

**Generation of a FHV-1 Viral Vaccine Against Gonadotropin Releasing Hormone for  
Immunocontraception of Felines**

**By**

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# **Generation of a FHV-1 Viral Vaccine Against Gonadotropin Releasing Hormone for Immunocontraception of Felines**

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## **(ABSTRACT)**

With approximately 3-4 million unwanted cats euthanized in the U.S. annually, convenient, cost effective methods of sterilization are greatly needed. Current spay/neuter techniques, such as surgery and hormonal intervention, are not satisfying this need due to their high cost, significant expertise required, and the need for feral cats to be collected and brought into clinics for treatment. The aim of this research is to develop a safe contraceptive vaccine that could be delivered to the feral cat population in bait without compromising non-feline species. Feline Herpes Virus (FHV) is a feline specific virus. The USDA has approved the immunization of cats with an attenuated, non-pathogenic strain of FHV expressing foreign antigens. In our research, we have partially replaced Glycoprotein I of FHV to express a fusion protein of Flagellin (FliC), Enhanced Green Fluorescent Protein (EGFP), and Gonadotropin Releasing Hormone (GnRH). FliC has been shown to stimulate a heightened antibody response when antigens are expressed as fusion proteins with it. GnRH, a major reproductive hormone responsible for the development of testes and ovaries in felines, is the target of our vaccine vector. Expression of EGFP will allow tracking of the viral vector. The expression of the fusion protein (FliC-EGFP-GnRH) is expected to stimulate an antibody and cell mediated immune response directed towards feline GnRH, which will provide an immunocontraceptive effect specific to cats.

Funding for this project was provided by the Winn Feline Foundation and the VA-MD Regional College of Veterinary Medicine.

## **DEDICATION**

I would like to dedicate this work to my family and friends for their endless support, spirit-boosting, and love. Thank you all so much.

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## LIST OF ABBREVIATIONS

Amp = ampicillin  
APC = antigen presenting cell  
bp = basepair  
C = Celsius  
CMV = cytomegalovirus  
CPE = cytopathic effect  
CRFK = Crandell Reese Feline Kidney  
Da = Dalton  
DC = dendritic cell  
DNA = deoxyribonucleic acid  
EBV = Epstein-Barr virus  
EG = EGFP::GnRH  
EGFP = enhanced green fluorescent protein  
FEG = FliC::EGFP::GnRH fusion protein  
FeLV = feline leukemia virus  
FHV = feline herpes virus  
FIV = feline immunodeficiency virus  
*fliC* = flagellin (nucleic acid)  
FliC = flagellin (protein)  
FRV = feline rhinotracheitis virus  
FSH = follicle stimulating hormone  
gD = glycoprotein D  
gE = glycoprotein E  
GFP = green fluorescent protein  
gI = glycoprotein I  
GnRH = gonadotropin releasing hormone  
hCG = human chorionic gonadotropin  
HCMV = human cytomegalovirus  
hpi = hours post-infection  
HRP = horse radish peroxidase  
HSV-1 = human herpesvirus-1  
IFA = immunofluorescence assay  
kb = kilobase  
kDa = kilodalton  
KLH = keyhole limpet hemocyanin  
KU = Klett unit  
LB = Luria broth  
LH = luteinizing hormone  
MCS = multiple cloning site  
MHC = major histocompatibility complex  
MOI = multiplicity of infection  
MyD88 = myeloid differentiation factor 88  
mZP3 = mouse zona pellucida-3  
NISV = non-ionic surfactant vesicles  
PAMP = pathogen associated molecular patterns

PCR = polymerase chain reaction  
Pen-Strep = penicillin-streptomycin  
PLGA = poly(lactide-co-glycolide)/triacetin  
RNI = reactive nitrogen intermediates  
ROI = reactive oxygen intermediates  
rpm = revolutions per minute  
SDS-PAGE = Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis  
TBS = tris-buffered saline  
TK = thymidine kinase  
TLR = toll-like receptor  
TNF $\alpha$  = tumor necrosis factor alpha  
 $\mu$ g = microgram  
 $\mu$ l = microliter  
UV = ultraviolet  
VBI = Virginia Bioinformatics Institute  
VZV = varicella zoster virus

## **CHAPTER 1: INTRODUCTION/LITERATURE REVIEW**

**Statement of Purpose:** The goal of this thesis research was to generate a species-specific, recombinant vaccine to be used for immunocontraceptive control of feral felines.

**Overview:** Towards this goal, we chose an attenuated strain of feline herpes virus (FHV) as the vaccine vector and gonadotropin-releasing hormone (GnRH) as an antigenic target in cats. In order to increase the immunogenicity of GnRH, we fused a 14 repeat sequence of GnRH to flagellin (*fliC*). The fusion protein also contained the Enhanced Green Fluorescent Protein (EGFP) for ease of visualization in cultured cells used to grow the FHV.

### **1.1 The Problem: Feline Overpopulation**

Feral cats are those cats that are living on their own in the wild. Some of these cats may have been a pet at one time, but were either abandoned or otherwise separated from their owners; others are the offspring of already feral cats. These undomesticated cats must learn to fight for survival and compete with each other for the limited resources in an area. Often, these populations, or cat colonies, are supported in part by well-meaning humans who provide food. However, the additional supply of food only widens the niche in any given area, permitting the population to likewise expand.

Each pair of cats and their offspring can produce as many as 420,000 cats within 7 years (HSUS, 2005). Alley Cats Allies, a national feral cat advocacy organization, estimates that there are approximately 30-60 million feral cats living in the United States alone while the American Veterinary Medical Association estimates another approximately 70 million cats housed and maintained as pets (AVMA, 2002; Holton & Manzoor, 1993). Although roughly 75% of owned cats have been sterilized, an unknown, but large, portion of the feral cat population continues to contribute to the generation of offspring (Patronek & Rowan, 1995). Meanwhile, there are not nearly enough homes for the placement of so many cats. Instead, many of these animals live as

unwanted feral cats, fighting for territory and resources, transmitting disease, and are frequently considered to be pests. In an effort to control this feline population, approximately 3-4 million unwanted feral cats are euthanized every year in the United States alone (HSUS, 2005; Irwin, 2001). Unless a critical number of cats can be sterilized, the problem will continue to snowball.

Approaches to control feline overpopulation problems employed thus far have included: spay/neuter programs, hormone based contraceptive injections, and euthanasia. While each of these methods has found some degree of success, each continues to have complicating factors preventing their full implementation in the control of the feline overpopulation problem. Euthanasia is seen by many to be inhumane and attracts the negative attentions of animal welfare groups. Hormone based contraceptives must be administered periodically over the reproductive lifetime of the animal and thus pose significant difficulty for use in controlling feral cat populations. While currently the most widely used method, spay/neuter programs, require surgery that is costly in both time and money. Additionally, each of these methods requires capturing the cats and the assistance of a licensed veterinarian. Feral cats are notoriously difficult to capture as most shy away from direct human contact and often bite and/or scratch when handled, posing additional public health risks.

Development of a single-dose method of contraception that is affordable, effective, and does not require the capturing of cats would be immensely valuable in the effort to control feral cat populations. The approach should be species-specific so as to ensure that humans and other animals would not become the unintended target. Whereas a drug-based approach may present a risk to non-target species, a species-specific vaccine could be designed to be functional in only cats. Additionally, a long-lasting effect would be desirable, such that once sterilized, the cats would not need to be subjected to the procedure again. Towards this end, research is currently underway to develop such a “contraceptive vaccine”. Sterilization through vaccination would provide an economical, easy-to-administer, safe solution to address the feral cat overpopulation concern.

Additionally, the contraceptive vaccine could be used in veterinary practices as a non-surgical alternative to the other contraceptive methods currently available for pet cats.

In light of these proposals to control specific animal populations, it is important to keep in mind and consider the potential environmental, ecological, and moral impacts that such controlled contraceptive efforts may have. Our world is an interconnected network of life with each life dependent on many others. The decision to alter the balance of our ecosystem may have greater consequences than are readily apparent and therefore such choices should not be taken lightly. Should we choose to intervene in Nature's reproductive process, we have a responsibility to act with caution and careful consideration.

## **1.2 Rationale for Viral Vector**

### **1.2.1 Feline Herpes Virus and Species Specificity**

In the development of a contraceptive vaccine, safety must be a primary concern. It is critical that such a vaccine only affect cats and poses no significant health risk to cats receiving treatment. Development of a contraceptive vaccine, then, should be non-detrimental and species-specific for felids.

In recent years, a number of viruses have been used as vectors for molecular therapy and vaccine approaches. While many people generally associate viruses with disease, there are a number of non-pathogenic and even beneficial viruses in our environment (Csatary *et al.*, 1985; Fujiyuki *et al.*, 2004; Lederberg, 1957, 1997; Mi *et al.*, 2000; Stapleton & Chaloner, 2004). Furthermore, because many viruses possess unique methods of transmission, cell entry, and expression in host cells, some viruses previously only associated with disease can now be genetically manipulated for beneficial purposes. In this sense, viruses can be thought of as tools, possessing characteristics that may be useful in achieving valuable goals.

A number of viruses such as vaccinia virus, vesicular stomatitisvirus, adenovirus, and some lentiviruses, have already been used as vectors for various gene therapy attempts (Hermonat & Muzyczka, 1984; Mackett *et al.*, 1982; Schnell *et al.*, 1996; Tabin *et al.*, 1982). These viruses often are not species-specific, but rather are used for transmission of genetic information to a wide variety of cell types. Consequently, a contraceptive viral vaccine not designed to be species-specific, would potentially raise safety and ecological issues for non-target mammalian vertebrates. For the purposes of a species-specific contraceptive vaccine, a virus with a more restrictive host range is desirable. By choosing a virus with a narrow host range, the possibility of unintentionally affecting non-target animals is minimized.

One group of viruses that has recently been explored as potential vectors is the *Herpesviridae* family (Kit *et al.*, 1985; Lowe *et al.*, 1987; Meignier & Roizman, 1989; Thomsen *et al.*, 1987; Whealy *et al.*, 1988). Viruses in the *Herpesviridae* family have evolved closely with their host species over time, resulting in a range of viruses that only productively infect their specific host species (Huemer *et al.*, 1993). Cytomegalovirus (CMV), a member of the *Betaherpesvirinae* subfamily, has been used for species-specific transmission of foreign antigens (Lloyd *et al.*, 2003; Redwood *et al.*, 2005; Smith *et al.*, 2005) and more specifically, for immunocontraceptive control of mouse populations in Australia (Smith *et al.*, 2005). While CMV can gain entrance into the cells of non-host species, transcriptional blocks and the inability to evade non-host immune systems prevents the virus from progressing to productive infection. However, it may be possible for protein expression to induce antibody responses in non-host species (Smith *et al.*, 2005).

In a study conducted by Smith *et al.* (2005), mouse CMV (mCMV) containing sequence encoding for mouse zona pellucida-3 (mZP3), administered to rats resulted in the production of antibodies against both mCMV and mZP3. However, these antibodies were not cross-reactive with the endogenous rat ZP3, which shows some degree of species-specificity at the level of the ZP glycoprotein, but not necessarily at the level of viral entry or protein expression. The contraceptive vaccine, designed to be effective in

mice, was shown to be non-effective in non-target animals, rats, despite the antibodies produced against mZP3. This study demonstrates the need for not only multiple levels of safety (i.e. restrictive viral vector and highly specific antibodies) in a contraceptive vaccine, but also for appropriate follow-up studies to confirm species-specificity of a developed vaccine.

Feline Herpesvirus (FHV), also referred to as feline rhinotracheitis virus (FRV), is a member of the *Alphaherpesvirinae* subfamily and was first isolated by Crandell and Maurer in 1957 (Crandell & Maurer, 1958). Having no known non-felid reservoir or host, FHV-1 is considered to be species specific. Because there is no evidence for *in-utero* transmission, it is believed that FHV-1 can only be spread horizontally from cat to cat as mediated by the virulence genes, such as glycoproteins I and E (R. Gaskell & Willoughby, 1999; R. M. Gaskell & Povey, 1982; Mijnes & de Groot, 1999; Mijnes *et al.*, 1999; Povey, 1979; M.J. Willemse *et al.*, 1996a).

An attenuated strain of FHV-1 was later developed for administration to felines for the prevention and/or reduction of feline viral rhinotracheitis disease symptoms and has been in public use since the late 1970s (Scott, 1977). This attenuated vaccine strain of FHV has been shown to produce few side effects when administered orally to cats, with somewhat greater side effects seen when administered nasally. Side effects from nasal administration include runny nose, ulcers, and minor respiratory problems. For this reason, oral administration of commercially available vaccines is typically suggested. Administration of the FHV vaccine strain in cats with prior exposure to FHV does not prevent disease, but diminishes frequency and severity of symptoms in infected cats. For a detailed description of the benefits and limitations of FHV-1 vaccines, see the 1999 review by Gaskell *et al.* (R. Gaskell & Willoughby, 1999). Other attenuated FHV strains, with deletions in critical glycoproteins to reduce side effects of vaccination, are also being commercially developed for use as improved vaccines strains (Kruger *et al.*, 1996; Sussman *et al.*, 1997; Sussman *et al.*, 1995; USDA, 2004).

Like CMV, FHV-1 has also been used in a number of immunization studies. FHV-1 has been used to deliver foreign antigens such as feline immunosuppressive virus (FIV) and the feline leukemia virus (FeLV) *env* and *gag* proteins in an effort to vaccinate felines against wild type FIV (Murphy *et al.*, 2000; Sato *et al.*, 2001; Sato *et al.*, 2000) and FeLV (Cole *et al.*, 1990; Wardley *et al.*, 1992). In 2004, the USDA (United States Department of Agriculture) approved a modified strain of FHV-1, developed by Pfizer, with additional gene deletions and expressing FIV antigens, for field-testing, to prevent disease caused by wild type FHV and FIV (USDA, 2004). FHV based vaccines have also been developed against feline calicivirus (Yokoyama *et al.*, 1998). And finally, researchers are exploring the possibility of constructing an immunocontraceptive vaccine, using FHV, and targeting important reproductive proteins, such as specific regions of the zona pellucida. Throughout these studies, the safety of using FHV-1 as the basis for vaccines has been repeatedly tested and, thus far, it has stood the test successfully, having only mild clinical side effects (Kruger *et al.*, 1996). FHV vaccines have been tested in non-feline animals, such as minks, guinea pigs, dogs, canaries, chickens, and mice, to assess environmental safety issues as well. The Purevax RCP vaccine, produced by Merial (Lyons, France), is a commercially available FHV based vaccine designed to protect felines against FHV and calicivirus. The vaccine was tested in cats, minks, guinea pigs, dogs, canaries, chickens, and mice and found safe prior to commercial release.

### **1.2.2 Role of gD, gI, gE (Rationale for Deletion of gI)**

Human herpesvirus-1 (HSV-1) has served as the model for the study of other animal herpesviruses. The glycoproteins in these animal herpesviruses have been given appropriate names corresponding to their HSV-1 counterparts (Roizman & Sears, 1993). Seven of the 11 HSV-1 glycoproteins are known to have counterparts in FHV-1 (See Table 1) (Spatz & Maes, 1993; Spatz *et al.*, 1994).

The roles of the various FHV-1 glycoproteins (Table 1) have been described as well as the consequences of deletion and/or replacement of some of the glycoprotein

genes with foreign genes (Burgener & Maes, 1988; Dingwell *et al.*, 1994; Dingwell *et al.*, 1995; Dingwell & Johnson, 1998; Fawl & Roizman, 1994; Fitzpatrick & Bielefeldt-Ohmann, 1991; R. Gaskell & Willoughby, 1999; Limcumpao *et al.*, 1991; Maeda *et al.*, 1998; Wisner *et al.*, 2000). While glycoprotein D (gD) is required for virion formation, attachment, and penetration, glycoprotein I (gI) and glycoprotein E (gE) are involved in cell-to-cell transmission across cell junctions and are considered non-essential genes for *in vitro* growth (Dingwell *et al.*, 1994; Dingwell, Doering, & Johnson, 1995; Dingwell & Johnson, 1998; Kruger, Sussman, & Maes, 1996; Mijnes & de Groot, 1999; Mijnes *et al.*, 1998; Roizman & Sears, 1993; Spatz, Rota, & Maes, 1994; Sussman, Maes, Kruger, Spatz, & Venta, 1995; Wisner, Brunetti, Dingwell, & Johnson, 2000). The gI and gE genes of FHV-1 encode 370- and 532- amino-acid proteins, respectively (Spatz *et al.*, 1994). Together, gI and gE form a complex responsible for migration of the virus into higher order neurons from the original site of infection (Enquist *et al.*, 1994; Mulder *et al.*, 1994). Portions of gI and/or gE have been deleted, resulting in further attenuation of the strain and, specifically, the generation of additional possible vaccine strains (Sussman *et al.*, 1997; Sussman *et al.*, 1995).

Deletion of cytoplasmic portions of gI results in the formation of plaques that are 15-35% smaller than wildtype FHV-1 in cell culture. The region of gI encoding for the N-terminal portion, particularly the first 95 residues, of the glycoprotein is the most critical portion when determining effects of deletion and decrease in virulence. It is within this region that critical disulfide bonds are formed with gE, which are necessary for proper posttranslational modification and transport in the wildtype virus (Mijnes & de Groot, 1999; Mijnes *et al.*, 1998; Mijnes *et al.*, 1997). Deletion of gI and/or gE prevents formation of this complex, resulting in diminished cell-to-cell spread. Kruger *et al.* developed an attenuated vaccine strain, in which portions of gI and gE were deleted, which was safe (presented no significant negative clinical symptoms while maintaining immunogenicity) to deliver to cats oronasally (Kruger *et al.*, 1996).

| <b>Glycoprotein</b> | <b>Proposed Function</b>  |
|---------------------|---|
| gB                  | Essential, virus-cell membrane fusion, adsorption, and penetration (Maeda <i>et al.</i> , 1992; Spatz & Maes, 1993)                             |
| gC                  | Non-essential, membrane glycoprotein, heparin binding (Maeda <i>et al.</i> , 1997a; Maeda <i>et al.</i> , 1997b)                                |
| gD                  | Essential, hemagglutinating protein, penetration, cell-to-cell spread (Maeda <i>et al.</i> , 1995; Maeda <i>et al.</i> , 1994)                  |
| gE                  | Non-essential, virulence gene, cell-to-cell transmission, late gene, binds to gI (Mijnes & de Groot, 1999)                                      |
| gG                  | Non-essential, entry, egress, immunologically important (Spatz <i>et al.</i> , 1994)  |
| gH                  | Essential, virus-cell membrane fusion and penetration (Maeda <i>et al.</i> , 1993)  |
| gI                  | Non-essential, virulence gene, cell-to-cell transmission, late gene, binds to gE (Mijnes & de Groot, 1999; M.J. Willemse <i>et al.</i> , 1996a) |

**Table 1:** Functions of FHV-1 Glycoproteins

In addition to these genes, FHV also contains a thymidine kinase (TK) gene that is a potential site for insertion of foreign genes as it is in other viruses. The protein encoded by the TK gene is responsible for phosphorylating thymidine for incorporation into DNA. It has been shown to be non-essential and has successfully been replaced in a number of viruses with genes encoding various foreign antigens. Some of these viruses include: FHV (Cole *et al.*, 1990; Sato *et al.*, 1998), vaccinia (Chakrabarti *et al.*, 1985),

and varicella zoster virus (VZV) (Lowe *et al.*, 1987). Currently, FHV vaccines using the TK gene as a site of insertion may have limited efficacy, requiring a number of boosters to reach protective antibody titers (Wardley *et al.*, 1992). This may be remedied in future efforts by increasing the immunogenicity of the inserted vaccinating cassette.

