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## Determining gene copy number in transfected caprine fibroblast cells

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**DETERMINING GENE COPY NUMBER IN TRANSFECTED CAPRINE  
FIBROBLAST CELLS**

**A Thesis**

**Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science**

**in**

**The Interdepartmental Program in  
the School of Animal Sciences**

**by  
Jessica A. Wilson  
B.S., Oregon State University, 2006  
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## LIST OF ABBREVIATIONS

AI.....	Artificial Insemination
ANOVA.....	Analysis of Variance
BAC.....	Bacterial Artificial Chromosome
bFGF.....	Bovine Fibroblast Growth Factor Basic
CGH.....	Comparative Genomic Hybridization
CF1.....	Caprine Fibroblast Cells
C <sub>T</sub> .....	Threshold Cycle
DMEM.....	Dulbecco's Modified Eagle Medium
dPBS.....	Dulbecco's Phosphate-Buffered Saline
EMA.....	European Medicines Agency
FBS.....	Fetal Bovine Serum
FISH.....	Fluorescence In Situ Hybridization
G418.....	Geneticin
HAC.....	Human Artificial Chromosome
ICSI.....	Introcrytoplasmic Sperm Injection
IF-1.....	Inhibitory Factor
IVF.....	In Vitro Fertilization
LTR.....	Long Terminal Repeats
MAPH.....	Multiplex Amplifiable Probe Hybridization
MLV.....	Murine Leukemia Virus
NLS.....	Nuclear Localization Signal
NPC.....	Nuclear Pore Complex
NT.....	Nuclear Transfer
PAC.....	P1-based Artificial Chromosome
Q-PCR.....	Quantitative Polymerase Chain Reaction
RCL.....	Replication-Competent Lentivirus
RT-PCR.....	Reverse Transcriptase Polymerase Chain Reaction
SCNT.....	Somatic Cell Nuclear Transfer
SEM.....	Standard Error of the Mean
SMGT.....	Sperm-Mediated DNA Transfer
TGMT.....	Testis-Mediated Gene Transfer
μF.....	Microfarads
YAC.....	Yeast Artificial Chromosome

## ABSTRACT

Transgene expression in stably transgenic organisms is affected by many factors, including the copy number of the transgene in the genome, and by interactions between the transgene and flanking DNA sequences. Transgene copy number has also been shown to effect genetic stability in transgenic plants. Two commonly used methods for transfecting cells are liposome-mediated transfection and electroporation. Little is known about the mean transgene copy number or variability of the copy number with these techniques. Quantitative PCR (Q-PCR) has been shown to be an effective method for determining transgene copy number.

The objective of this study was to determine transgene copy number after liposome mediated transfection and electroporation. The mean transgene copy number and variability between individual integration events have been determined.

Q-PCR conditions were optimized for primer annealing temperature and concentration when amplifying a region of the plasmid hEGFP used for transfection. The quantitative nature of the Q-PCR reaction was confirmed by amplifying 10-fold dilutions of the plasmid and plotting the threshold cycle ( $C_T$ ) value against the log of the plasmid concentration. A correlation coefficient of 1.00 and a calculated PCR efficiency of 93.3% were obtained from this analysis. Caprine fibroblasts were transfected by electroporation or FuGENE<sup>®</sup> HD reagent with either a circular or linearized hEGFP plasmid and plated at low density in medium containing Geneticin<sup>®</sup>. After 10 days of culture, single cell colonies were isolated and expanded. When cultures reached 1-2 million cells, genomic DNA was isolated. Transgene copy number was determined by amplifying genomic DNA from individual clones representing  $1 \times 10^5$  cells with Q-PCR. Transgene copy number was calculated by comparing  $C_T$  values to a standard curve. The transgene copy number for electroporation circular was  $2.7 \pm 0.75$  (n=32) and  $1.3 \pm 0.65$  (n=19) when using a linear DNA construct. FuGENE<sup>®</sup> HD using a circular plasmid construct generated a gene copy number of  $0.5 \pm 0.11$  (n=14) and  $0.64 \pm 0.13$  (n=16) for the linear

plasmid construct. There were significant differences when comparing electroporation circular to all other treatments, however, there were no differences when comparing electroporation linear, FuGENE<sup>®</sup> HD circular and FuGENE<sup>®</sup> HD linear to each other.

