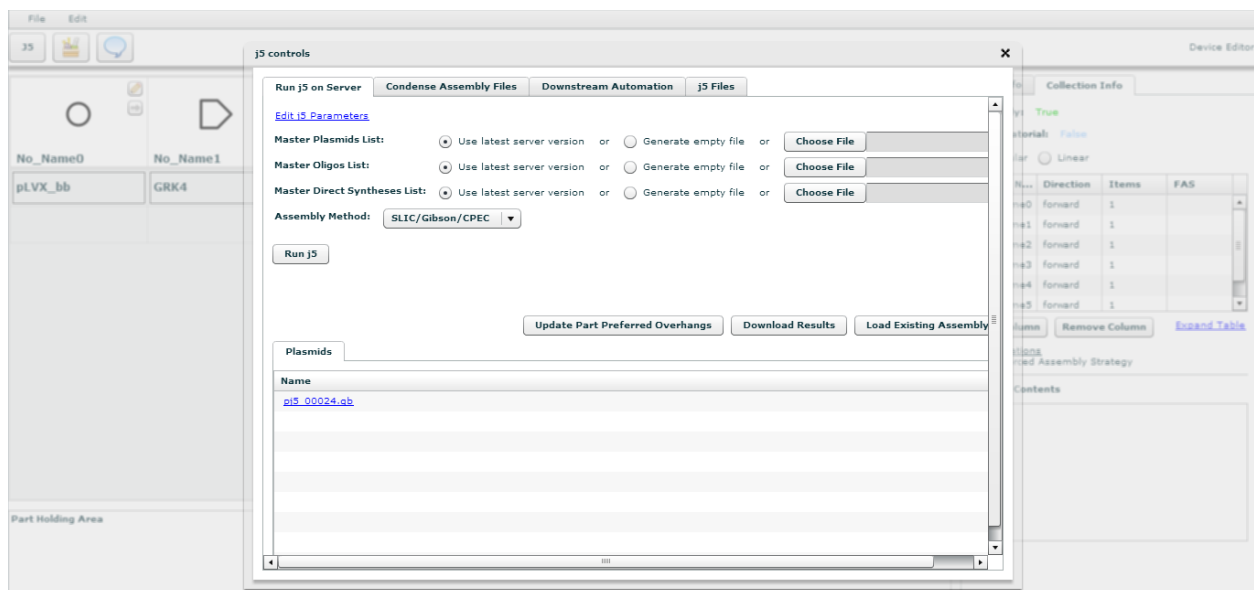


Figure S4: Screenshot of Device Editor the icon j5 is pressed when all pieces of pLVX-GZC2 are ready to be assembled. The green “True” in the upper right side of the window on the line stating j5 Ready indicates that the pieces are added in a manner that the program can assemble them into a vector. Changing Assembly Method from Mock Assembly to SLIC/Gibson/CPEC allows for a file to be created with the protocol, assembly method, and primers required along with an image of the vector. Once changed, pressing “Run j5,” will run the j5 program to assemble the vector. The zip file can be downloaded by clicking on Download Results, and the image can be viewed on vector editor by clicking on the blue line that appears under the Plasmids tab once j5 has finished processing (12).