Alternatively, FHV gI, a non-essential gene, has also been suggested as a potential site of insertion for heterologous sequences. Furthermore, deletions in portions of gI result in decreased virulence making the potential viral vaccine safer in terms of the possibility of inadvertently inducing disease symptoms (Sussman *et al.*, 1995; M. J. Willemse *et al.*, 1996b).

Murata *et al.* (1999) compared the efficacy of the TK promoter of bovine herpesvirus type 1, another alphaherpesvirus, to the gB and gC promoters. Their findings suggest that late viral genes, such as gB, gC, gD, gI and gE, express higher quantities of proteins than some of the earlier genes, such as TK (Sato *et al.*, 2000; Yokoyama *et al.*, 1998). Greater protein production could increase the efficacy of recombinant vaccines. Other studies have explored increasing protein expression through the use of a constitutive promoter from CMV in FHV vectors. However, no felid-CMV has yet been reported and non-felid promoters, such as those from human cytomegalovirus (HCMV), would necessitate further studies addressing safety before meeting approval for field-testing (Sato *et al.*, 2000; Sussman *et al.*, 1995). Lowe *et al.* have tested the endogenous gI viral promoter from VZV, for use in viral vaccines (1987). In their study, the VZV gI promoter plus a leader sequence was fused with an Epstein-Barr Virus (EBV) protein and expressed in cell culture (Lowe *et al.*, 1987). Based on the findings of these studies, the endogenous FHV gI promoter was chosen to express a contraceptive antigen for this thesis work.

Gene regulation in alphaherpesviruses is tightly controlled and occurs in a cascade fashion. Genes are loosely divided into three groups ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) based on timing of expression and corresponding roughly to early, intermediate, and late, although expression in alphaherpesviruses occurs more in a continuous fashion rather than in

specific stages (Costa *et al.*, 1981; Jones & Roizman, 1979). gI and gE are  $\gamma$  genes, expressed late in the virus life cycle (approximately 10-20 hours post-infection) (Fields *et al.*, 1996). Expression of  $\gamma$  genes depends on *cis*- and *trans*-acting factors as well as viral DNA synthesis. For this reason, it is suggested that  $\gamma$  genes be studied in the presence of a productive viral infection (Fields *et al.*, 1996). The current study uses gI plus a leader sequence, which is thought to contain the necessary *cis*-acting element, fused to our target sequence.

### **1.3 Rationale for Target Protein: GnRH**

Thus far, immunocontraception has been used to target gamete production by limiting requisite hormones (GnRH, FSH, and LH), inhibit gamete function (sperm and egg proteins), and interrupt maintenance of pregnancy (hCG). Currently much of the research being done for humans is focusing on gamete function by targeting gamete glycoproteins: either eppin on the sperm or zona pellucida on the egg (Gupta *et al.*, 2004; O'Rand M *et al.*, 2004; Sivapurapu *et al.*, 2005). It is rationalized that by targeting the human gametes, the reproductive hormones responsible for sexual development and drive will be unaffected. For animals, on the other hand, much of the recent focus has been on targeting the reproductive hormones responsible not only for gamete production, but also for sexual behavior.

Gonadotropin-releasing hormone (GnRH), a decapeptide, is produced and excreted into the blood by the medial basal hypothalamus. In the anterior pituitary, GnRH stimulates the release of gonadotropins, such as luteinizing hormone (LH) and follicle stimulating hormone (FSH). These hormones in turn support the growth, maintenance, and functioning of the vertebrate reproductive system. High frequency pulses of GnRH into the bloodstream stimulate the release of LH, while low frequency pulses stimulate the release of FSH. LH, in turn, is responsible for the production of testosterone and estrogen in males and females. Likewise, FSH supports maturation of ovarian follicles in females and sperm from Sertoli cells in males. Each of these hormones serves as a potential point of interference for contraceptive approaches (Bowen, 1998).

Researchers are exploring the possibility of targeting each of these hormones and their receptors with agonists and antagonists. For instance, in humans, agonists have been particularly useful in the treatment of sex hormone induced cancers (Dondi *et al.*, 2006; Parkinson *et al.*, 2004). However, the body is also capable of interfering with these hormones through the action of specific antibodies. When antibodies are induced against GnRH in cats, for instance, testosterone levels drop and the reproductive system ceases to function productively (Baker *et al.*, 2004; Bertschinger *et al.*, 2001; Bertschinger *et al.*, 2002; Levy *et al.*, 2004). This decrease in hormone and consequent loss of fertility is what is referred to as “immunocontraception”. The success of immunocontraceptive studies is typically measured by production of neutralizing antibody titers and/or suppression of reproductive ability. As antibody titers fall over time, in the absence of boosters, the contraceptive effect may fade. GnRH vaccines, agonists, and antagonists developed for contraceptive use thus far appear to be reversible once boosters are discontinued. However, it is unclear what happens with long-term use and/or use in some prepubescent animals. The reversibility of such contraceptive vaccines depends largely on the intensity and duration of antibody response to the vaccine. The reversibility of GnRH vaccines for mares has been reviewed by Stout and Colenbrander (2004). For recent reviews on advances in immunocontraception, see Thompson (2000); Delves (2004); Naz and Rajesh (2004); and Naz, *et al.*, (2005).

Immunocontraception has been available as a non-surgical alternative for unwanted fertility in vertebrate species for a number of years now (Delves, 2004; Ferro & Mordini, 2004; Suri, 2004, 2005). Henry Baker and Bruce Smith at Auburn University conducted studies with a 14-mer repeat of GnRH crosslinked to leukotoxin for the immunization of cats (Baker *et al.*, 2004). In these experiments, the polypeptide coupled to leukotoxin as an adjuvant was injected directly into cats. Cats with significant GnRH antibody titers had no measurable sperm production, diminished testicular size, and consequently were not able to reproduce (Baker *et al.*, 2004; Levy *et al.*, 2004).

Likewise, a number of other investigators have tested GnRH and other reproductive protein targets coupled to various adjuvants, for the purposes of inducing an immune response. When measured by antibody production against GnRH, the availability of viable sperm and eggs or their interaction was limited and thus triggered infertility (Ferro *et al.*, 2004a; Ferro *et al.*, 2004b; Ferro & Mordini, 2004; Levy *et al.*, 2004). These studies have helped to confirm GnRH as a favorable target protein for immunocontraception. GnRH vaccines have been designed for use in rats (Miller *et al.*, 1997), pigs (Meloan *et al.*, 1994; Oonk *et al.*, 1998), rodents (Delves & Roitt, 2005), white-tailed deer (Miller *et al.*, 2000), fish (Delves, 2004; Delves & Roitt, 2005), horses (Stout & Colenbrander, 2004), bison (L. A. Miller *et al.*, 2004), cats (Baker *et al.*, 2004; Levy *et al.*, 2004; Robbins *et al.*, 2004), mice (Ferro *et al.*, 2004a; Hannesdottir *et al.*, 2004), dogs (Ferro & Mordini, 2004), rams (Ferro & Mordini, 2004; Janett *et al.*, 2003), rhesus monkeys (Siler-Khodr *et al.*, 2004), and humans (Delves, 2004; Jinshu *et al.*, 2005).

GnRH is highly conserved across vertebrate-species with antibodies cross-reacting across species lines. Thus far, fifteen types of GnRH have been identified. (Table 2) The physiological roles of GnRH-II and GnRH-III have not been determined. Mammalian GnRH-I has been successfully used in various immunocontraceptive studies. However, GnRH alone has limited immunogenicity because of its small size (~1200 Da). For this reason, most studies have employed the use of various adjuvants such as oil, CoVaccine, Carbopol, keyhole limpet hemocyanin (KLH), leukotoxin, *Mycobacterium tuberculosis* hsp70, Ribi, incomplete Freund's adjuvant (IFA), non-ionic surfactant vesicles (NISV), aluminium hydroxide, Quil A, poly(lactide-co-glycolide)/triacetin (PLGA), and Quil A/PLGA. While NISV appear to be non-toxic, many of these adjuvants are known to cause side effects or have toxic effects negatively impacting the health of the animals (Ferro & Stimson, 1996).

| GnRH Source          | Sequence                              | Known Species Cross-Reactivity/Notes  |
|----------------------|---------------------------------------|---|
| GnRH-I: Mammalian    | EHWSYGLRPG                            | This is the synthesized repeating sequence we are using. Rats, Mice, Sheep (non-permanent), Dogs (minor, non-permanent) (Ferro et al., 2004b) |
| GnRH-I: Guinea Pig   | E <u>Y</u> WSYGV <u>R</u> PG          |   |
| GnRH-I: Chicken      | EHWSYGL <u>Q</u> PG                   |   |
| GnRH-II: Chicken     | EHWS <u>H</u> G <u>W</u> Y <u>P</u> G | Implantation, embryo regulation, kidney maintenance   |
| GnRH-III: Salmon     | EHWSYGW <u>L</u> PG                   | Salmon, Bovine, Human   |
| GnRH: Seabream       | EHWSYGL <u>S</u> PG                   |   |
| GnRH: Madaka/Pejerry | EHWS <u>F</u> GL <u>S</u> PG          |   |
| GnRH: Herring        | EHWS <u>H</u> GL <u>S</u> PG          |   |
| GnRH: Catfish        | EHWS <u>H</u> GL <u>N</u> PG          |   |
| GnRH: Dogfish        | EHWS <u>H</u> G <u>W</u> LPG          |   |
| GnRH-I: Tunicate     | EHWS <u>D</u> Y <u>F</u> KPG          |   |
| GnRH-III: Tunicate   | EHWS <u>L</u> CH <u>A</u> PG          |   |
| GnRH: Frog           | EHWSYGL <u>W</u> PG                   |   |
| GnRH-I: Lamprey      | EH <u>Y</u> S <u>L</u> E <u>W</u> KPG |   |
| GnRH-III: Lamprey    | EHWS <u>H</u> D <u>W</u> KPG          |   |

**Table 2:** GnRH Isoforms – Bold underlined letters indicate variations in amino acid sequence from mammalian GnRH-I (Grove-Strawser *et al.*, 2002).

In addition to adjuvants, conjugates have also been explored, such as ovalbumin and tetanus toxoid. Ferro *et al.*(2002) investigated the difference between attaching conjugates to the N-terminal end versus the C-terminal end of GnRH. Both products induced significant levels of antibody production, however functional antibodies, as measured by a decrease in GnRH, testosterone, and testicular size, were only produced when the C-terminal end was unaltered. These results may explain some of the less effective immunocontraceptive efforts attempted so far. Future efforts at development of

contraceptive vaccines should take these results into consideration. For this reason, we have chosen to attach the non-GnRH components of our vaccine to the N-terminal end of the GnRH peptide sequence. For a comparison of adjuvants and conjugates, please refer to Ferro *et al.* (1998).

Another means of avoiding the use of chemical adjuvants and conjugates while increasing the immunogenicity of a peptide is by creating a repeating polymer of peptide repeats. Baker *et al.* found that 8 or greater repeats of the GnRH decapeptide, crosslinked to keyhole lymphocyte hemocyanin, were sufficient to stimulate a contraceptive immune response whereas fewer repeats were not effective (2004).

Likewise, Cuadros *et al.* (2004) found that coupling a non-immunogenic peptide sequence, such as Enhanced Green Fluorescent Protein (EGFP), to the highly immunogenic *Salmonella* flagellin (*fliC*) sequence enabled murine antigen presenting cells to produce anti-EGFP antibodies. Hsu *et al.* (2000) created a fusion protein containing *Pseudomonas* exotoxin A followed by a 12-GnRH repeat for use in rabbits. The high titers of GnRH antibodies produced in rabbits resulted in degeneration of the ovaries. The approach for the current thesis research has been to create a fusion protein encoding for FliC::EGFP::GnRH in which the GnRH portion of the sequence contains 14 repeats of the decapeptide. We expect that, when expressed in felines, the immunogenic qualities of FliC and the 14 GnRH repeats will, together, stimulate a strong antibody response against GnRH resulting in lower GnRH titers and subsequent downstream reproductive hormones, and ultimately in a contraceptive effect.

No studies to date have been found that explore the expression of GnRH in a herpesvirus. Thus far, studies exploring GnRH as a target for immunocontraception have focused on the injection of protein rather than expression in bacteria or viruses (Baker, Griffin, Smith, Braden, & Robbins, 2004; Baker, Griffin, & Smith, 2002; Delves, 2002, 2004; Delves, Lund, & Roitt, 2002a, 2002b; Delves & Roitt, 2005; Ferro, Costa *et al.*, 2004; Ferro *et al.*, 2001; Ferro, Khan *et al.*, 2004; Levy *et al.*, 2004; Robbins, Jelinski, & Stotish, 2004). However, protein expression in a vector that can be administered orally

and/or nasally has the benefit of not requiring protein purification or injection of the product. We expect that the vaccine strain of feline herpesvirus, which is currently used for vaccination against FHV, will provide a useful and safe vehicle for expression of the nucleic acid encoding our fusion protein.

#### **1.4 Rationale for Immunogen: Flagellin**

Toll receptors were first identified in *Drosophila* (Stein *et al.*, 1991). They were shown to be important for anti-fungal and anti-microbial defense mechanisms (Lemaitre *et al.*, 1996). Subsequently, mammalian counterparts of *Drosophila* Toll receptors were found and referred to as Toll-Like Receptors (TLR). Ten mammalian TLR have been identified, responsible for recognizing various pathogen associated molecular patterns (PAMP) (Akira *et al.*, 2001; Zarembler & Godowski, 2002).

While TLR are specifically part of the innate immune system, they link the innate and adaptive immune responses through stimulatory mechanisms. TLR are responsible for recognizing a number of PAMP. As the name suggests, PAMP represent portions of pathogens that are not recognized as ‘self’ by the host system. When PAMP bind to the TLR on antigen presenting cells (APC), host defense pathways are triggered to combat the perceived infection (Medzhitov & Janeway Jr., 1997). TLR stimulation leads to the induction of reactive oxygen and nitrogen intermediates (ROI and RNI, respectively) as part of the innate adaptive system response.

At the same time, the adaptive immune system is activated through the production of pro-inflammatory cytokines and the recruitment of T-helper cells by dendritic cells (DC). When TLR on DC bind PAMP, the DC produce chemokines in secondary lymphoid organs to recruit natural killer cells and T-cells (McSorley *et al.*, 2002). In general, TLR stimulated DC result in a Th1 immune response, however, TLR4 has been shown to be involved with, and may be required, in the stimulation of a Th2 immune response (Dabbagh *et al.*, 2002).

Flagellin (FliC) produced by *Salmonella enteritidis* is specifically recognized by TLR5 on DC (Hayashi *et al.*, 2001). Through TLR5, FliC stimulates myeloid differentiation factor 88 (MyD88) and NFκB, which in turn result in production of tumor necrosis factor alpha (TNFα), IL-12, RNI, and maturation of DC (Eaves-Pyles *et al.*, 2001; Gewirtz *et al.*, 2001; Hayashi *et al.*, 2001). Although the mechanism for maturation of the DC in response to FliC is not yet clear, it has been determined that while TNFα promotes maturation of DC, it is not necessary (Honko *et al.*, 2006). Mature DC migrate to draining lymphatic tissue where they display increased levels of major histocompatibility complex (MHC), which in turn helps to prime naïve T cells. Because of the ability of FliC to stimulate a pro-inflammatory response and induce maturation of naïve T cells, resulting in production of an antibody response, FliC is considered a functional adjuvant (Cuadros *et al.*, 2004).

Cuadros *et al.* (2004) have created a FliC::EGFP fusion protein for use as proof of concept that FliC can be used to mount an immune response against a weakly antigenic protein, EGFP. Mice inoculated with the FliC::EGFP fusion protein developed a pro-inflammatory response, EGFP specific cytotoxic CD8<sup>+</sup> T cells, and EGFP specific CD4<sup>+</sup> T cells. This data supports the notion that FliC, combined as a fusion protein, could serve to enhance vaccines. In this regard, it is important to consider a possible anti-FliC response that would hinder future vaccination efforts. An anti-FliC response could be expected in the case of prior exposure to flagellin either through natural means (natural flora or infection) or through prior vaccinations that include flagellin as a component. However, studies by both Ben-Yedidia and Arnon (1998) and Cuadros *et al.*, (2004) suggest that little to no FliC specific antibodies are produced on vaccination with FliC. They propose that this may be the result of the body's natural tolerance to FliC stemming from the presence of flagellin containing bacteria in the natural flora. Whatever may be determined as the reason for the lack of production of FliC specific antibodies, lack of such a response can be used to our advantage.

Because of its small size (1200 Da), GnRH is not highly antigenic. One to four copies in tandem of the GnRH decapeptide injected alone is not enough to stimulate an

adequate antibody response for immunocontraception without an adjuvant. While 5-8 copies are enough to trigger a small response, more than 8 repeats are typically needed (B. Smith, Auburn University, personal communication). The current project involves the expression of a 14-mer GnRH multimer repeat as one method to address the concern of immunogenicity. Additionally, we have chosen to fuse the GnRH polypeptide with FliC in order to ensure a strong adaptive immune response (Cuadros et al., 2004; A. N. Honko & Mizel, 2004). Although FliC expressed on *Salmonella* elicits a Th1 response, soluble FliC has been shown to direct a Th2 response (Cunningham *et al.*, 2004; Honko et al., 2006). This research will be the first known study expressing FliC as an adjuvant in a herpesvirus vector.

### **1.5 Rationale for Visualization: EGFP**

Cuadros *et al.* (2004) demonstrated that a FliC::EGFP fusion protein induced an immune response against the weakly antigenic EGFP. This observation suggests that additional antigenic epitopes may also be beneficial in the generation of recombinant vaccines (Cuadros et al., 2004). The combination of FliC and EGFP together as a fusion product has the added benefit of conferring fluorescence based visibility of cells expressing the fusion protein. The current thesis builds upon this concept, introducing a GnRH repeat as the added epitope (FliC::EGFP::GnRH). We expect that the presence of EGFP may stimulate an anti-EGFP immune response in addition to the anti-GnRH response, but do not expect this to pose any risk to animals. Future studies may be conducted which may exclude the EGFP portion of the fusion for comparison.