## CHAPTER I

### INTRODUCTION

There is high demand for the production of recombinant DNA for utilization in therapeutic drugs. The use of transgenic animals is one method that has been proposed to supply this need. Nuclear transfer (NT) allows researchers to manipulate the genome of livestock, resulting in offspring with desired genetic modifications, however, NT has an extremely low success rate ranging from 0.2% to 3.4% of manipulated embryos giving rise to live offspring (Yanagimichi, 2002). There have been several hypotheses that have examined why NT and other in vitro manipulated embryos have low development rates, including: abnormal expression of transcription factors, epigenetic changes such as DNA methylation, reprogramming of the somatic cell nucleus, chromatin remodeling and the culture environment. Due to the limited success of NT embryos and the many factors affecting the development of embryos in culture, it is important to optimize a transfection protocol to ensure high gene expression levels in transgenic animals.

It is theorized that there is a high correlation between gene expression and transgene copy number in transgenic animals. It is imperative for transgenes to be successful; they need to integrate into the genome, not only intact, but without mutation, however, recent evidence indicates that DNA transferred by a number of chemical methods is subject to mutations and rearrangements, possibly as a result of passage through the lysosomal compartment (Toneguzzo et al. 1988). Transgene expression level in stably transgenic organisms is affected by many factors; in particular by the promoter driving the transgene, the copy number of the transgene in the genome, and by interaction between transgene and flanking sequence DNA (Rahman et al., 2000). We hypothesize that gene expression levels can be increased when using a transfection method that does not alter or mutate the transgene.

The majority of research looking at transgene copy number has been performed in transgenic plants. Even though the plant model is drastically different from the domestic animal

model, there are some important similarities, such as the design and transformation of the transgene. Transgenic plants are usually produced by one of two methods: particle bombardment and agrobacterium. These methods are considerably effective in transgenic plant production; however, these methods are not effective in the production of transgenic animals.

The overall goal of this project is to improve *in vivo* expression and stability of transgenes introduced into the genome by NT. More specifically, we hope to determine how the method of transfection affects gene copy number. The results of this study will aid in the production of therapeutic drugs and other transgenic animal applications.

## CHAPTER II

### LITERATURE REVIEW

#### Production of Transgenic Animals

A transgenic animal is defined as an animal containing recombinant DNA molecule in its genome that was introduced by intentional human intervention (Wall, 1996). The limited success of producing transgenic livestock does not reflect the need for this technology but rather the high costs and low efficiencies of these procedures. Transgenesis provides the ability to study models for single gene function and human diseases, production of pharmaceutical proteins such as humanized recombinant antibodies, hormones, enzymes, vaccines, etc., improvement of animal production and health, and the adaption of pig organs or tissues for transplantation in humans (Bacci, 2007).

Transgenesis presents a mechanism by which economically important traits can be attained more rapidly than by selective breeding without concern of propagating associated, possibly undesirable, genetic characteristics (Wall, 1996). Genes can be transferred across species boundaries and can be modified to function very differently than they do in their native form. Gene products, tissue specificity, and the timing of expression can all be altered. The ability to redirect expression of genes initiated the production of transgenic animals for use in the biomedical industry and for food production (Wall, 1996).

There are two different forms of modifying the genome of an animal; gain of function or loss of function. A gain of function is the production of a new protein, or the expression of an existing protein at a higher level or in a different range of cells. The loss of function or “knockout” is elimination of a gene or the deletion of a functional domain of the protein. “Knockout” animals are produced by homologous recombination that completely removes one or more exons from a gene, resulting in the production of a mutated protein or no protein at all.

The new genetic information is introduced into the genome of animals by means of a transgene. Transgenes are composed of a genetic regulatory element also known as promoters

that determine the tissue in which the gene is to be expressed, the timing of expression, and the degree of expression (Wall, 1996). The regulatory element can be controlled externally by the addition of a switch, allowing the transgene to be turned on and off. The second addition to the transgene construct is the structural component of the transgene, which includes the DNA sequence that encodes for the desired protein. A majority of early research in this area focuses on the insertion of genes that encode for growth hormones.