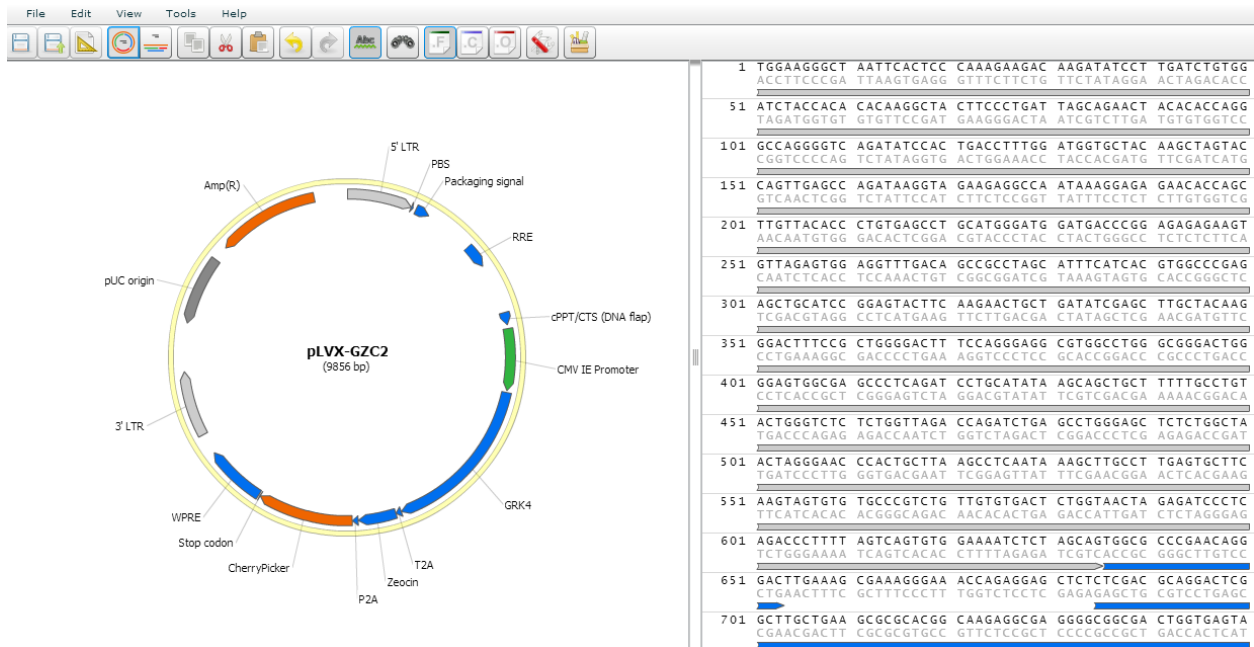


Figure S5: Screenshot of pLVX-GZC2 from Vector Editor once the user has clicked on the plasmid name in Device Editor. The vector image can be saved as a Genbank file or be printed. The left side of the window illustrates the vector with the desired features and overall size of the vector along with features indicated from DNA fragments that were Genbank files (.gb). On the right hand of the window, contains the whole sequence of the vector and have highlighted portions based on if they belong to a feature. Clicking on the icon with a C with a blue line over it will show all restriction enzyme sites located within the plasmid. Clicking on the O with a red line over it will show with small arrows the ORF and the directions they are in. These arrows will also appear on the sequence side of the window (14).

eugeneruleslist.eug	EUG File	1 KB	No	1 KB	0%	6/27/2014 10:55 AM
j5parameters.csv	Microsoft Excel Comma S...	2 KB	No	5 KB	63%	6/27/2014 10:56 AM
masterdirectsyntheselist.csv	Microsoft Excel Comma S...	1 KB	No	1 KB	0%	6/27/2014 10:55 AM
masteroligolist.csv	Microsoft Excel Comma S...	2 KB	No	6 KB	77%	6/27/2014 10:55 AM
masterplasmidlist.csv	Microsoft Excel Comma S...	10 KB	No	159 KB	95%	6/27/2014 10:56 AM
mastersequences.csv	Microsoft Excel Comma S...	1 KB	No	1 KB	24%	6/27/2014 10:55 AM
masterzippedsequences.zip	Compressed (zipped) Fol...	6 KB	No	6 KB	5%	6/27/2014 10:55 AM
partlist.csv	Microsoft Excel Comma S...	1 KB	No	1 KB	40%	6/27/2014 10:56 AM
pj5_00017.csv	Microsoft Excel Comma S...	8 KB	No	45 KB	84%	6/27/2014 10:56 AM
pj5_00017.gb	GB File	6 KB	No	15 KB	65%	6/27/2014 10:56 AM
targetpartsorder.csv	Microsoft Excel Comma S...	1 KB	No	1 KB	40%	6/27/2014 10:55 AM

Figure S6: Screenshot of files that are contained in the zip file from the downloaded results from Device Editor. The file that contains all the DNA pieces added together along with assembly methods, primers and temperatures, and potential issues with assembly is pj5_00017.csv. The file name will be relatively similar for the change will only be in the number based on the number of times the user has run j5 on Device Editor (12).

SLIC/Gibson/CPEC j5 v2.7.3 assembly 6/27/2014
Please cite: Hillson, N.J., Rosengarten, R.D., and Keasling J.D. (2012) j5 DNA Assembly Design Automation Software. ACS Synthetic Biology 1 (1), 14-21. DOI: 10.1021/sb2000116

Assembly Parameters:
MASTERO MASTERPI GIBSONO\ GIBSONO\ GIBSONO\ MAXIMUM MINIMUM GOLDENG GOLDENG GOLDENG MAXIMUM OLIGOSYN OLIGOPAC OLIGOMA MINIMUM DIRECTSY\ DIRECTSY\ PRIMER_G PRIMER_N PRIMER_N_DEFAULT
5 5 26 60 70 110 250 4 GGTCTC CACACCA 2 0.1 40 60 100 0.39 159 2 18 36

Note: sequence pLVX-CherryPicker2 is implied to be circular...