EGFP (BD Biosciences; San Jose, CA) is an improved version of the original green fluorescent protein (GFP). Proteins expressing GFP fluoresce green when exposed to ultraviolet light. In a similar manner, EGFP fluoresces green when exposed to visible light. EGFP has been shifted slightly towards the red end of the spectrum (excitation maximum 488 nm; emission maximum 509 nm) to facilitate visualization using fluorescent microscopes. EGFP has been optimized for brighter fluorescence and greater expression in mammalian cells. Additionally, the codon usage of our synthetic gene

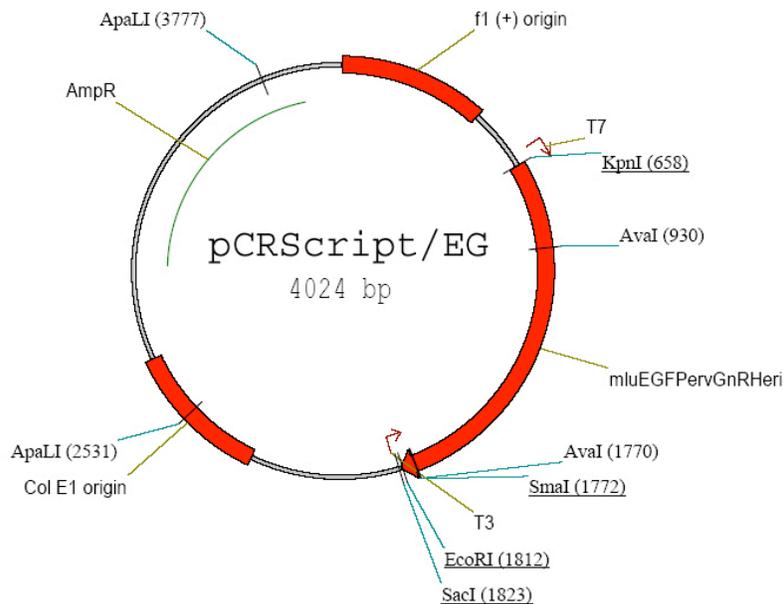
encoding the FliC::EGFP::GnRH fusion has been further optimized for expression in feline cells.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Generation of Clones

#### 2.1.1 pCRScript/EGFP/GnRH

Drs. Henry Baker and Bruce Smith, of Auburn University (Auburn, AL), supplied plasmid p5.1.39 containing a 14.5 X repeat of mammalian GnRH-I based on rabbit. The sequence data for EGFP was derived from pEGFP-C1 (BD Biosciences; San Jose, CA). Using the sequence data provided, an EGFP::GnRH fusion construct was designed. The EGFP portion of the construct corresponds to basepairs 616-1407 of pEGFP-C1. The GnRH portion of the fusion construct encodes for 14 repeats of mammalian GnRH (EHWSYGLRPG) followed by two consecutive stop codons. The EGFP::GnRH coding sequence was optimized for feline codon usage and commercially synthesized by GeneArt (Regensburg, Germany). The EGFP::GnRH fusion gene was designed with an *EcoRV* site separating the two genes and an *EcoRI* site following the stop codons. The construct was delivered as pCRScript/EGFP/GnRH. (Fig. 1)



**Figure 1:** pCRScript/EG: Delivery vector containing synthesized EGFP/GnRH sequence.

### 2.1.2 pRSetA/*fliC*/EGFP/GnRH (pRSetA/FEG)

The pET29a/*fliC* plasmid containing the flagellin gene (*fliC*) was graciously provided by Dr. S. Mizel at Wake Forest University (Winston-Salem, NC). The *fliC* gene was amplified by polymerase chain reaction (PCR) using modified primers to introduce 5' *Xho*I and 3' *Bam*HI sites. Primers were designated *fliC*\_For\_*Xho*I and *fliC*\_Rev\_*Bam*HI (*fliC*\_For\_*Xho*I: GGGCTCGAGGCACAAGTCATTA; *fliC*\_Rev\_*Bam*HI: GGGGGATCCACGCAGTAAAGAGAG). PCR was set up in an Eppendorf Mastercycler Gradient Thermal Cycler using Platinum PCR Supermix (Invitrogen; Carlsbad, CA), pET29a/*fliC* as template, and primers (10 pmol) in a 50  $\mu$ l total volume. PCR parameters were: 95°C for 2 minutes; 40 cycles of 95°C for 1 minute, 59.5°C for 30 seconds, and 72°C for 2 minutes; 72°C for 10 minutes; and a 4°C hold. These primers amplified a 1.5 kb fragment, designated *fliC* (XB), which was gel purified with the QIAquick Gel Extraction Kit from Qiagen (Valencia, CA) using the standard protocol described in the user manual.

The EGFP::GnRH fusion gene was amplified with PCR from, pCRScript/EGFP/GnRH, using modified primers to introduce a 5' *Bam*HI and a 3' *Pst*I site. Primers were designated EG\_For\_*Bam*HI and EG\_Rev\_*Pst*I (EG\_For\_*Bam*HI: AAAGGATCCACGCGTGTGTCCAAGGGCGAG; EG\_Rev\_*Pst*I: GGGCTGCAGGAATTCTCATCAGCCGGGTCT). PCR was set up in an Eppendorf Mastercycler Gradient Thermal Cycler using Platinum PCR Supermix (Invitrogen; Carlsbad, CA), pCRScript/EG template, and primers (10 pmol) in a 50  $\mu$ l total volume. PCR parameters were the same as those used to amplify *fliC*. These primers amplified a 1.2 kb fragment, designated EG (BP) which was gel purified with the QIAquick Gel Extraction Kit from Qiagen (Valencia, CA) using the standard protocol described in the user manual.

pRSetA (Invitrogen; Carlsbad, CA) was double digested with *Xho*I and *Pst*I (Promega, Madison, WI) and the 2.9 kb fragment was gel purified with the QIAquick Gel Extraction Kit from Qiagen (Valencia, CA) using the standard protocol described in the

user manual. The eluted digested plasmid was subsequently treated with Shrimp Alkaline Phosphatase at 37°C overnight to prevent recircularization of any incompletely digested plasmid.

The *fliC* (XB) and EG (BP) fragments were likewise double digested with *XhoI* and *BamHI* or *BamHI* and *PstI* (Promega, Madison, WI), respectively, and gel purified as described above. pRSetA (XP), *fliC* (XB), and EG (BP) were set up in a tripartite ligation reaction, in a 1:2:2 ratio, at room temperature for 6 hours. Subsequently, 1µl of the ligation reaction was electroporated into *Escherichia coli*. After recovery, cells were plated onto LB-Amp plates and grown overnight at 37°C; 30-40 colonies grew on the plates and 20 of these colonies were transferred to a new Luria Broth (LB) patch plate containing 100µg/µl ampicillin (Amp) patch plate for screening.

Potential positive clones were identified by a quick screen procedure as having inserts of various sizes. Quick screen consisted of transferring a monoclonal patch of cells to phenol:chloroform and vortexing for 1 minute. Samples were then centrifuged at 15,000 x g for 5 minutes. The supernatant was transferred to a fresh tube and extracted by vortexing with an equal volume of chloroform followed by centrifugation at 15,000 x g for 5 minutes. The supernatant was harvested for analysis. The plasmid extraction was then run on a gel to check for presence and comparison of size of plasmids. Clones containing plasmids of potential interest were then grown in 10ml overnight LB-Amp (100ug/ml) liquid culture for further screening. 7.5% glycerol stocks of these cultures were made using a portion of the culture and the remaining culture was used for plasmid extraction using the standard protocol for the QiaSpin Miniprep (Qiagen, Valencia, CA). DNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop; Wilmington, DE) as per manufacturer's protocol.

Plasmid preparations from various pRSetA/FEG clones were analyzed by a series of restriction enzyme digestions using *XhoI* – *PstI* (double), *XhoI* – *HindIII* (double), and *BamHI* (Promega, Madison, WI). Clones pRSetA/FEG #28 and #31 were submitted to Virginia Bioinformatics Institute (VBI, VA Tech; Blacksburg, VA) for sequence

confirmation. Glycerol stocks of all pRSetA/FEG clones were prepared with a final concentration of 7.5% glycerol and stored at  $-80^{\circ}\text{C}$ .

### 2.1.3 pRSetA/EGFP/GnRH (pRSetA/EG)

pRSetA/FEG was digested with *Bam*HI (Promega, Madison, WI) to drop out the 1.5 kb *fliC* fragment from the plasmid leaving the pRSetA/EG backbone. pRSetA/EG was gel purified and re-ligated to circularize. The resulting plasmid was transformed into *E. coli* DH5- $\alpha$  cells and confirmed by restriction enzyme digestion.

Colonies of pRSetA/EG clone #2, and pRSetA were streaked onto LB-Amp plates containing 100 $\mu$ l of 100mg/ml IPTG and 60 $\mu$ l of Bluo-gal (GibcoBRL; Carlsbad, CA) in order to induce expression of the fusion construct. Plates were grown at  $37^{\circ}\text{C}$  overnight and stored at  $4^{\circ}\text{C}$ . Colonies were visibly green after storage at  $4^{\circ}\text{C}$  for 24-48 hours.

### 2.1.4 pFHV/*fliC*/EGFP/GnRH (pFHV/FEG)

*fliC* was amplified by PCR from pET29a/*fliC* using modified primers to introduce 5' *Spe*I and 3' *Bam*HI sites. Primers were designated *fliC*\_For\_*Spe*I and *fliC*\_Rev\_*Bam*HI (*fliC*\_For\_*Spe*I: GGGACTAGTGCACAAGTCATTA; *fliC*\_Rev\_*Bam*HI: GGGGGATCCGCACAAGTCATTA). PCR was set up in an Eppendorf Mastercycler Gradient Thermal Cycler using Platinum PCR Supermix (Invitrogen, Carlsbad, CA), pET29a/*fliC* template, and primers (10 pmol) in a 50  $\mu$ l total volume. These primers amplified a 1.5 kb fragment, designated *fliC* (SB), which was gel purified with the QIAquick Gel Extraction Kit from Qiagen (Valencia, CA) using the standard protocol described in the user manual. The EGFP::*GnRH* fusion gene, EG (BP), was generated by PCR, as described above for pRSetA/FEG.

The parental plasmid pFHV- $\Delta$ gILZ was kindly provided by Drs. de Groot, Horzinek, Mijnes, and van Anken (Netherlands). This plasmid, approximately 12 kb in size, contains the feline herpes viral glycoprotein genes gD, gI, and gE on a pUC20

ampicillin resistant backbone with gI largely replaced with an IRES-βgal element (~4.1 kb). This plasmid is further described in Spatz (Spatz et al., 1994). pFHV-ΔgILZ, minus the IRES-βgal element, was PCR amplified using a 5' primer to introduce a *SpeI* site just upstream of the IRES-βgal element (pFHV\_For\_*SpeI*: GGGACTAGTTACTGGTAATGGTTGGTCGTC) and a 3' primer to introduce a *PstI* site just downstream of the IRES-βgal element (pFHV\_Rev\_*PstI*: GGGCTGCAGATACGCAAGCATATAATGGTC). The approximately 8 kb amplicon was gel purified with the QIAquick Gel Extraction Kit from Qiagen (Valencia, CA) as described in the user manual. The amplicon was then double digested with *SpeI* and *PstI* (Promega, Madison, WI) and purified again, to remove components of the digestion buffer, using the same kit and protocol.

The *fliC* (SB) amplicon was double digested with *SpeI* and *BamHI* while the EG (BP) amplicon was digested with *BamHI* and *PstI* (Promega, Madison, WI). The pFHV (SP), *fliC* (SB), and EG (BP) fragments were set up in a tripartite ligation reaction, in a 1:2:2 ratio, at room temperature for 6 hours.

Subsequently, 1 μl of the ligation reaction was electroporated into *E. coli* DH5-α. After recovery, cells were plated onto LB-Amp plates and grown overnight at 37°C. 20 colonies were transferred to a fresh LB-Amp patch plate for screening. Clones containing inserts were identified by rapid screen as described above for pRSetA/FEG. Liquid cultures were grown for plasmid extractions as described above for pRSetA/FEG. *SpeI* - *PstI* (Promega, Madison, WI) double digests were done on 10 of the clones to further analyze the size of the inserts. Glycerol stocks of all pFHV/FEG clones containing inserts were prepared with a final concentration of 7.5 % glycerol and stored at -80°C.

DNA concentration was determined using a NanoDrop Spectrophotometer. *SpeI* and *PstI* (Promega, Madison, WI) double digests were performed on possible clones to confirm the presence of the *fliC*, EGFP, and GnRH elements. Extracted plasmid DNA from clones pFHV/FEG #11 and #17 were sent to VBI for sequence confirmation.

### 2.1.5 pFHV/EGFP/GnRH (pFHV/EG)

The EGFP::GnRH fusion gene was amplified with PCR, from pCRScript/EGFP/GnRH, using modified primers to introduce 5' *SpeI* and 3' *PstI* sites. Primers were designated EG\_For\_*SpeI* and EG\_Rev\_*PstI*HI (EG\_For\_*SpeI*:AAAAC TAGTACGCGTGTGTCCAAGGGCGAG; EG\_Rev\_*PstI*:GGGCTGCAGGAATTCTCATCAGCCGGGTCT). PCR was set up to amplify a 1.2 kb fragment, EG (SP), as previously described for pRSetA/FEG. The EG (SP) fragment was gel purified with the QIAquick Gel Extraction Kit from Qiagen (Valencia, CA) using the standard protocol described in the user manual.

The pFHV backbone for this construct was amplified by PCR as described above for pFHV/FEG. The ~8 kb pFHV (SP) and EG (SP) amplicons were then double digested with *SpeI* and *PstI* (Promega, Madison, WI) and gel purified a second time, to remove components of the digestion buffer. The pFHV (SP) and EG (SP) fragments were set up in a ligation reaction, in a 1:2 ratio, at room temperature for 6 hours.

Subsequently, 1  $\mu$ l of the ligation reaction was electroporated into *E. coli* DH5- $\alpha$  cells. After recovery, cells were plated onto LB-Amp plates and grown overnight at 37°C. Clones #1-3 were grown as liquid cultures and plasmid was extracted as described above. Glycerol stocks of pFHV/EG #2 was prepared with a final concentration of 7.5 % glycerol and stored at -80°C.

### 2.1.6 Sequencing of Constructs: pRSetA/FEG, pRSetA/EG, pFHV/FEG, and pFHV/EG

Primers were synthesized commercially by Sigma-Genosys (Woodlands, TX) to sequence clones for confirmation of inserts. (See Table 3.)

| Primer Name      | Primer Sequence        |
|------------------|------------------------|
| pFHVgDFor        | CATACAACCTCACCATCCTTA  |
| T7 For           | AATACGACTCACTATA       |
| <i>fliC</i> 400  | GACAACCAGATGAAAATCCAGG |
| <i>fliC</i> 1000 | TACATCTGTAGTGAACGGTCAG |
| EG40             | CCTGGTCGAGCTGGACGGC    |
| EG600            | AACCACTACCTGAGCACCCAGT |
| EG1000           | CATTGGTCATATGGACTACGG  |
| pRSetRev         | CTAGTTATTGCTCAGCGGTGG  |
| pFHVgERev        | GGTAACAAGCAGTCCCAT     |

**Table 3:** Primers used for sequence confirmation of clones.

Clones pRSetA/FEG #28 and 31 and pFHV/FEG #11 and 17 were sequenced at the VBI core facility to confirm the sequence of the *fliC*, EGFP, and GnRH elements. Likewise, clones pRSetA/EG#2 and pFHV/EG#2 were sequenced at the VBI core facility to confirm the sequence of the EGFP and GnRH elements.

### 2.1.7 BL-21 Stocks: pRSetA/FEG, pRSetA/EG, pFHV/FEG, and pFHV/EG

*E. coli* colonies containing pRSetA/FEG (#28 and 31), and pRSetA were later restreaked from frozen glycerol stocks onto LB-Amp plates containing 100µl of 100mg/ml IPTG and 60µl of Bluo-gal (GibcoBRL, Carlsbad, CA) in order to induce expression of the fusion construct. Plates were grown at 37°C overnight and stored at 4°C. Colonies were visibly green after storage at 4°C for 24-48 hours.

Chemically competent *E. coli* BL-21 cells were obtained from Stratagene (La Jolla, CA). 1µl each of plasmids pRSetA/FEG clones (#21, 26, 28, 29, 31, 36, and 37), pFHV/FEG clones (#11, 12, 13, and 17), or pRSetA was mixed with 5µl of competent *E. coli* cells. Cells were incubated on ice for 30 minutes followed by a 30 second heat shock at 42°C and a 2 minutes recovery on ice. Cells were then shaken at 220 rpm at 37°C for 1 hour. The entire transformation was plated out onto LB-Amp (100ug/ml) plates and

incubated overnight at 37°C. Glycerol stocks of all clones were prepared using a final concentration of 7.5% glycerol and stored at -80°C.

## **2.2 Verification of Protein Expression**

### **2.2.1 Western Blot: GnRH, FliC, and EGFP Expression from pRSetA/FEG #31**

5 ml of LB-Amp was inoculated with pRSetA or pRSetA/FEG #31 and grown overnight shaking at 37°C and 4 ml of this culture was used to inoculate 96 ml of LB-Amp. Once the large cultures reached  $OD_{600} = 40$  Klett units (KU), the cultures were incubated on ice for 10 minutes to halt growth. 3 ml of culture was removed in 1 ml aliquots for analysis. The remaining culture was induced to express the fusion protein with a final concentration of 1mM IPTG. The culture was allowed to continue to grow shaking at 37°C and three 1 ml aliquots were removed every hour for 4 hours. The density of the culture was noted at each collection point. Once removed from the large culture, the aliquots were immediately centrifuged at 10,000 x g for 2 minutes to pellet cells, supernatant was discarded and the remaining pellet frozen at -20°C for later analysis.

The frozen cell pellets taken at 0 to 4 hours were subsequently resuspended by vortexing in 100 µl of Tris-HCl, pH 8.0 and 100 µl of Sample Buffer, 2 x Laemli (Sigma, St. Louis, MO) for SDS-PAGE. Samples were boiled for 5 minutes and centrifuged at 15,000 x g for 10 minutes to spin down debris. 20 µl of the supernatant for the 0 hour time point (40 KU) was loaded onto matching NuPage 10% Bis-Acrylamide gels (Invitrogen, Carlsbad, CA). A volume of supernatant, adjusted for cell density based on KU, for time points 1-4 hours was also loaded along with a molecular weight marker for size comparison. The gels were run for 1 hour at 165V. After rinsing the gels three times in dH<sub>2</sub>O for 5 minutes, one of the gels was stained with Coomassie Blue for 30 minutes, destained, and photographed.

The matching gel was used for Western Blot analysis. Protein was transferred to nitrocellulose using a Transblot SD electrotransblotter (BioRad, Hercules, CA) at 15V for

45 minutes. The blot was stained with Ponceau S solution to confirm protein transfer. The membrane was then blocked with StartingBlock T20 (TBS) Blocking Solution Buffer (Pierce, Rockford, IL) for 10 minutes and probed with the primary antibody, rabbit anti-GnRH (1:2500), at room temperature overnight. The primary antibody was then removed, the membrane washed three times with TBS/Tween for 5 minutes, and the secondary antibody, goat anti-rabbit-HRP (1:500), incubated with the membrane for 45 minutes at room temperature. The membrane was again rinsed with TBS/Tween and a peroxidase solution (Pierce, Rockford, IL) was applied to visualize probed proteins.

In a subsequent Western Blot comparing extracts from *E. coli* containing either pRSetA or pRSetA/FEG #31 (4 hours post-induction), the following changes were made: 3 $\mu$ l of the protein supernatant was loaded on the gel, the proteins were transferred for 1 hour to the membrane, and alternate primary antibodies were used: rabbit anti-FliC (1:10,000) or mouse anti-GFP (1:2,000). Secondary antibodies were goat anti-rabbit-HRP (1:500) or goat anti-mouse-HRP (1:500). Antibodies against the His tag, produced in an unknown source and at an unknown concentration, were also tested on the protein, but did not yield visible results. At a later date, Tyler Noble confirmed recognition of the protein using new mouse-anti-His.

An experiment was conducted to determine optimal concentrations of primary and secondary antibodies. 2 $\mu$ l of extract from *E. coli* containing pRSetA/FEG #31 was spotted onto each of 18 - 1 inch by 1 inch pieces of nitrocellulose. These mini-blot, in individual pouches, were blocked and hybridized with various combinations of concentrations of primary and secondary antibodies to determine the ideal concentration for each antibody in Western Blotting.

## **2.3 Generation of Recombinant Virus**

### **2.3.1 Propagation of CRFK Cells**

Crandell-Reese Feline Kidney (CRFK) cells were generously provided by Dr. Richard Sutton (Baylor College of Medicine, Houston, TX). CRFK cells were

maintained at 37°C/5% CO<sub>2</sub> in either MEM/BSS (Hyclone, Logan, UT), 10% horse serum, 1% penicillin-streptomycin, non-essential amino acids, sodium pyruvate, and L-glutamine or RPMI 1640, 10% inactivated fetal bovine serum, and 1% penicillin-streptomycin.

When cells were confluent in a T-75 flask, they were split between 1:4 and 1:10. To split cells, the media was removed and 1 ml of 0.05% trypsin/0.53mM EDTA (Cellgro – Mediatech, Herndon, VA) was added. The flask was rocked until cells were visibly detaching from the flask. Cells were then resuspended in fresh growth media and passaged into new flasks.

To generate frozen stocks of CRFK cells for future use, cells were grown to confluence in T-75 flasks. Cells were detached using 1 ml of 0.05% trypsin/0.53mM EDTA (Cellgro – Mediatech, Herndon, VA), resuspended in 9 ml of media, and transferred to a 15 ml conical tube. The cells were centrifuged for 5 minutes at 1000 rpm so that the supernatant containing the trypsin could be removed. The cell pellet was resuspended in 10 ml Cell Freezing Media DMSO (Sigma, St. Louis, MO) and 1-2 ml aliquots were placed into cryovials. Cells were stored at –80°C overnight in an ethanol bath and subsequently transferred to liquid nitrogen for long-term storage.

### **2.3.2 Propagation of FHV-1, FHV/FEG, and FHV/EG**

FHV was propagated in CRFK cells. Initially, a frozen virus stock, previously prepared by the lab, was snap thawed at 37°C and re-frozen with liquid nitrogen three times to release virus from any intact cells. Growth media was removed from the cells and the cells were rinsed once with low serum media (containing only 2% FBS). In later experiments, however, it was found that virus titers were better preserved if thawed in cold tap water and frozen at –80°C. 0.5ml of viral supernatant was then used to inoculate a flask of 90% confluent CRFK cells and the culture was incubated at 37°C/5% CO<sub>2</sub> for 1 hr. After 1 hr., 12 ml of growth media was added to the flask and the cells were incubated 24-72 hours, until >50% of cells were lysed by the propagating virus. At 50% cell lysis,

the contents of the flask was frozen at  $-80^{\circ}\text{C}$  and subsequently thawed in room temperature water. For longer-term storage, the lysate was transferred to either a 50 ml conical tube or a cryovial at  $-80^{\circ}\text{C}$ . Cultures were frozen and thawed two more times prior to using the lysate to infect new cells. Propagated virus was titered as described below.

### 2.3.3 Titering of Virus

Propagated parental (pFHV) and generated recombinant (pFHV/FEG and pFHV/EG) viruses were titered as follows. CRFK cells in a confluent T-75 flask were trypsinized as previously described and resuspended in 2.5 ml total volume using growth media with 2% FBS. Cells were seeded into each well of a 24-well plate containing 1ml of growth media. Cells were allowed to recover and grow at  $37^{\circ}\text{C}/5\% \text{CO}_2$  until 90% confluent (approximately  $0.2 \times 10^6$  cells per well). Frozen supernatant containing virus was used to make serial dilutions from  $10^{-1}$  to  $10^{-6}$  in low serum media. Virus dilutions were added in quadruplicate to CRFK cells that had been washed once with low serum media. Cells were incubated with diluted virus for 1 hour at  $37^{\circ}\text{C}/5\% \text{CO}_2$ , rinsed with growth media, and incubated with 1ml of fresh growth media per well. Cells were observed for cytopathic effect (CPE) at 24, 48, and 72 hours post-infection (hpi). From these results, viral titer was calculated.

Titers of viruses were calculated 72 hpi. Virus titers were estimated using the Karber method as follows (Schmidt & Emmons, 1989):

$$\text{Log TCID}_{50} = L - d (s - 0.5)$$

Where L is the log of the lowest dilution, d is the difference between dilution steps, and s is the sum of the proportion of positive tubes.

Multiplicity of Infection (M.O.I) was calculated as follows:

$$\text{M.O.I} = \text{number of infectious units/number of cells}$$

### 2.3.4 Transient Transfection and Co-Transfection

For transient transfections, CRFK cells were grown to 90% confluence in a 24-well plate and rinsed with low serum media, containing only 2% FBS, prior to transfection. 1 µg of plasmid (pFHV/FEG #11, pFHV/EG#2, or pEGFP-C1) bound with Lipofectamine 2000 (Invitrogen) was incubated with the cells overnight at 37°C/5% CO<sub>2</sub> as per the manufacturer's instructions. Cells were observed for expression of EGFP 24-72 hpi.

For co-transfections, CRFK cells were grown to 90% confluence in a 6-well plate, rinsed with low serum media, containing 2% FBS, and infected with 400 µl of FHV in 100 µl of MEM/EBSS serum media (Hyclone, Logan, UT). Virus was incubated with cells for 1 hour at 37°C/5% CO<sub>2</sub>. After removing the virus solution from the cells and rinsing once with PBS, 4 µg of plasmid (pFHV/FEG #11, pFHV/EG #2, or pEGFP-C1) bound with Lipofectamine 2000 was incubated with the cells overnight at 37°C/5% CO<sub>2</sub> as per manufacturer's instructions. Cells were observed for expression of EGFP under a fluorescent microscope and CPE noted for 24-72 hpi. At 50% cell lysis, 0.5 ml of viral supernatant was transferred to a fresh T-75 flask of CRFK cells to allow for expansion of the viral population. Remaining viral supernatant was transferred into cryovials and stored at -80°C for long-term storage. Expanded viral populations were likewise harvested at 50% CPE and stored at -80°C.

Recombinant virus was also tested for using PCR to detect DNA sequences encoding *fliC*, EGFP, and/or GnRH in the viral supernatant. *FliC\_For\_Spe* and *FliC\_Rev\_Bam* or *EG\_For\_Bam* and *EG\_Rev\_PstI* were used as primers. 3 µl of viral supernatant was used from frozen viral stocks as template in test reactions while pFHV/FEG plus 2 µl of cell culture media was used as a positive control. Reactions were run at 95°C for 2 minutes, followed by 40 cycles of 95°C for 1 minute, 56°C for 30 seconds, and 68°C for 2 minutes. The reactions were permitted to run to completion at 68°C for 10 minutes and then remained at 4°C until transferred to -20°C for storage. 5 µl of each reaction were run on a 0.8% agarose TBE gel.

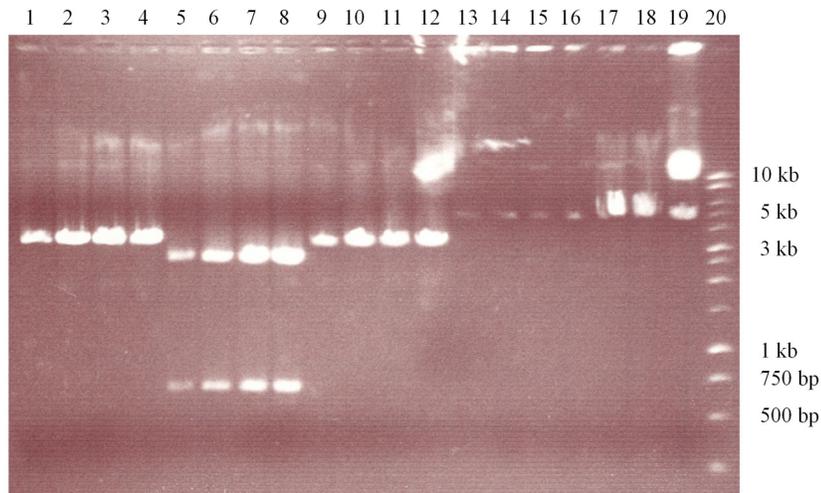
A protocol to test for recombinant protein expression from virus (FHV/FEG and FHV/EG) via Western was designed for future testing. The protocol to confirm recombinant virus involves immunoblotting using the viral supernatant of infected CRFK cells. At 50% lysis, the flask is frozen at  $-80^{\circ}\text{C}$  and thawed in room temperature water. The viral supernatant is harvested using a cell scraper and the sample centrifuged at 7500 x g for 10 minutes to remove debris. The remaining supernatant is transferred to a 30% sucrose cushion and centrifuged for 2 hours at 27000 rpm in a Beckman SW-27 rotor ( $\sim 131,400 \times g$ ). The protein pellet that forms in the tube is used for immunoblotting. Duplicate gels using the protein supernatant should be run at 165V for 1 hour. One gel should be stained with Coomassie blue to visualize total protein. The second gel should be transferred for 1 hour to nitrocellulose membrane, and probed with primary antibodies: rabbit anti-FliC (1:10,000) and mouse anti-GFP (1:2,000) and followed by secondary antibodies: goat anti-rabbit-HRP (1:500) or goat anti-mouse-HRP (1:500), respectively.

## CHAPTER 3: RESULTS

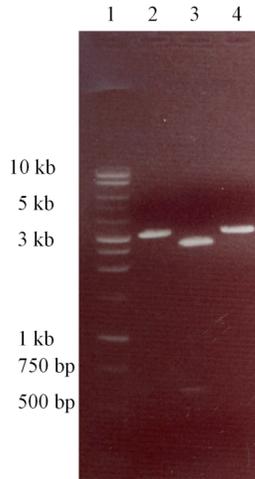
### 3.1 Generation of Clones

#### 3.1.1 pCRScript/EGFP/GnRH

Plasmid from four *E. coli* DH5- $\alpha$  clones, containing p5.1.39, was digested with *SalI*, and/or *BglII* to confirm approximate plasmid size and restriction mapping. All four clones had identical digestion patterns. Single digestion with *SalI* or *BglII* linearized the plasmid at ~3150 bp, while a *SalI* - *BglII* double digestion produced two fragments (0.65 kb and 2.5 kb), comprised mainly of the 14.5 X GnRH repeat and the plasmid backbone, respectively (Fig. 2). Likewise, single digestion with *SalI* or *NotI*, linearized the plasmid at ~3150 bp, while a *SalI* - *NotI* double digestion produced a band corresponding to the GnRH repeat (0.6 kb) and the plasmid backbone (2.55 kb) (Fig. 3).



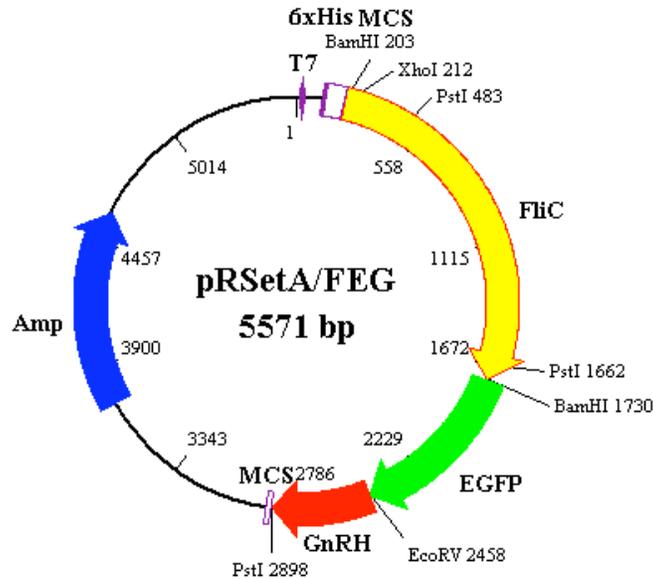
**Figure 2:** Restriction enzyme digest of p5.1.39. Lanes 1-4: p5.1.39 clones #1-4 digested with *SalI*; Lanes 5-8: p5.1.39 clones #1-4 digested with *SalI* and *BglII*; Lanes 9-12: p5.1.39 clones #1-4 digested with *BglII*; Lane 13, 15, 17: pEGFP clones #1-3 digested with *SalI*; Lane 14, 16, 18, 19: pEGFP clones #1-4 digested with *BglII*; Lane 20: 1 kb ladder (Promega, Madison, WI).



**Figure 3:** Restriction enzyme digest of p5.1.39. Lane 1: 1 kb ladder (Promega, Madison, WI); Lane 2: p5.1.39 digested with *SalI*; Lane 3: p5.1.39 digested with *SalI* and *NotI*; Lane 4: p5.1.39 digested with *NotI*.

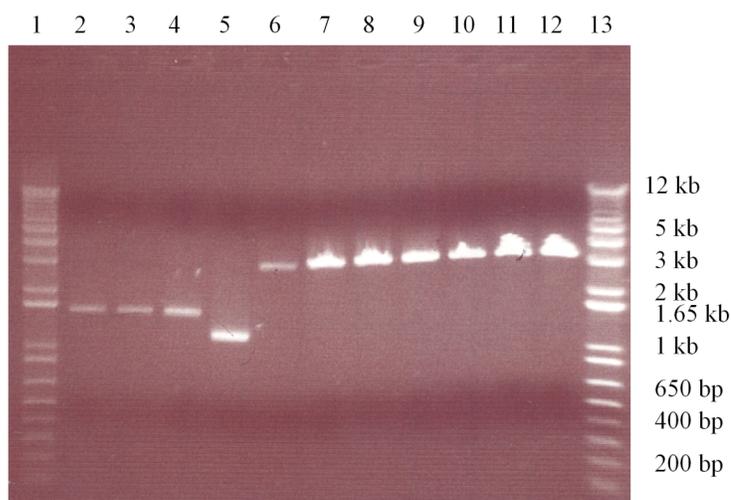
Using the sequence data for p5.1.39, the sequence for the EGFP::*GnRH* construct was designed. GeneArt optimized the sequence for feline codon usage, synthesized the construct, and delivered the product as plasmid pCRScript/EGFP/*GnRH* (Appendix A). On receipt of pCRScript/EGFP/*GnRH*, the plasmid was transformed into *E. coli* DH5- $\alpha$  and four clones were digested with *EcoRI* and/or *MluI* to confirm approximate size and restriction mapping. Single digests produced a linearized fragment at ~4.0 kb, while the double digest produced two fragments corresponding to the 2.9 kb backbone and ~1.15 kb EGFP::*GnRH* insert. Frozen glycerol stocks of pCRScript/EGFP/*GnRH* clone #1-4 were frozen down for storage at  $-80^{\circ}\text{C}$ .

### 3.1.2 pRSetA/*fliC*/EGFP/GnRH (pRSetA/FEG)



**Figure 4:** Map of pRSetA/FEG

In order to construct pRSetA/FEG (Fig. 4), the *fliC* fragment was amplified from pET29a/*fliC* by PCR, using custom primers to add *XhoI* and *BamHI* sites at the beginning and end, respectively. The PCR reaction was run on a 0.8% agarose gel to visualize the 1.5 kb product, designated *fliC* (XB). No bands were detected in control samples not containing template DNA. The EGFP::*GnRH* fusion gene was likewise amplified by PCR using modified primers to introduce a 5' *BamHI* and a 3' *PstI* site. The primers amplified a 1.2 kb fragment, designated EG (BP). No bands were detected in control samples not containing template DNA (Fig. 5).



**Figure 5:** Comparison of concentration of digested and gel purified PCR fragments prior to tripartite ligation. Lane 1 and 13: 1 kb+ Ladder (Invitrogen, Carlsbad, CA); Lanes 2-3: *fliC* (XB) digested with *XhoI* and *BamHI* in Buffer B; Lane 4: *fliC* (SB) digested with *SpeI* and *BamHI* in Buffer E; Lane 5: EG (BP) digested with *BamHI* and *PstI* in Buffer H; Lane 6: pRSetA digested with *XhoI* and *PstI* in Buffer H; Lanes 7-12: (positive controls) pBluescript digested with *SpeI* in Buffer E, *BamHI* in Buffer E, *XhoI* in Buffer B, *BamHI* in Buffer B, *PstI* in Buffer H, and *BamHI* in Buffer H.

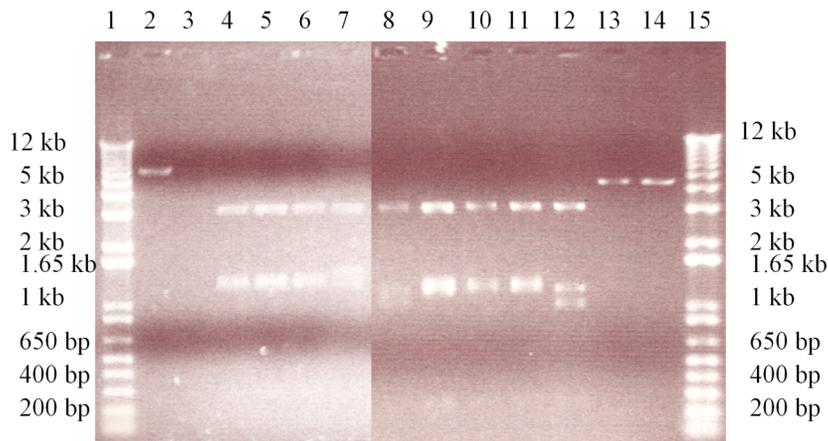
After ligation of the pRSetA, *fliC*, and EGFP/GnRH fragments and transformation of the plasmid construct into competent *E. coli* DH5- $\alpha$ , clones #21, 24, 25, 26, 28, 29, 31, 36, and 37 were identified, by quick screen, as containing inserts of various sizes. Plasmid extractions were performed on the clones for further analysis. *BamHI*, *XhoI* - *PstI*, and *XhoI* - *HindIII* restriction enzyme digests indicated that while the clones contained all of the anticipated elements, some of them had deletions in the GnRH portion of the sequence. Restriction enzyme digest revealed that clone #28 appeared to have one of the largest deletions in the GnRH sequence while clone #31 appeared to be equivalent to the predicted size.