Non-degenerate Part IDs and Sources

Part ID	Name	Location	Source Pl	Reverse C	Start (bp)	End (bp)	Size (bp)	Sequence
0	pLVX_bb	pLVX-Che	FALSE		4350	2802	6847	ACGGGCTGGAAACAATCAACCTCTGGATTACAAAATTTGAAAAGATTGACTGGTATTTCTTAACATGTGTCTCTTTACGCTATGGATACGCTGCTTAATGC
1	GRK4	GRK4	FALSE		1	1596	1596	ATGGAGCTCGAGAAACATCGTGGCCAACTCGCTGCTGCTGAAAGCGCGTCAAGGAGGATATGGCAAAAAAGTGGTGTAGTAAAAATGGAAGGAGATACTG
2	T2A	T2A	FALSE		1	54	54	GAGGGCAGAGGAAGTCTTCAACATGCGGTGACGTGGAGGAGAAATCCGGCCCT
3	Zeocin	Zeocin	FALSE		1	369	369	GCCAAACTCACCTCCGCTGTCCGGTGTGACCGCAGCAGACGTTGACGAGGACTGTTGAGTTTTGGACTGATCGCTCGGATTCACAGAGACTTTGTGAAGAC
4	P2A	P2A	FALSE		1	57	57	GCTACTAACTCAGCCTGTGAAGCAGGCTGGAGACGTGGAGGAGAACCTGGACCT
5	CherryPic2	pLVX-Che	FALSE		3417	4349	933	ATGGGAATGGTGGACGCGCAACACGCCACTGGAGATGAAGCTGGCCGTGGACGAGGAAGAGAAACCCGCAACAACACCAAGGCCAACCTGACCAAGCC

Figure S7: Screenshot of the protocol file generated by j5. This part of the file is to summary portion of the file indicating what pieces are coming together and if there are some issues with the primer design. This file is using pLVX-GZC2 as an example with all the DNA fragments used to create the vector listed above (13).

Target Part Ordering/Selection/Strategy

Order	ID Numbe	Name	Direction	Strategy
0	0	pLVX_bb	forward	PCR
1	1	GRK4	forward	PCR
2	2	T2A	forward	Embed_in_primer_reverse
3	3	Zeocin	forward	PCR
4	4	P2A	forward	Embed_in_primer_reverse
5	5	CherryPic2	forward	PCR

Assembly Piece 1 CPEC primer design: Warning: Primer3 could not find a reasonable set of primers. Right primer explanation: considered 11; high tm 11; ok 0. Primer Pair explanation: considered 0; ok 0. Ignor
Assembly Piece 2 CPEC primer design: Warning: Primer3 could not find a reasonable set of primers. Left primer explanation: considered 11; high tm 11; ok 0. Primer Pair explanation: considered 0; ok 0. Ignorin
Assembly Piece 1 CPEC left primer design (with respect to Assembly Piece 0): Warning: mispriming (seq: TATGGAGTCAAAAACAGCGTG; bps: 6804..6824; strand: -1; query seq: TATGGAGCTCGAGAAATCGTG; que

Incompatibilities between Assembly Pieces:

Assembly Left End	Right End
0 NONE	0
1 NONE	NONE
2 NONE	NONE
3 NONE	NONE

Figure S8: Screenshot of the protocol file generated by j5. This part of the file is to summarize the strategy adding the DNA pieces together. The 2A peptide sequences were specifically designed to be embedded in the primers due to their small size and to reduce the number of pieces coming together. Even without specifically assigning the strategy, j5 resulted in a similar strategy (13).

Suggested Assembly Piece Contigs For Hierarchical Assembly:

Contig	Assembly Pieces Contained
0	0 1 2 3

Oligo Synthesis

ID Numbe	Name	First Targe	Last Targe	Length	Tm	Tm (3' onl	Cost	Sequence
0	j5_00046	0	0	39	71.506	68.928	0	GCTGTACAAATGAACGCTCTGGAAACAATCAACCTCTGG
1	j5_00004	0	0	50	70.242	66.044	0	CACGATGTTCTCGAGCTCCATACCGGAAATAGATCCTCTAGTAGAGTCGG
2	j5_00035	1	1	40	74.008	69.938	0	GGATCTATTTCCGGTATGGAGCTCGAGAAACATCGTGCCA
3	j5_00052	1	2	97	76.31	69.58	0	GAGGTGAGTTTGGCAGGGCCGGATTCTCTCCACGTCACCGCATGTTAGAAGACTTCTCTGCCCTCGCATGTCTGGGTTCCACTCTCTCT
4	j5_00053	3	3	35	79.664	68.708	0	GAATCCCGCCCTGCCAAACTCACCTCCGCTGTGC
5	j5_00054	3	4	88	76.82	64.984	0	CCACCTATCCCATAGTCCAGGGTTCTCTCCACGCTCCAGCCTGCTTCAGCAGGCTGAAGTTAGTAGCCTGCTCTCGGCGAC
6	j5_00051	5	5	33	76.121	65.126	0	AGAACCTGGACCTATGGGAATGGTGGACGGCG
7	j5_00037	5	5	37	75.075	65.393	0	GTTCAGACGCTTCATTTGACAGCTGTCATGCC

Figure S9: Screenshot of the protocol file generated by j5. This part of the file generates the sequences of the primers and the Tm's of the primers that are need for the DNA assembly protocol chosen (Gibson protocol). These primers contain the overhangs needed for annealing two pieces of DNA together along with added sequences embedded in the primer (13).

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