*XhoI* - *PstI* digestion of the pRSetA/FEG clones resulted in 4 bands. The predicted sizes of digestion products for the target construct were: 2.9 kb, 1.2 kb, 1.1 kb, and 270 bp, however, three variations in the sizes of the four bands generated were found among the clones: 1.) 2.9 kb, 1.2 kb, 1.1 kb, and 270 bp; 2.) 2.9 kb, 1.3 kb, 1.1 kb, and 270 bp; and 3.) 2.9 kb, 1.1 kb, 1.0 kb and 270 bp. (Table 4)

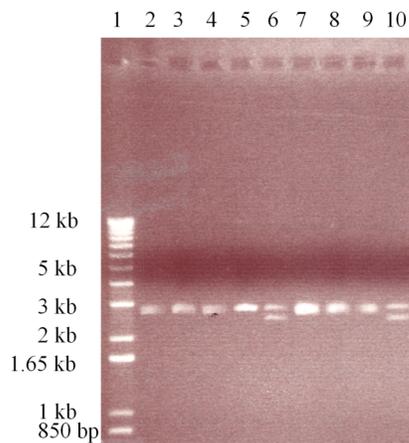
| Type/Size of Bands                | Clones                 |
|-----------------------------------|------------------------|
| 1: 2.9 kb, 1.2 kb, 1.1 kb, 270 bp | 21, 24, 25, 29, 31, 36 |
| 2: 2.9 kb, 1.3 kb, 1.1 kb, 270 bp | 26                     |
| 3: 2.9 kb, 1.1 kb, 1.0 kb, 270 bp | 28, 37                 |

**Table 4:** Digestion products of pRSetA/FEG cut with *XhoI* and *PstI*

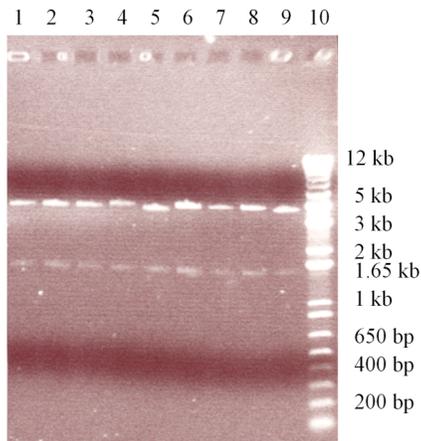
A second diagnostic digestion with *XhoI* and *HindIII* was expected to yield two fragments (2.7 kb and 2.9 kb). When the clones were double digested with *XhoI* and *HindIII*, three categories were again observed. A single digest of clones #28 and #31 with *BamHI*, to drop out the *fliC* gene, indicated that the variation in the clones was occurring in the EGFP::*GnRH* portion of the construct. The restriction enzyme digest indicated that while the clones contained all of the anticipated elements, some of them had deletions in the *GnRH* portion of the sequence (Fig. 6). Clone pRSetA/FEG #28 appeared to have one of the largest deletions in the *GnRH* sequence while clone pRSetA/FEG #31 appeared to be equivalent to the predicted size of 4.1 kb and 1.5 kb.



**Figure 6A:** pRSetA/FEG digested with *XhoI* and *PstI*. Lanes 1 and 15: 1 kb+ Ladder (Invitrogen, Carlsbad, CA); Lane 2-3: pFHV/FEG clones #4 and 6 digested with *SpeI* and *PstI*; Lanes 4-7: pRSetA/FEG clones #21, 24, 25, 26 digested with *XhoI* and *PstI*; Lanes 8-14: pRSetA/FEG clones #28, 29, 31, 36, 37, 23, 35 digested with *XhoI* and *PstI*.



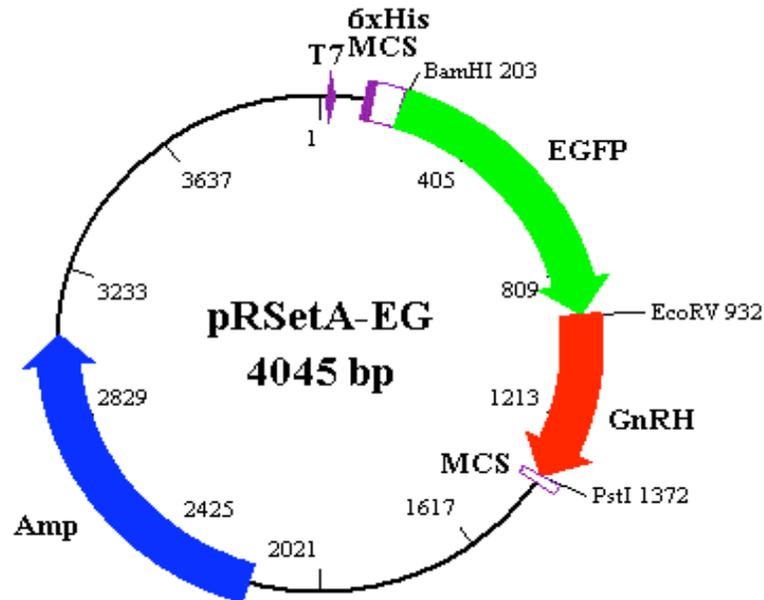
**Figure 6B:** pRSetA/FEG digested with *XhoI* and *HindI*. Lane 1: 1 kb+ Ladder (Invitrogen, Carlsbad, CA); Lanes 2-10: pRSetA/FEG clones #21, 24, 25, 26, 28, 29, 31, 36, 37 digested with *XhoI* and *HindIII*.



**Figure 6C:** pRSetA/FEG digested with *Bam*HI. Lanes 1-9: pRSetA/FEG clones #21, 24, 25, 26, 28, 29, 31, 36, 37 digested with *Bam*HI; Lane 10: 1 kb+ Ladder (Invitrogen, Carlsbad, CA).

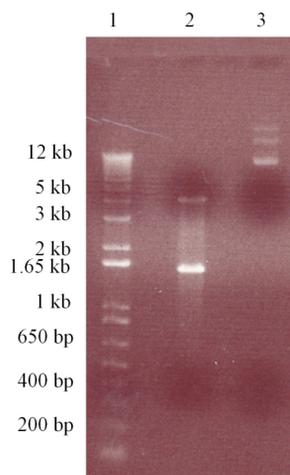
When cultures of pRSetA/FEG #28, 31, and pRSetA were restreaked from frozen glycerol stocks onto LB-Amp plates containing 0.1 M IPTG and 60 $\mu$ l of Blueo-gal, colonies were not green after the initial overnight incubation. However, the pRSetA/FEG #28 and #31 colonies subsequently turned green on storage at 4°C. In comparison, *E. coli* containing pRSetA alone never expressed green color. Clone pRSetA/FEG #31 grew the best with small, plentiful colonies. Clone #28 grew more slowly than #31 and pRSetA and the colonies were larger than those of #31 (similar in size to pRSetA).

### 3.1.3 pRSetA/EGFP/GnRH (pRSetA/EG)



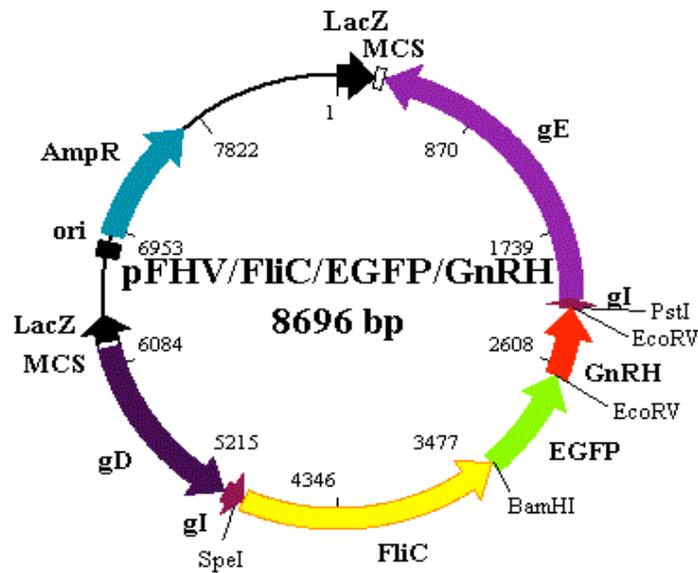
**Figure 7:** Map of pRSetA/EG

Digestion of pRSetA/FEG with *Bam*HI dropped a 1.5 kb fragment corresponding to the *fliC* fragment. The pRSetA/EG (Fig. 7) backbone was el purified for recircularization and transformation (Fig. 8). Clones pRSetA/EG #1 and 2 in *E. coli* DH5- $\alpha$  were frozen as glycerol stocks. pRSetA/EG was also transformed into *E. coli* BL-21 and stored as a glycerol stock.



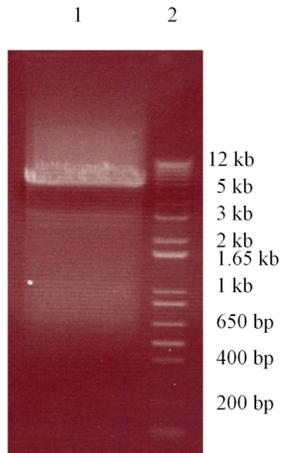
**Figure 8:** pRSetA/FEG #31 digested with *Bam*HI to drop *fliC* fragment. Lane 1: 1 kb+ Ladder (Invitrogen, Carlsbad, CA); Lane 2: pRSetA/FEG #31 digested with *Bam*HI; Lane 3: pRSetA/FEG undigested negative control.

### 3.1.4 pFHV/*fliC*/EGFP/GnRH (pFHV/FEG)

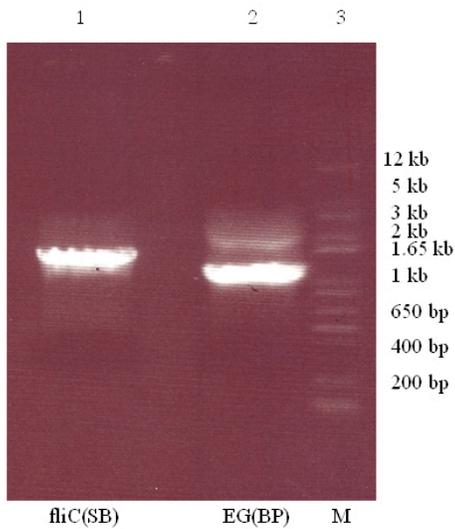


**Figure 9:** Map of pFHV/FEG

pFHV/FEG (Fig. 9) was constructed in a tripartite ligation similar to pRSetA/FEG. PCR produced a 1.5 kb fragment, designated *fliC* (SB), containing 5' *SpeI* and 3' *BamHI* sites, a 1.2 kb designated EG (BP) containing 5' *BamHI* and 3' *PstI* sites, and a 8 kb fragment designated pFHV (SP) containing 5' *SpeI* and 3' *PstI* sites (Fig. 10). Each of these three fragments was digested with the appropriate enzyme and gel purified prior to ligation (Fig. 11).



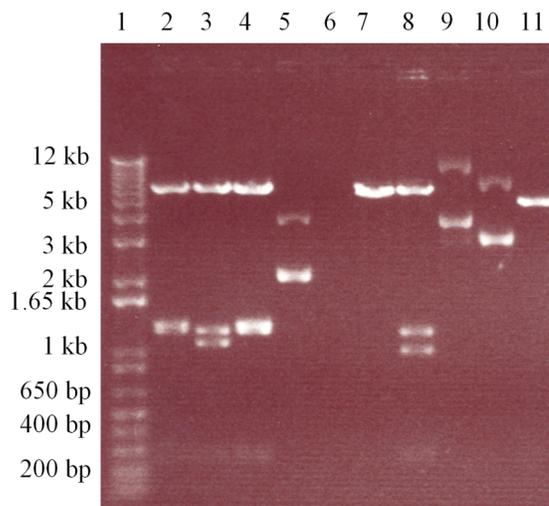
**Figure 10:** PCR amplicon of pFHV (SP). Lane 1: pFHV (SP) PCR amplicon; Lane 2: 1 kb+ Ladder (Invitrogen, Carlsbad, CA).



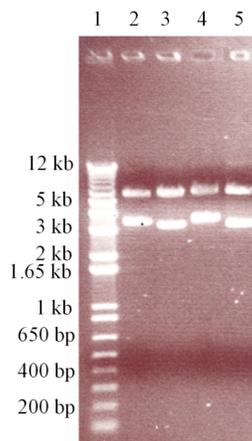
**Figure 11:** PCR amplicons in preparation for gel purification. Lane 1: *fliC* (SB); Lane 2: EG (BP); Lane 3: 1 kb+ Ladder (Invitrogen, Carlsbad, CA).

Electroporation of the tripartite ligation product resulted in approximately 30-40 colonies on the LB-Amp-Bluo-Gal plate. Control plates containing either no DNA or parental pFHV grew no colonies or too many to count, respectively, indicating that the transformation was successful. 18 of 20 clones screened by rapid screen were determined

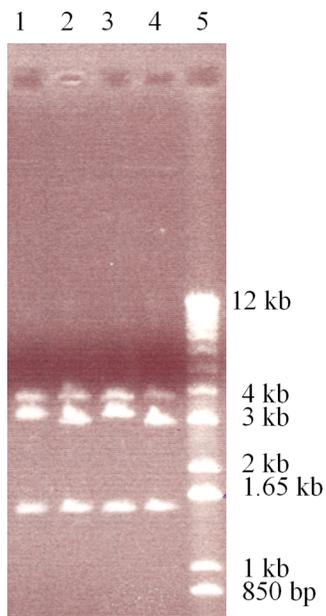
to have having inserts of various sizes. Plasmid extractions were performed for additional analysis. DNA concentration was determined using a NanoDrop Spectrophotometer and found to range from 0.2  $\mu\text{g}/\mu\text{l}$  to 1.0  $\mu\text{g}/\mu\text{l}$ . *Bam*HI, *Bam*HI - *Spe*I, and *Spe*I - *Pst*I digests of 10 of the clones indicated that there were deletions in the GnRH repeat portion of some of the clones (Fig. 12). The predicted digestion products of a *Spe*I - *Pst*I double digest were four fragments: 5.5 kb, 1.2 kb, 1.1 kb, and 270 bp. Clones #11, 12, 13, and 17 had inserts approximately corresponding to these sizes. Clones #11 and 13 contained GnRH repeats approximately equal in size, and  $\sim$ 100bp larger than #12 and 17, which were also approximately equal in size. As with the pRSetA/FEG clones, deletions of varying lengths were found in the GnRH portion of screened clones #12 and #17, as determined by digestion and later confirmed by sequencing.



**Figure 12A:** Restriction Enzyme Digest. Lane 1: 1 kb+ Ladder (Invitrogen, Carlsbad, CA); Lanes 2-11: pFHV/FEG clones #11, 12, 13, 14, 15, 16, 17, 18, 19, 20 digested with *Spe*I and *Pst*I.



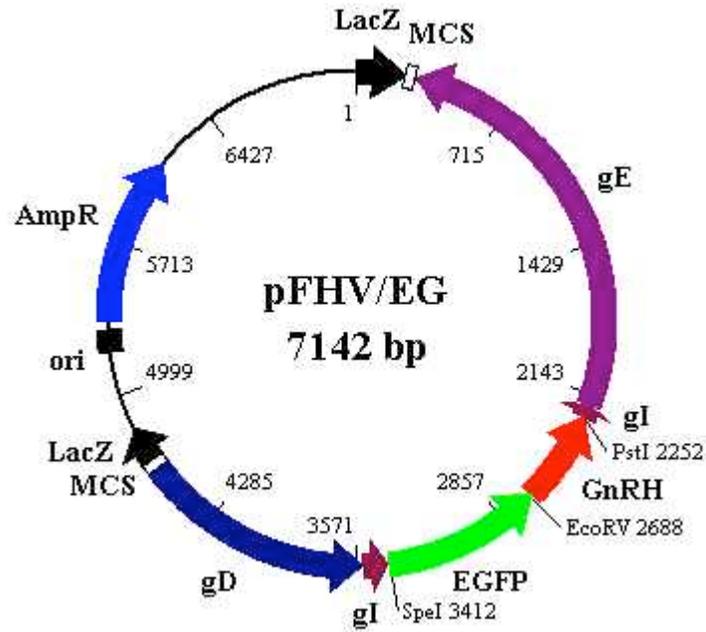
**Figure 12B:** Restriction Enzyme Digest. Lane 1: 1 kb+ Ladder (Invitrogen, Carlsbad, CA); Lane 2-5: pFHV/FEG clones #11, 12, 13, 17 digested with *Bam*HI.



**Figure 12C:** Restriction Enzyme Digest. Lanes 1-4: pFHV/FEG clones #11, 12, 13, 17 digested with *Bam*HI and *Spe*I; Lane 5: 1 kb+ Ladder (Invitrogen, Carlsbad, CA).

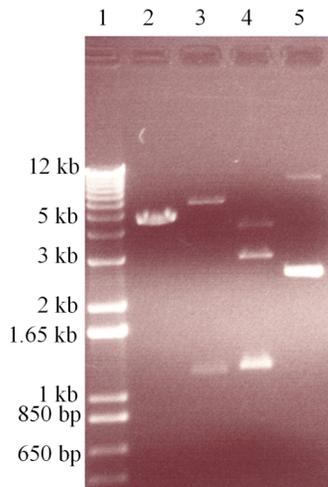
While clone #12 was not sequence confirmed, we expect that #12 will contain a similar deletion as that found in #17. Clone #13 appears to be close in size to #11 and so would be a good alternative clone to #11 if needed. Towards this end, clone #13 will need to be sequenced to confirm an absence of mutations.

### 3.1.5 pFHV/EGFP/GnRH (pFHV/EG)



**Figure 13:** Map of pFHV/EG

pFHV/EG was constructed in a similar manner to pRSetA/FEG (Fig. 13). PCR produced two amplicons: pFHV(SP) (~8 kb) and EG (SP) (~1.2 kb). Transformation of the ligation product into *E. coli* yielded only 2 colonies. pFHV/EG Clone #2 was confirmed to contain the insert by *SpeI* and *PstI* restriction enzyme digestion (Fig. 14).



**Figure 14:** Restriction Enzyme Digest. Lane 1: 1 kb+ Ladder (Invitrogen, Carlsbad, CA); Lane 2-5: pFHV/EG clones #1-4 digested with *SpeI* and *PstI*.

### 3.1.6 Sequencing of Constructs: pRSetA/FEG, pRSetA/EG, pFHV/FEG, and pFHV/EG

Clones pRSetA/FEG #28 (small insert) and 31 (large insert) and pFHV/FEG #11 (large insert) and 17 (small insert) were sequenced at Virginia Bioinformatics Institute (VBI). Clones pFHV/FEG #17 and pRSetA/FEG #28 were found to contain deletions in the GnRH portion of the clone, which would result in premature termination of the GnRH repeat if expressed. Clone pRSetA/FEG #31 and pFHV/FEG #11 were confirmed to be correct with no mutations, deletions, or frame shifts.

Likewise, pRSetA/EG #2 and pFHV/EG #2 were sequence confirmed at VBI. In both pFHV/FEG and pFHV/EG, the EGFP::GnRH fusion construct was found to be in frame with the ATG start codon of the FHV gI. However, pFHV/EG #2 contains two mutations. The first mutation is within gI, upstream of the fusion construct, and is a T → C nucleotide substitution (nt 81), which does not result in an amino acid substitution. The second mutation is in the last of the 14 GnRH repeats (nt 1334) and is a G → A

substitution resulting in a change in the final amino acid from G → D. Because there are 13 prior repeats of GnRH, we do not anticipate that this substitution will pose further experimental problems. However, future studies should be considered in light of this substitution. (See Appendix A for sequences of these clones.)

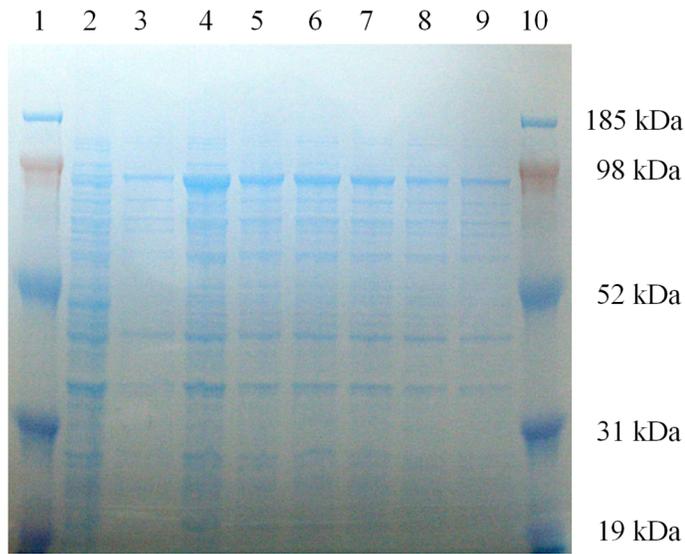
### **3.1.7 *E. coli* BL-21 Stocks: pRSetA/FEG, pRSetA/EG, pFHV/FEG, and pFHV/EG**

*E. coli* BL-21 cultures containing either pRSetA or pRSetA/FEG #31 were induced to express protein from the plasmid with IPTG. After 4 hours of incubation shaking at 37°C, the collected pellets from the 1ml aliquots were only occasionally visibly green despite confirmation of EGFP expression through Western Blotting. Often the intensity of the expression of EGFP was further enhanced by longer-term storage. Colonies streaked on LB-Amp plates containing IPTG were also sometimes visibly green with the intensity being further enhanced by storage at 4°C.

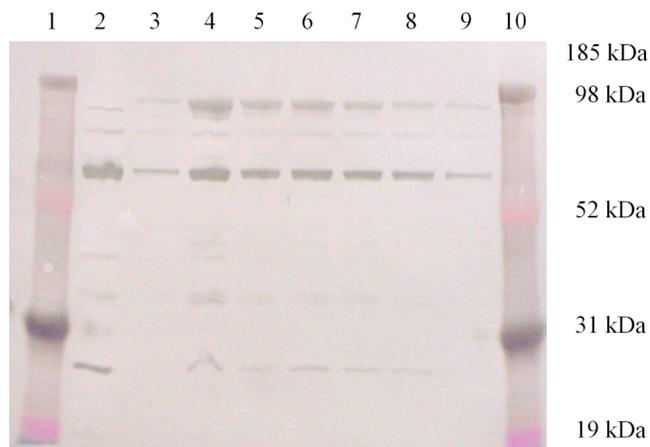
## **3.2 Verification of Protein Expression from pRSetA/FEG #31 and pRSetA/EG #2**

### **3.2.1 Western Blot: GnRH, FliC, and EGFP**

Results of the first Western Blot using anti-GnRH (AbCam, Cambridge, MA) show a band (~95 kDa) produced in the pRSetA/FEG #31 clone not found in the pRSetA control clone. This band is seen to accumulate over the course of incubation of the culture with IPTG during the 4 hours, suggesting that the protein is being induced over time. There are other smaller bands throughout the sample, which suggest that either degradation or incomplete expression of the protein may be occurring (Fig. 15).

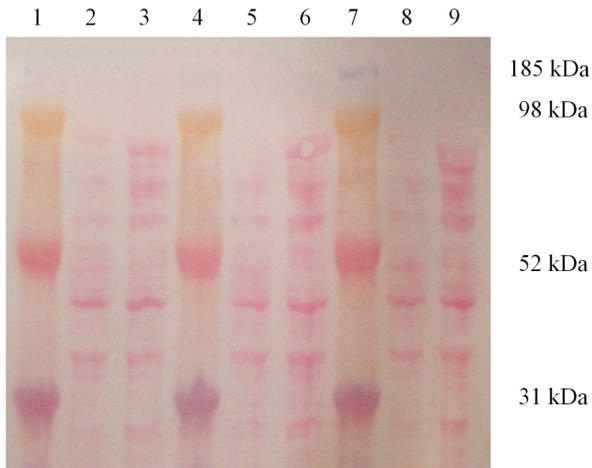


**Figure 15A:** Coomassie Stain on 10% Bis-Tris Acrylamide Gel: Lanes 1 and 10: Multimark Multi-Colored Standard (Invitrogen, Carlsbad, CA); Lanes 2: pRSetA pellet stored at 4°C overnight; Lane 3: pRSetA/FEG #31 at 0 hours post-induction; Lane 4: pRSetA/FEG #31 at 4 hours post-induction and pellet stored at 4°C overnight; Lane 5-9: pRSetA/FEG #31 at 4, 3, 2, 1, and 0 hours post-induction.

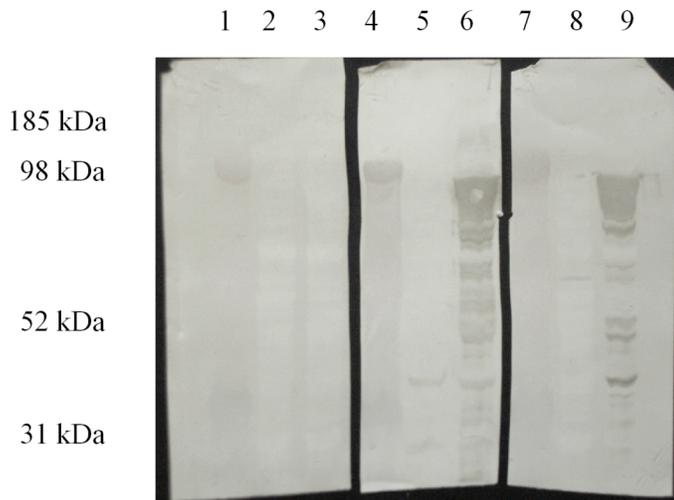


**Figure 15B:** Western Blot of pRSetA/FEG #31 protein extraction probed with polyclonal rabbit GnRH antibody (AbCam, Cambridge, MA): Lanes 1 and 10: Multimark Multi-Colored Standard (Invitrogen, Carlsbad, CA); Lanes 2: pRSetA pellet stored at 4°C overnight; Lane 3: pRSetA/FEG #31 at 0 hours post-induction; Lane 4: pRSetA/FEG #31 4 hours post-induction and pellet stored at 4°C overnight; Lane 5-9 pRSetA/FEG #31 at 4, 3, 2, 1, and 0 hours post-induction.

A second immunoblot to test for recognition of the fusion protein with anti-His, anti-FliC, or anti-GFPuv serum was performed (Fig. 16). Again, a protein band was visualized at approximately 95 kDa with a number of smaller products also being recognized in the sample using rabbit-anti-FliC (courtesy A. Honko), and mouse-anti-GFPuv (AbCam, Cambridge, MA). The anti-His used was of unknown origin and unknown concentration. No bands were visualized with the anti-His previously diluted in our lab and using either an anti-mouse secondary antibody, or an anti-rabbit secondary antibody on a subsequent blot. However, Tyler Noble later confirmed antibody recognition of a 95 kDa protein product recognized by newly purchased mouse-anti-His (Sigma, St. Louis, MO). Reversed bands (white on dark background) were seen with all three primary antibody probes, indicating too much primary and/or secondary antibody being used.



**Figure 16A:** Ponceau S Staining: Lanes 1, 4, and 7: Multimark Multi-Colored Standard (Invitrogen, Carlsbad, CA); Lanes 2, 5, and 8: pRSetA; Lanes 3, 6, and 9: pRSetA/FEG #31 at 4 hours post-induction.



**Figure 16B:** Western Blot of pRSetA and pRSetA/FEG #31 probed with unknown source-anti-His (Lane 1-3), rabbit-anti-FliC (Lanes 4-6), and mouse-anti-GFPuv (Lanes 7-9). Lanes 1, 4, and 7: Multimark Multi-Colored Standard (Invitrogen, Carlsbad, CA); Lanes 2, 5, and 8: pRSetA; Lanes 3, 6, and 9: pRSetA/FEG #31 at 4 hours post-induction.

A third immunoblot was performed as a dot blot to optimize the concentration of antibodies used. See Table 5 for ideal concentration of antibodies as determined by optimization experiment.

| Antibody        | Source | Ideal Concentration |
|-----------------|--------|---------------------|
| Anti-GnRH       | Rabbit | 1:2500              |
| Anti-FliC       | Rabbit | 1:10,000            |
| Anti-GFP        | Mouse  | 1:2,000             |
| Anti-rabbit-HRP | Goat   | 1:1,000             |
| Anti-mouse-HRP  | Goat   | 1:1,000 – 1:2,000   |

**Table 5:** Optimized Concentrations of Primary and Secondary Antibodies

Tyler Noble, an undergraduate honors student in the Boyle lab, subsequently purified, by Ni<sup>2+</sup> affinity chromatography, the FliC/EGFP/GnRH fusion protein expressed in *E. coli* BL-21. This recombinant protein is visibly green in daylight and fluoresces under ultra-violet light.

### 3.3 Generation of Virus

#### 3.3.1 Propagation of CRFK Cells

In a healthy culture, the CRFK cells are somewhat spiky in appearance. The cells tend to be somewhat plumper at a low density and become elongated when crowded. If too sparse, the cells will grow more slowly; they grow best at higher density. If left at 100% confluence, their growth will slow even when media is replenished and they may take additional time to recover when split. When splitting the CRFK cells, they tend to grow better when transferred to a fresh flask rather than continuing the line in the same flask repeatedly. If grown in the same flask more than 2-3 times, the cells have some trouble staying attached and tend to form dense clumps. The cells appeared to grow slightly more slowly in RPMI than in DMEM, however, they tended to be more robust and healthier looking when grown in the RPMI.

### **3.3.2 Propagation of FHV-1, FHV/FEG, and FHV/EG**

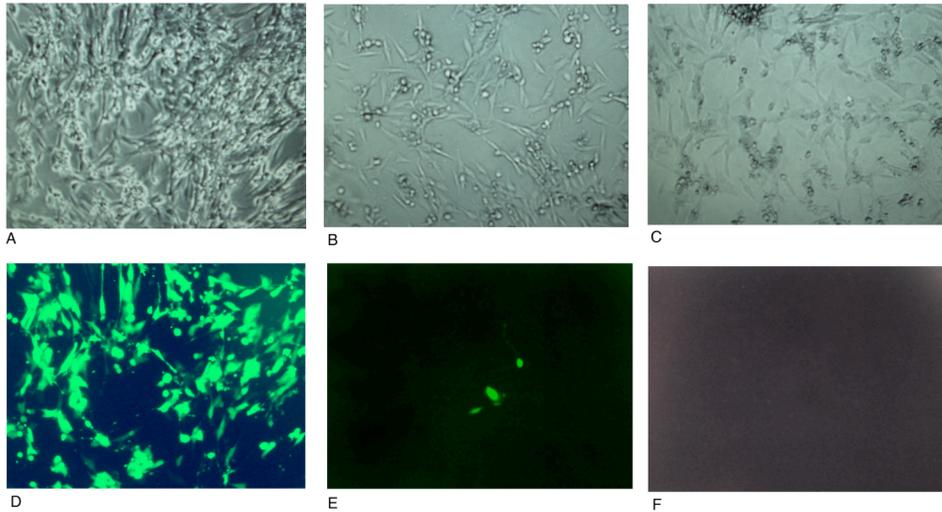
When CRFK cells were infected with FHV (M.O.I. = 0.3), visible cytopathic effect (CPE) was observed beginning at approximately 24-48 hours post-infection (hpi). The patches in the cell lawn continued to increase in size and number until the virus had lysed almost all cells. Total cell lysis typically occurred by 72 hpi.

### **3.3.3 Titering of Virus: FHV-1, FHV/FEG, and FHV/EG**

Titers of parental FHV-1 virus preparations ranged from  $1 \times 10^4$  to  $1 \times 10^6$ . Viral cultures permitted to continue beyond total cell lysis at  $37^\circ\text{C}/5\% \text{CO}_2$  tended to have lower titers presumably due to denaturation of the virus. Additionally, the presence of trypsin interfered with the growth of the virus and resulted in lower viral titers.

### **3.3.4 Transient Transfection and Co-Transfection**

CRFK cells transiently transfected with the pEGFP-C1 plasmid, displayed EGFP activity when viewed with a fluorescent microscope 48 hpi, while those that were mock-transfected without plasmid did not fluoresce. Cells transfected with pEGFP-C1 served as a positive control for transient transfection experiments with pFHV/FEG #11. CRFK cells transfected with pFHV/FEG gave inconsistent results. In the first experiment, 5-6 cells, plated at  $4 \times 10^6$ , were positive for EGFP activity (Fig. 17). However, in a second follow-up transient transfection experiment, these results could not be repeated.



**Figure 17:** Transient Transfection of CRFK cells: (A-C) Bright light; (D-F) Fluorescent light; (A, D) pEGFP-C1; (B, E) pFHV/FEG; (C, F) Mock transfection.

During co-transfection experiments, EGFP expression was observed in positive control samples. However, no EGFP activity was visible in wells transfected with pFHV/FEG #11 or pFHV/FEG#2. All wells infected with FHV-1 virus showed CPE by approximately 24-48 hpi. Virus from these wells was harvested for further experiments. These viral samples were later titered and expanded in T-75 flasks of CRFK cells. Virus was then screened by PCR for detection of recombinant DNA and protein expression.

Although positive and negative controls confirmed successful PCR reactions, recombinant virus could not be detected via PCR using primers to amplify the EGFP::GnRH fragment (data not shown).

## CHAPTER 4: DISCUSSION

With 30-60 million homeless feral cats in the United States alone, the need for contraception is clear. Surgical sterilization is only effective where there are the time and economic resources to support the approach. Currently, funding for such efforts is minimal, and consequently, so have the results been in the efforts to control feral cat populations. In recent years, researchers have explored the possibilities of alternative methods of sterilization such as hormone-based therapy and immunocontraception. Safety, efficacy, affordability, and convenience are the main driving factors guiding such research. Immunocontraceptive vaccines provide a potential answer for such a need.

The products in development through this research offer two possible alternatives in the immunocontraceptive approach. As a first step, purified FliC::EGFP::GnRH recombinant protein may serve as an injectable vaccine. Secondly, recombinant FHV expressing the recombinant protein may provide the added benefit of a non-injectable, possibly edible, vaccine.

Our results support previous findings that FHV with deletions of gI and gE produce viable virus with reduced cell-to-cell transmission. We have replaced genes gI and gE with a recombinant sequence encoding for FliC/EGFP/GnRH (FEG) with the goal of stimulating a strong immune response against endogenous GnRH, a hormone at the top of the reproductive cascade, and one that has shown promise in a number of other immunocontraceptive efforts.

For this research project, four clones were constructed for the production of recombinant protein: pRSetA/FEG, pRSetA/EG, pFHV/FEG, and pFHV/EG. The recombinant pRSetA clones were designed for the production of recombinant EGFP::GnRH protein, with and without FliC, in bacteria for protein purification. The recombinant proteins expressed from the pRSetA backbone are His-tagged for ease of purification. The purified fusion protein has been shipped to Dr. H. Baker, of Auburn University, for testing as an injectable contraceptive product in cats. We expect that the

presence of FliC, an exceptionally strong stimulator of the immune system, will enhance the activity of the EG protein. The pFHV/FEG and pFHV/EG clones were likewise designed to create live, attenuated recombinant FHV virus that will produce recombinant protein in infected cells.

We observed variable EGFP activity in the protein produced by pRSetA/FEG in *E. coli* BL-21. We believe this to be an effect of the strain of *E. coli* rather than of the plasmid or protein itself. Transformed *E. coli* BL-21 are often somewhat unstable and exhibit variable protein expression.

In the generation of the plasmid constructs, we found that the 14 GnRH repeats posed some hurdles. PCR primers specific to the region could not be used due to the repeating nature of the construct and the potential for mispriming. Similarly, we observed that a number of clones constructed contained significant deletions in the GnRH repeat portion of the recombinant sequence. We believe that this resulted through a combination of mutations and deletion during the PCR and ligation processes, and possibly during the plasmid replication process with the bacteria.

Western Blots of the recombinant FEG protein probed with antibodies against GnRH, His, FliC, or GFPuv show the presence of an approximately 95 kDa recombinant protein that is not detected in protein preparations from *E. coli* containing only pRSetA. However, the polyclonal GnRH antibodies produced a number of non-specific bands. This effect should be minimized by binding the antibody to protein extractions from CRFK cells prior to use in a Western Blot. Protein extractions probed with FliC and GFP antibodies revealed a number of smaller bands specifically recognized by the antibodies and not found in the pRSetA control. We believe that these smaller bands are products of degradation and/or incomplete transcription.

Although we attempted to generate the recombinant FHV/FEG and FHV/EG viruses a number of times, our results were inconclusive. EGFP activity was only detected in a very small number of cells on one occasion and so the possibility of cross-

contamination with the positive control must be considered. However, we continue to attempt generation of the virus and to detect recombinant DNA sequences in CRFK cells through PCR and Western Blots of viral protein.

Although we were not able to confirm the presence of recombinant DNA in our generated virus, it is possible that recombinant virus may have been below the detectable limits of PCR in these samples or that recombination did not successfully occur.

Alternatively, we must consider the possibility that the pFHV- $\Delta$ gILZ plasmid we are currently using is not suitable for generation of recombinant virus from parental FHV-1. Other pFHV recombination vectors should be considered. Future attempts to generate the recombinant virus should include a control for recombination. For this purpose, we suggest using the original pFHV- $\Delta$ gILZ plasmid in conjunction with the parental FHV-1 virus. In a co-transfection experiment, recombinant FHV should produce blue plaques when grown in the presence of  $\beta$ -gal as a result of the Lac Z gene from the plasmid. The successful generation of virus producing blue plaques would confirm the functionality of the current co-transfection protocol and reagents.

The project described is unique in that it brings together a number of recent findings that may offer new insights into immunocontraceptive approaches. In this work, we aimed to enhance the immunogenicity of a non-antigenic polypeptide by expressing it as a multimer fused to flagellin. Additionally, the fusion product contains EGFP to facilitate visualization of protein expression in cell culture. It is our expectation that this research can be more fully developed and provides some hope to control the millions of homeless cats that face euthanasia annually.

## CHAPTER 5: FUTURE WORK

Additional experiments are currently underway to confirm generation of the recombinant FHV/FEG and FHV/EG viruses. The recombinant FEG and EG proteins may also be confirmed by N-terminal sequencing. Recombinant FEG and EG protein purified from *E.coli* BL-21 expressing pRSetA/FEG and pRSetA/EG will be tested for contraceptive efficacy in mice, in the Boyle lab, this year. Additionally, Dr. Henry Baker, at Auburn University, will conduct contraceptive trials using the recombinant protein in cats this year. These studies should provide further support for the immunocontraceptive effectiveness of these potential vaccines. Because GnRH is well conserved among mammals, we expect that these injectable protein vaccines may be effective not only in cats, but in other animals as well, such as cattle, rodents, and horses.

Efforts to produce the feline specific recombinant vaccines, FHV/FEG and FHV/EG, will continue as well. Isolation of the recombinant viruses may be facilitated by using end-point dilution to isolate and subsequently expand recombinant viruses with GFP activity. We hope to complete the generation of these viruses shortly and to test their efficacy in felines with Dr. Baker later this year. The safety of both protein and viral GnRH vaccines in a variety of animals and pregnancy status will have to be carefully tested prior to widespread use.

Because there has been some concern about cross-reactivity among the different GnRH isoforms, future animal studies will have to explore the safety and possible side effects of such vaccines closely. However, it is possible to decrease cross-reactivity through amino acid substitutions within GnRH, if necessary (Ferro *et al.*, 2001; Turkstra *et al.*, 2005). Further modifications to future GnRH viral vaccines may include the use of an alternative insertion site, such as the thymidine kinase site as opposed to our gI site, or alternative promoters, such as CMV rather than our viral gI promoter. Little is currently known about the activity of the gI promoter other than it is considered a promoter for a gamma-protein, or “late” protein. Other viral promoters that have higher expression

and/or earlier expression that do not depend on the expression of early viral genes may be more effective.

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## **APPENDIX A**

The following pages contain annotated DNA sequences for some of the clones used and generated in this project. These files were generated using Lasergene v6.1.4 (DNASTar, Madison, WI) from the sequences provided by Virginia Bioinformatics Institute. All sequence files are available through Dr. Stephen Boyle's lab (Lab Computer: Molecular Genetics Lab/Former Users/Kerry/Seqs/Thesis Files).

Created: Thursday, August 10, 2006 3:51 PM

1-420: GnRH repeat

421-435: multiple cloning site

```
      10      20      30      40      50      60
┌──────────┴──────────┬──────────┴──────────┬──────────┴──────────┬──────────┴──────────┬──────────┴──────────┬──────────┴──────────┐
TCCCGGCCGTAGTCCATATGACCAATGTTCTCCCGGCCGTAGTCCATATGACCAATGTTC 60
TCCCGGCCGTAGTCCATATGACCAATGTTCTCCCGGCCGTAGTCCATATGACCAATGTTC 120
TCCCGGCCGTAGTCCATATGACCAATGTTCTCCCGGCCGTAGTCCATATGACCAATGTTC 180
TCCCGGCCGTAGTCCATATGACCAATGTTCTCCCGGCCGTAGTCCATATGACCAATGTTC 240
TCCCGGCCGTAGTCCATATGACCAATGTTCTCCCGGCCGTAGTCCATATGACCAATGTTC 300
      310      320      330      340      350      360
┌──────────┴──────────┬──────────┴──────────┬──────────┴──────────┬──────────┴──────────┬──────────┴──────────┬──────────┴──────────┐
TCCCGGCCGTAGTCCATATGACCAATGTTCTCCCGGCCGTAGTCCATATGACCAATGTTC 360
TCCCGGCCGTAGTCCATATGACCAATGTTCTCCCGGCCGTAGTCCATATGACCAATGTTC 420
CATGGCGGCCGCGCC 435
```

pCRScript\_EG

Created: Thursday, August 10, 2006 4:09 PM

1-16: pCRScript vector

17-22: MluI

23-736: EGFP-C1

737-742: EcoRV

743-1162: GnRH

1163-1168: Two stop codons

1169-1174: EcoRI

1175-1195: pCRScript vector

Features

```

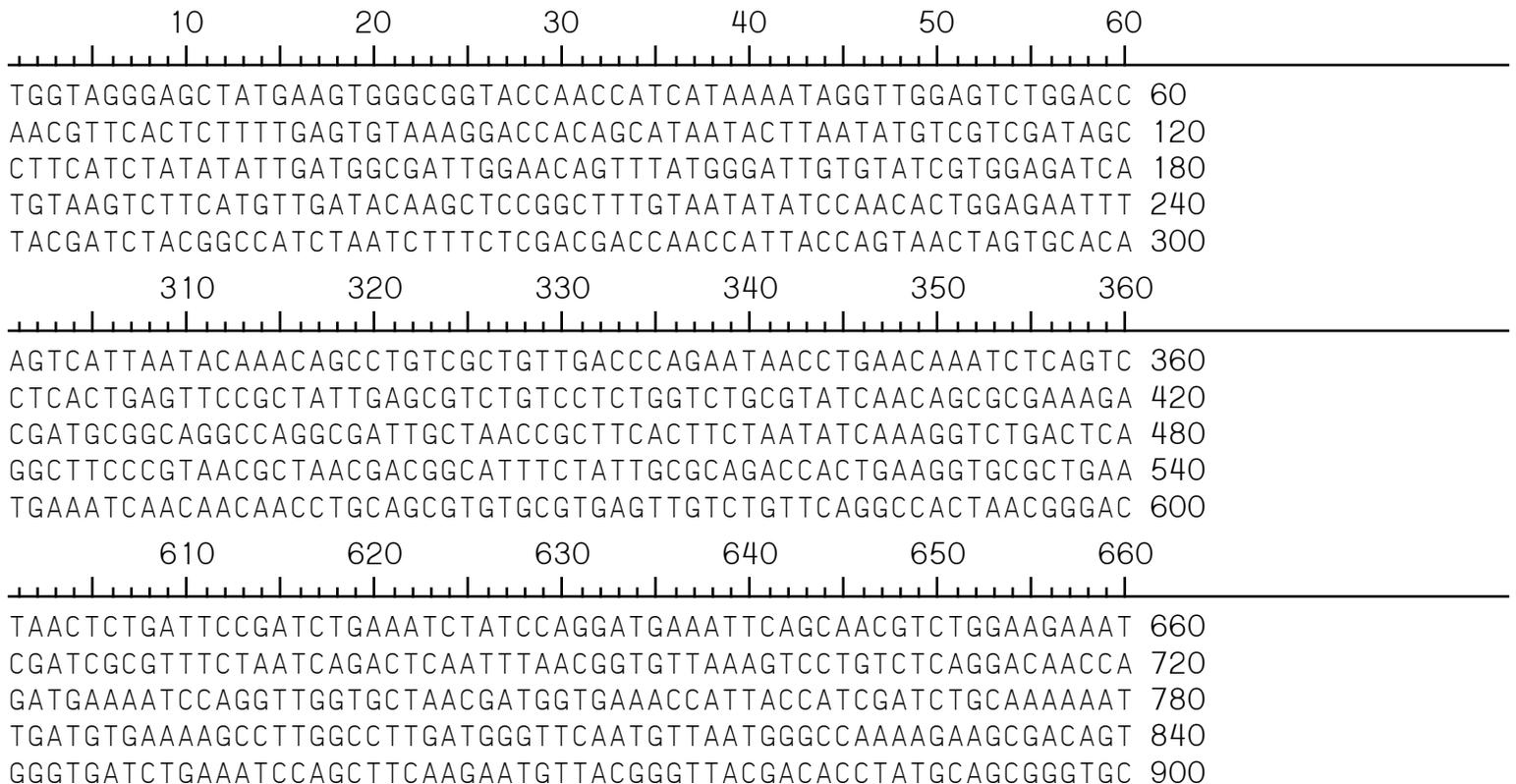
      10      20      30      40      50      60
      |-----|-----|-----|-----|-----|-----|
GGGCGAATTGGGTACCACGCGTGTGTCCAAGGGCGAGGAGCTGTTACCGGCGTGGTGCC 60
CATCCTGGTGGAGCTGGACGGCGACGTGAACGGCCACAAGTTCTCCGTGTCCGGCGAGGG 120
CGAAGGGCGACGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGTACCACCGGCAAGCT 180
GCCCGTGCCCTGGCCCACCCTGGTGACCACCCTGACCTACGGCGTGCAGTGCTTCTCCAG 240
ATACCCCGACCATATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAGGGCTACGT 300
      310      320      330      340      350      360
      |-----|-----|-----|-----|-----|-----|
GCAGGAGAGGACCATCTTCTTCAAGGACGACGGCAACTACAAGACCAGGGCCGAGGTGAA 360
GTTCGAAGGCGACACCCTGGTGAACAGGATCGAGCTGAAGGGCATCGACTTCAAAGAGGA 420
CGGAAACATCCTGGGCCACAAGCTGGAGTACAACACTCCACAAACGTGTACATCAT 480
GGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCAGGCACAACATCGAGGA 540
CGGCTCCGTGCAGCTGGCCGACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCGT 600
      610      620      630      640      650      660
      |-----|-----|-----|-----|-----|-----|
GCTGCTGCCTGACAACCACTACCTGTCCACCCAGTCCGCCCTGTCCAAGGACCCCAACGA 660
GAAGAGGGACCACATGGTGCTGCTGGAGTTTCGTGACCGCTGCTGGCATCACCTGGGCAT 720
GGACGAGCTGTACAAGGATATCGAGCACTGGTCCTACGGCCTGAGACCCGGCGAACACTG 780
GAGCTATGGGCTGAGGCCCTGGAGAACACTGGTCTTACGGGCTGAGGCCAGGGGAGCACTG 840
GAGCTATGGACTGAGACCCGGAGAGCACTGGTCTTATGGCCTGAGGCCAGGCGAACATTG 900
      910      920      930      940      950      960
      |-----|-----|-----|-----|-----|-----|
GTCATACGGACTGAGACCTGGGGAGCATTGGAGCTACGGCCTGAGGCCCTGGGGAGCATTG 960
GTCATATGGACTGAGGCCCTGGCGAGCACTGGAGCTACGGGCTGAGACCAGGCGAACATTG 1020
GTCTTATGGCCTGAGACCAGGAGAGCACTGGTCCTACGGGCTGAGGCCCGGAGAGCACTG 1080
GTCTTACGGACTGAGACCTGGCGAGCACTGGTCATATGGACTGAGACCCGGGGAACATTG 1140
GTCTTACGGGCTGAGACCCGGCTGATGAGAATTCGGAGCTCCAGCTTTTGTTCCC 1195
    
```

pFHVFEg11

- 1-289: pFHV vector - gI
- 269-295: pFHV\_Spe\_For primer binding site (amplify 3' to 5':  
GGGACTAGTTACTGGTAATGGTTGGTCGTC)
- 290-295: SpeI (ACTAGT)
- 296-1807: FliC
- 1808-1813: BamHI (GGATCC)
- 1814-2533: EGFP-C1
- 2534-2539: EcoRV (GATATC)
- 2540-: GnRH
- 2960-2965: Two stop codons
- 2966-2971: EcoRI (GAATTC)
- 2972-2977: PstI (CTGCAG)
- 2978-3871: pFHV vector - gI

Contig 1:

|                          |             |
|--------------------------|-------------|
| Contig Length:           | 3871        |
| bases                    |             |
| Average Length/Sequence: | 1018 bases  |
| Total Sequence Length:   | 7129 bases  |
| Top Strand:              | 6 sequences |
| Bottom Strand:           | 1 sequences |
| Total:                   | 7 sequences |



910 920 930 940 950 960  
CGATAAATATCGTGTAGATATTAATTCCGGTGCTGTAGTACTGATGCAGCAGCACCGGA 960  
TAAAGTATATGTAATGCAGCAAACGGTCAGTTAACAACGACGATGCGGAAAATAACAC 1020  
TGCGGTTGATCTCTTTAAGACCACTAAATCTACTGCTGGTACCGCTGAAGCCAAAGCGAT 1080  
AGCTGGTGCCATTAAGGTGGTAAGGAAGGAGATACCTTTGATTATAAAGGCGTGACTTT 1140  
TACTATTGATACAAAACCTGGTGATGACGGTAATGGTAAGGTTTCTACTACCATCAATGG 1200

1210 1220 1230 1240 1250 1260  
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TACCTTACAATCAAGCAAAAATGTTTATACATCTGTAGTGAACGGTCAGTTTACTTTTGA 1320  
TGATAAAACCAAAAACGAGAGTGCGAAACTTTCTGATTTGGAAGCAAACAATGCTGTAA 1380  
GGGCGAAAGTAAAATTACAGTAAATGGGGCTGAATATACTGCTAACGCCACGGGTGATAA 1440  
GATCACCTTAGCTGGCAAAACCATGTTTATTGATAAAACAGCTTCTGGCGTAAGTACATT 1500

1510 1520 1530 1540 1550 1560  
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TTCTGCATTGTCAAAGTGGACGCAGTTCGTTCTTCTCTGGGGCAATTCAAAACCGTTT 1620  
TGATTCAGCCATTACCAACCTTGGCAATACGGTAACCAATCTGAACTCCGCGCGTAGCCG 1680  
TATCGAAGATGCTGACTATGCAACGGAAGTTTCTAATATGTCTAAAGCGCAGATTCTGCA 1740  
GCAGGCTGGTACTTCCGTTCTGGCGCAGGCTAACCGAGTTCCGCAAAACGTCTCTCTTT 1800

1810 1820 1830 1840 1850 1860  
ACTGCGTGGATCCACGCGTGTGTCCAAGGGCGAGGAGCTGTTACCGGCGTGGTGCCCAT 1860  
CCTGGTGGAGCTGGACGGCGACGTGAACGGCCACAAGTTCTCCGTGTCCGGCGAGGGCGA 1920  
AGGCGACGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGTACCACCGGCAAGCTGCC 1980  
CGTGCCCTGGCCCACCCTGGTGACCACCCTGACCTACGGCGTGCAGTGCTTCTCCAGATA 2040  
CCCCGACCATATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAGGGCTACGTGCA 2100

2110 2120 2130 2140 2150 2160  
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CGAAGGCGACACCCTGGTGAACAGGATCGAGCTGAAGGGCATCGACTTCAAAGAGGACGG 2220  
AAACATCCTGGGCCACAAGCTGGAGTACAACCTACAACCTCCACAACGTGTACATCATGGC 2280  
CGACAAGCAGAAGAACGGCATCAAGGTGAACCTTCAAGATCAGGCACAACATCGAGGACGG 2340  
CTCCGTGCAGCTGGCCGACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCGTGCT 2400

2410 2420 2430 2440 2450 2460  
GCTGCCTGACAACCGCTACCTGTCCACCCAGTCCGCCCTGTCCAAGGACCCCAACGAGAA 2460  
GAGGGACCACATGGTGTGCTGGAGTTCGTGACCGCTGCTGGCATCACCTGGGCATGGA 2520  
CGAGCTGTACAAGGATATCGAGCACTGGTCTACGGCTGAGACCCGGCGAACACTGGAG 2580  
CTACGGGCTGAGGCCTGGAGAACACTGGTCTTACGGGCTGAGGCCAGGGGAGCACTGGAG 2640  
CTATGGACTGAGACCCGGAGAGCACTGGTCTTATGGCCTGAGGCCAGGCGAACATTGGTC 2700

2710 2720 2730 2740 2750 2760  
ATACGGACTGAGACCTGGGGAGCATTGGAGCTACGGCCTGAGGCCTGGGGAGCATTGGTC 2760  
ATATGGACTGAGGCCTGGCGAGCACTGGAGCTACGGGCTGAGACCAGGCGAACATTGGTC 2820  
TTATGGCCTGAGACCAGGAGAGCACTGGTCCTACGGGCTGAGGCCCGGAGAGCACTGGTC 2880  
TTACGGACTGAGACCTGGCGAGCACTGGTCATATGGACTGAGACCCGGGGAACATTGGTC 2940  
TTACGGGCTGAGACCCGGCTGATGAGAATTCTGCAGATACGCAAGCATATAAATGGTCTG 3000  
3010 3020 3030 3040 3050 3060  
TGCTGGGCGGCGGATCTATATACCAAACAACGATGGGCGACCATCAACGGAAATGACACG 3060  
GTTTACTCGCCAGACTAAACCATCGAATTCGAGTTCCAAGTCCCTACTCGATGTCCCCAG 3120  
ATCTTCGAATTCACCCCAACCGATGGCGTCTCTAGAAGTCAGTTAACCGTAGTTAACGA 3180  
AGAAACCTAATATATTTATAAACAATAAAATACTTTTTAAAAATGGATATCTGGTCATGT 3240  
GTAATGTTGACGCATAGTGGGTGGTGACCTAAGATTATATTAATAATGTAGAAGGTTTTAT 3300  
3310 3320 3330 3340 3350 3360  
GCCCAGTTCACAGTATCTACTGTGACCTACCCCGGGGTGGTAATAACAATACTATCGAAT 3360  
AGCCAACAATGGGACTGCTTGTACCATCCTCGTGATATTATTGATTGTTACTTCATCAA 3420  
GTTCTACTATTCATCAAGTAACGATGACAGAAGGTGCCGCACTTTTATACGATGGTGATG 3480  
GGATCGACCCACCTTTAAACTAACTTCACATTTTTTTGCGAGGTTGGACATTTTCTAGAG 3540  
ACTCCCGAAAGGATGTACAGGATAGGTGAGTGTTCTAAAAGTATGTATTAGATCGTGGGG 3600  
3610 3620 3630 3640 3650 3660  
GTATGTCCGGATGATATCGTTATAAATAACAGAAGTGGGTACACAATGCTGGAAACCCC 3660  
ACTTAGCGTTGGCGGATTTGGGCATTTCTAAAAGTTCTCTCATCCGAACCAAAGGACTAT 3720  
TATTTTCGTGGAATAGAACGTGTTTCAAATTCCTCACCCCGAAAAAGTAGGCCTTGGTAT 3780  
TCAGGGTGGCCACTATGATAATATCCGGGATATATACCCCTGGCTGGAGCCCGGTGATAA 3840  
TGGGAGGCAGTCCATCATCCATACTTTTTTTT 3871

pFHVEG2.seq

- 1-183: pFHVgI up to point of insertion
- 184-189: SpeI added
- 190-195: MluI added
- 196-909: EGFP
- 910-915: EcoRV
- 916-1335: GnRH
- 1336-1341: Two stop codons
- 1342-1347: EcoRI
- 1348-1353: PstI added using EG\_Rev\_Pst Primer
- 1327-1353: EG\_Rev\_Pst primer binding site

^^: 353,1960

"Contig 1" (1,1967)

Contig Length: 1967 bases  
 Average Length/Sequence: 1084 bases  
 Total Sequence Length: 6507 bases  
 Top Strand: 5 sequences  
 Bottom Strand: 1 sequences  
 Total: 6 sequences

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      10      20      30      40      50      60
      |-----|-----|-----|-----|-----|-----|
ATGTCGTCGATAGCCTTCATCTATATATTGATGGCGATTGGAACAGTTTATGGGATTGTG 60
TATCGTGGAGATCATGTAAGYCTTCATGTTGATAACAAGCTCCGGCTTTGTAATATATCCA 120
ACACTGGAGAATTTTACGATCTACGGCCATCTAATCTTTCTCGACGACCAACCATTACCA 180
GTAAGTAGTACGCGTGTGTCCAAGGGCGAGGAGCTGTTACCGGCGTGGTGCCCATCCTG 240
GTGGAGCTGGACGGCGACGTGAACGGCCACAAGTTCTCCGTGTCCGGCGAGGGCGAAGGC 300
      310      320      330      340      350      360
      |-----|-----|-----|-----|-----|-----|
GACGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGTACCACCGGCAAGCTGCCCGTG 360
CCCTGGCCCACCTGGTGACCACCCTGACCTACGGCGTGAGTGCTTCTCCAGATACCCC 420
GACCATATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAGGGCTACGTGCAGGAG 480
AGGACCATCTTCTTCAAGGACGACGGCAACTACAAGACCAGGGCCGAGGTGAAGTTCGAA 540
GGCGACACCCTGGTGAACAGGATCGAGCTGAAGGGCATCGACTTCAAAGAGGACGGAAAC 600
      610      620      630      640      650      660
      |-----|-----|-----|-----|-----|-----|
ATCCTGGGCCACAAGCTGGAGTACAACACTACAACCTCCCACAACGTGTACATCATGGCCGAC 660
AAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCAGGCACAACATCGAGGACGGCTCC 720
GTGCAGCTGGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTG 780
CCTGACAACCACTACCTGTCCACCCAGTCCGCCCTGTCCAAGGACCCCAACGAGAAGAGG 840
GACCACATGGTGTGCTGGAGTTCGTGACCGCTGCTGGCATCACCTGGGCATGGACGAG 900
    
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910 920 930 940 950 960

CTGTACAAGGATATCGAGCACTGGTCCTACGGCCTGAGACCCGGCGAACACTGGAGCTAT 960  
GGGCTGAGGCCTGGAGAACAACACTGGTCTTACGGGCTGAGGCCAGGGGAGCACTGGAGCTAT 1020  
GGACTGAGACCCGGAGAGCACTGGTCTTATGGCCTGAGGCCAGGCCAACATTGGTCATAC 1080  
GGACTGAGACCTGGGGAGCATTGGAGCTACGGCCTGAGGCCTGGGGAGCATTGGTCATAT 1140  
GGACTGAGGCCTGGCGAGCACTGGAGCTACGGGCTGAGACCAGGCCAACATTGGTCTTAT 1200

1210 1220 1230 1240 1250 1260

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GGCCTGAGACCAGGAGAGCACTGGTCCTACGGGCTGAGGCCCGGAGAGCACTGGTCTTAC 1260  
GGACTGAGACCTGGCGAGCACTGGTCATATGGACTGAGACCCGGGGAACATTGGTCTTAC 1320  
GGGCTGAGACCCGACTGATGAGAATTCCTGCAG 1353

1-211: pRSetA vector  
211-216: XhoI (CTCGAG)  
217-1728: FliC  
1729-1734: BamHI (GGATCC)  
1735-2454: EGFP  
2455-2460-: EcoRV (GATATC)  
2461-2880: GnRH  
2881-2886: Two stop codons  
2887-2892: EcoRI (GAATTC)  
2893-2898: PstI (CTGCAG)  
2899-5571: pRSetA vector

Contig 1:

Contig Length: 5571 bases  
Average Length/Sequence: 1774 bases  
Total Sequence Length: 10646 bases  
Top Strand: 5 sequences  
Bottom Strand: 1 sequences  
Total: 6 sequences

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      10      20      30      40      50      60      70
+-----+
GATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATT 70
TTGTTTAACTTTAAGAAGGAGATATACATATGCGGGGTTCTCATCATCATCATCATGGTATGGCTAG 140
CATGACTGGTGGACAGCAAATGGGTTCGGGATCTGTACGACGATGACGATAAGGATCGATGGGGATCCGAG 210
CTCGAGGCACAAGTCATTAATAACAACAGCCTGTGCGCTGTTGACCCAGAATAACCTGAACAAATCTCAGT 280
CCTCACTGAGTTCGGCTATTGAGCGTCTGTCTCTGGTCTGCGTATCAACAGCGCGAAAGACGATGCGGC 350
      360      370      380      390      400      410      420
+-----+
AGGCCAGGCGATTGCTAACCGCTTCACTTCTAATATCAAAGGTCTGACTCAGGCTTCCCGTAACGCTAAC 420
GACGGCATTCTATTGCGCAGACCACTGAAGGTGCGCTGAATGAAATCAACAACAACCTGCAGCGTGTGC 490
GTGAGTTGTCTGTTTCAGGCCACTAACGGGACTAACTCTGATTCCGATCTGAAATCTATCCAGGATGAAAT 560
TCAGCAACGTCTGGAAGAAATCGATCGCGTTTCTAATCAGACTCAATTTAACGGTGTAAAGTCCTGTCT 630
CAGGACAACCAGATGAAAATCCAGGTTGGTGCTAACGATGGTGAAACCATTACCATCGATCTGCAAAAAA 700
      710      720      730      740      750      760      770
+-----+
TTGATGTGAAAAGCCTTGGCCTTGATGGGTTCAATGTTAATGGGCCAAAAGAAGCGACAGTGGGTGATCT 770
GAAATCCAGCTTCAAGAAATGTTACGGGTTACGACACCTATGCAGCGGGTGCCGATAAATATCGTGTAGAT 840
ATTAATTCGGTGCTGTAGTGACTGATGCAGCAGCACCGGATAAAGTATATGTAAATGCAGCAAACGGTC 910
AGTTAACAACACTGACGATGCGGAAAATAAACAACACTGCGGTTGATCTCTTTAAGACCACTAAATCTACTGCTGG 980
TACCGCTGAAGCCAAAGCGATAGCTGGTGCCATTAAGGTGGTAAGGAAGGAGATACCTTTGATTATAAA 1050
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1060 1070 1080 1090 1100 1110 1120  
GGCGTGACTTTTACTATTGATACAAAACTGGTGATGACGGTAATGGTAAGGTTTCTACTACCATCAATG 1120  
GTGAAAAAGTTACGTTAACTGTCGCTGATATTGCCACTGGCGCGACGGATGTTAATGCTGCTACCTTACA 1190  
ATCAAGCAAAAATGTTTATACATCTGTAGTGAACGGTCAGTTTACTTTTGATGATAAAACCAAAAACGAG 1260  
AGTGCGAAACTTTCTGATTTGGAAGCAAACAATGCTGTTAAGGGCGAAAGTAAAATTACAGTAAATGGGG 1330  
CTGAATATACTGCTAACGCCACGGGTGATAAGATCACCTTAGCTGGCAAACCATGTTTATTGATAAAAC 1400

1410 1420 1430 1440 1450 1460 1470  
AGCTTCTGGCGTAAGTACATTAATCAATGAAGACGCTGCCGCAGCCAAGAAAAGTACCGCTAACCCACTG 1470  
GCTTCAATTGATTCTGCATTGTCAAAGTGGACGCAGTTCGTTCTTCTCTGGGGGCAATTCAAACCGTT 1540  
TTGATTCAGCCATTACCAACCTTGGAATACGGTAACCAATCTGAACTCCGCGCGTAGCCGTATCGAAGA 1610  
TGCTGACTATGCAACGGAAGTTTCTAATATGTCTAAAGCGCAGATTCTGCAGCAGGCTGGTACTTCCGTT 1680  
CTGGCGCAGGCTAACCCAGGTTCCGCAAACGTCCTCTCTTTACTGCGTGGATCCACGCGTGTGTCCAAGG 1750

1760 1770 1780 1790 1800 1810 1820  
GCGAGGAGCTGTTACCGGCGTGGTGCCCATCCTGGTGGAGCTGGACGGCGACGTGAACGGCCACAAGTT 1820  
CTCCGTGTCCGGCGAGGGCGAAGGGCGACGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGTACCACC 1890  
GGCAAGCTGCCCGTGCCCTGGCCACCCCTGGTGACCACCCCTGACCTACGGCGTGCAGTGCTTCTCCAGAT 1960  
ACCCCGACCATATGAAGCAGCAGACTTCTTCAAGTCCGCCATGCCCGAGGGCTACGTGCAGGAGAGGAC 2030  
CATCTTCTTCAAGGACGACGGCAACTACAAGACCAGGGCCGAGGTGAAGTTCGAAGGCGACACCCTGGTG 2100

2110 2120 2130 2140 2150 2160 2170  
AACAGGATCGAGCTGAAGGGCATCGACTTCAAAGAGGACGGAAACATCCTGGGCCACAAGCTGGAGTACA 2170  
ACTACAACCTCCACAACGTGTACATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGAT 2240  
CAGGCACAACATCGAGGACGGCTCCGTGCAGCTGGCCGACCACTACCAGCAGAACACCCCCATCGGCGAC 2310  
GGCCCCGTGCTGCTGCCTGACAACCACTACCTGTCCACCCAGTCCGCCCTGTCCAAGGACCCCAACGAGA 2380  
AGAGGGACCACATGGTGTGCTGGAGTTCGTGACCGCTGCTGGCATCACCCCTGGGCATGGACGAGCTGTA 2450

2460 2470 2480 2490 2500 2510 2520  
CAAGGATATCGAGCACTGGTCTTACGGCCTGAGACCCGGCGAACACTGGAGCTATGGGCTGAGGCCTGGA 2520  
GAACACTGGTCTTACGGGCTGAGGCCAGGGGAGCACTGGAGCTATGGACTGAGACCCGGAGAGCACTGGT 2590  
CTTATGGCCTGAGGCCAGGCCAACATTGGTTCATACGGACTGAGACCTGGGGAGCATTGGAGCTACGGCCT 2660  
GAGGCCTGGGGAGCATTGGTTCATATGGACTGAGGCCTGGCGAGCACTGGAGCTACGGGCTGAGACCAGGC 2730  
GAACATTGGTCTTATGGCCTGAGACCAGGAGAGCACTGGTCTTACGGGCTGAGGCCCGGAGAGCACTGGT 2800

2810 2820 2830 2840 2850 2860 2870  
CTTACGGACTGAGACCTGGCGAGCACTGGTTCATATGGACTGAGACCCGGGGAACATTGGTCTTACGGGCT 2870  
GAGACCCGGCTGATGAGAATTCCTGCAGCTGGTACCATGGAATTCGAAGCTTGATCCGGCTGCTAACAAA 2940  
GCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTA 3010  
AACGGGTCTTGAGGGGTTTTTGTGAAAGGAGGAACATATCCGGATCTGGCGTAATAGCGAAGAGGCC 3080  
CGCACCGATCGCCCTTCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCGCAT 3150

3160 3170 3180 3190 3200 3210 3220  
TAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCC 3220  
TTTCGCTTTCTTCCCTTCTTTCTCGCCACGTTTCGCCGGCTTTCCCGTCAAGCTCTAAATCGGGGGCTC 3290  
CCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCAAAAAACTTGATTAGGGTGATGGTTCAC 3360  
GTAGTGSYCATCGCCCTGATAGACGGTTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGG 3430  
ACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTG 3500  
3510 3520 3530 3540 3550 3560 3570  
CCGATTTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATAT 3570  
TAACGCTTACAATTTAGGTGGCACTTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTCTAA 3640  
ATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGA 3710  
AGAGTATGAGTATCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTTCGGGCATTTTGCCTTCTGTTTT 3780  
TGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATC 3850  
3860 3870 3880 3890 3900 3910 3920  
GAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTTCGCCCGAAGAACGTTTTTCCAATGATGAGCA 3920  
CTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTGCGCCG 3990  
CATACACTATTCTCAGAAATGACTTGGTTGAGTACTCACAGTACAGAAAAGCATCTTACGGATGGCATG 4060  
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CGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCG 4200  
4210 4220 4230 4240 4250 4260 4270  
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ATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGT 4480  
ATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAG 4550  
4560 4570 4580 4590 4600 4610 4620  
GTGCCTCACTGATTAAGCATTGGTAACCTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAA 4620  
ACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTTGATAATCTCATGACCAAATCCCTTAA 4690  
CGTGAGTTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTT 4760  
TTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACCACCGCTACCAGCGGTGGTTTTGTTTGCCTGATCA 4830  
AGAGCTACCAACTCTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTA 4900  
4910 4920 4930 4940 4950 4960 4970  
GTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCC 4970  
TGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACC 5040  
GGATAAGGCGCAGCGGTGCGGCTGAACGGGGGGTTCGTGCACACAGCCAGCTTGGAGCGAACGACCTAC 5110  
ACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGCGGACA 5180  
GGTATCCGGTAAGCGGCAGGGTTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTA 5250



pRSetAEG2

- 1-211: pRSetA vector
- 202-207: BamHI (GGATCC)
- 208-927: EGFP
- 928-933: EcoRV (GATATC)
- 934-2880: GnRH
- 1354-1359: Two stop codons
- 1360-1365: EcoRI (GAATTC)
- 1366-1371: PstI (CTGCAG)
- 1372-4044: pRSetA vector

Contig 1:

Contig Length: 5571 bases  
 Average Length/Sequence: 1774 bases  
 Total Sequence Length: 10646 bases  
 Top Strand: 5 sequences  
 Bottom Strand: 1 sequences  
 Total: 6 sequences

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      10      20      30      40      50      60
      |-----|-----|-----|-----|-----|
GATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCT 60
AGAAATAAATTTTGTAACTTTAAGAAGGAGATATACATATGCGGGTTCTCATCATCAT 120
CATCATCATGGTATGGCTAGCATGACTGGTGGACAGCAAATGGGTTCGGGATCTGTACGAC 180
GATGACGATAAGGATCGATGGGGATCCACGCGTGTGTCCAAGGGCGAGGAGCTGTTACCC 240
GGCGTGGTGGCCATCCTGGTGGAGCTGGACGGCGACGTGAACGGCCACAAGTTCTCCGTG 300
      310      320      330      340      350      360
      |-----|-----|-----|-----|-----|
TCCGGCGAGGGCGAAGGCGACGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGTACC 360
ACCGGCAAGCTGCCCCGTGCCCTGGCCACCCTGGTGACCACCCTGACCTACGGCGTGCAG 420
TGCTTCTCCAGATACCCCGACCATATGAAGCAGCAGACTTCTTCAAGTCCGCCATGCCC 480
GAGGGCTACGTGCAGGAGAGGACCATCTTCTTCAAGGACGACGGCAACTACAAGACCAGG 540
GCCGAGGTGAAGTTCGAAGGCGACACCCTGGTGAACAGGATCGAGCTGAAGGGCATCGAC 600
      610      620      630      640      650      660
      |-----|-----|-----|-----|-----|
TTCAAAGAGGACGGAACATCCTGGGCCACAAGCTGGAGTACAACACTACAACCTCCCAAC 660
GTGTACATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACCTTCAAGATCAGGCAC 720
AACATCGAGGACGGCTCCGTGCAGCTGGCCGACCACTACCAGCAGAACACCCCATCGGC 780
GACGGCCCCGTGCTGCTGCCTGACAACCACTACCTGTCCACCCAGTCCGCCCTGTCCAAG 840
GACCCCAACGAGAAGAGGGACCACATGGTGTCTGCTGGAGTTCGTGACCGCTGCTGGCATC 900
      910      920      930      940      950      960
      |-----|-----|-----|-----|-----|
ACCCTGGGCATGGACGAGCTGTACAAGGATATCGAGCACTGGTCCTACGGCCTGAGACCC 960
GGCGAACACTGGAGCTATGGGCTGAGGCCTGGAGAACACTGGTCTTACGGGCTGAGGCCA 1020
GGGGAGCACTGGAGCTATGGACTGAGACCCGGAGAGCACTGGTCTTATGGCCTGAGGCCA 1080
GGCGAACATTGGTCATACGGACTGAGACCTGGGGAGCATTGGAGCTACGGCCTGAGGCCT 1140
GGGGAGCATTGGTCATATGGACTGAGGCCTGGCGAGCACTGGAGCTACGGGCTGAGACCA 1200
    
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1210 1220 1230 1240 1250 1260  
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GGAGAGCACTGGTCTTACGGACTGAGACCTGGCGAGCACTGGTCATATGGACTGAGACCC 1320  
GGGGAACATTGGTCTTACGGGCTGAGACCCGGCTGATGAGAATTCCTGCAGCTGGTACCA 1380  
TGAATTTCGAAGCTTGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCT 1440  
GCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGT 1500

1510 1520 1530 1540 1550 1560  
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ATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCG 1620  
CATTAAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCC 1680  
TAGCGCCCGCTCCTTTTCGCTTTCTTCCCTTCCCTTCTCGCCACGTTCCGCCGGCTTTCCC 1740  
GTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCGGATTTAGTGCTTTACGGCACCTCG 1800

1810 1820 1830 1840 1850 1860  
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GAACAACACTCAACCCTATCTCGGTCTATTCTTTTGAATTTATAAGGGATTTTGCCGATTT 1980  
CGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAA 2040  
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2110 2120 2130 2140 2150 2160  
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2410 2420 2430 2440 2450 2460  
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CAACATGGGGGATCATGTAACCTGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCAT 2700

2710 2720 2730 2740 2750 2760  
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ATTAACGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGC 2820  
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TAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACG 3000

3010 3020 3030 3040 3050 3060  
AAATAGACAGATCGCTGAGATAGGTGCCCTCACTGATTAAGCATTGGTAACTGTCAGACCA 3060  
AGTTTACTCATATATACTTTAGATTGATTTAAAACCTTCATTTTTTAATTTAAAAGGATCTA 3120  
GGTGAAGATCCTTTTTGATAATCTCATGACCAAATCCCTTAACGTGAGTTTTTCGTTCCA 3180  
CTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCG 3240  
CGTAATCTGCTGCTTGCAAACAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGA 3300

3310 3320 3330 3340 3350 3360  
TCAAGAGCTACCAACTCTTTTTCCGAAGGTAACGGCTTCAGCAGAGCGCAGATACCAAA 3360  
TACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCC 3420  
TACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTG 3480  
TCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTGCGGGCTGAAC 3540  
GGGGGGTTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCT 3600

3610 3620 3630 3640 3650 3660  
ACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCC 3660  
GGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTG 3720  
GTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATG 3780  
CTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCT 3840  
GGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGA 3900

3910 3920 3930 3940 3950 3960  
TAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCG 3960  
CAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGC 4020  
GCGTTGGCCGATTCATTAATGCAG 4044

## **CURRICULUM VITAE**

Kerry was born on December 17, 1974, in Silver Spring, MD. She grew up in Annapolis, MD, graduating with a high school diploma from Key School in 1992.

Kerry was then accepted to Virginia Tech, in Blacksburg, VA, to pursue a Bachelor of Science in Biology, graduating in May 1996.

After graduation, Kerry worked for 2 years in the Department of Animal and Poultry Sciences at Virginia Tech followed by 2 years in the Department of Molecular Virology and Microbiology at Baylor College of Medicine in Houston, TX.

After returning to Virginia Tech to work for 1 year as a Research Assistant in the Department of Plant Pathology, Physiology, and Weed Science, Kerry was accepted to the Virginia-Maryland Regional College of Veterinary Medicine, to pursue a Masters of Science in Biomedical and Veterinary Sciences under Dr. Stephen Boyle in 2004. Kerry completed a concurrent Masters of Science in Health Promotion and Education in 2005 at Virginia Tech.

Upon completion of her Masters in Science in Biomedical and Veterinary Sciences, Kerry, her puppies (Maggie and Nico), and her cat (Thneed) plan to relocate to Chapel Hill, NC where they will venture forth into the next exciting stage of their life.