There are several different methods that can be used to produce transgenic animals: microinjection of DNA into the pronucleus, DNA transfer using retroviral vectors, somatic cell nuclear transfer and microchromosome transfer. All of these techniques are used to produce recombinant proteins in different animal systems including blood, urine, seminal plasma, egg whites, milk and even in the silk worm cocoon (Houdebine, 2000).

#### Pronuclear Microinjection

Microinjection was the first gene transfer technique specifically designed to produce germline transgenic animals (Gordon et al., 1980). However, pronuclear microinjection was not the first gene transfer technique used to introduce foreign DNA into embryos (Brackett et al., 1971). Microinjection of cloned DNA directly into a pronucleus was first defined in 1981, and remains the method of choice for prolific species such as the mouse, rat, rabbit and pig (Houdebine, 2000). Pronuclear microinjection has also been successful in sheep (Hammer et al., 1985), goats (Ebert et al., 1991) and cows (Bondioli et al., 1991). After injection, the multiple DNA molecules arrange into a concatomer, which will stably integrate into the host genome by homologous recombination (Jaenisch, 1988). It has also been proposed that random chromosome breaks, possibly caused by repair enzymes that are induced by free ends of the injected DNA molecules, may serve as integration sites of the transgene (Brinster et al., 1985). If the transgene is integrated at the pronuclear stage, then all cells of the resulting animal will contain the exogenous DNA. Microinjection of DNA into the host genome does not always result in stable integration at the pronuclear stage, but at one or more cellular divisions later. This



usually results in a mosaic animal, meaning that not all the cells contain the transgene (Palmiter et al., 1984). When integration does occur it can cause rearrangements, deletions, duplications, or translocations at the site of integration (Mark et al., 1985; Mahon et al., 1988). Unfortunately with microinjection, not all animals produced, or their offspring, will have the transgene because of the random integration of the transgene into the genome.

The first biopharmaceutical product produced by a transgenic animal to receive European Medicines Agency (EMA) approval was ATryn, a recombinant form of human antithrombin. ATryn was produced in the milk of transgenic goats created by pronuclear microinjection.

### DNA Transfer Using Viral Vectors

The first successful foreign DNA transfer in a mammal was accomplished using viral DNA (Jaenisch et al., 1974). There are two types of viral vectors that have been developed for the production of transgenic animals: vectors derived from the genome of prototypic retrovirus such as Murine leukemia virus (MLV) and vectors derived from the genome of a more complex retrovirus; such as lentiviruses. The main difference between the prototypic retroviruses and lentiviruses: when used as vectors the lentiviral genome can be actively transported into the nucleus, allowing it to integrate transgenes to non-dividing cell types (Follenzi et al., 2000).

MLV vectors have some major disadvantages even though they have been extremely successful in producing transgenic animals in a wide variety of species, including livestock. First, the transgenes delivered by retroviral vectors cannot be expressed in transgenic animals (Jahner et al., 1985). Silencing of the transgene is induced when the promoter and enhancer elements of the retroviral long terminal repeats and the subsequent hypermethylation of the viral promoter sequences by de novo DNA methylations recruits gene repression machinery (Jahner et al., 1985). Second, the amount of exogenous DNA that can be inserted into a prototypic viral vector is limited to 10 kb, making it impossible to deliver larger genes (Brem, 1993). Third, the long terminal repeats (LTR) can interfere with the mammalian promoters, which can suppress or

misdirect the expression in the host genome (Wells et al., 2000). Lastly, the injection with prototypic viruses requires the breakdown of the nuclear envelope to enter the nucleus. If there is a delay in the timing of integration a mosaic animal will be produced (Robl et al., 2007).

The use of the lentiviral vectors broadens the range of cell types and the ability to use cells at different developmental stages for transgenesis. This breakthrough has made the lentiviral vector the most efficient method for producing transgenic animals, including livestock and poultry (Naldini et al., 1996; Poeschla et al., 1996). The prospect of using lentiviral vectors has brought forward many concerns including the recombination of the virus during production to generate a replication-competent lentivirus (RCL), the recombination with the wild type in HIV+ patients, and the possibility of insertional activation of cellular oncogenes by random integration of the vector provirus in the host genome. These concerns were the driving force behind the development of preparing the lentiviral vectors into two different constructs; the vector and the packaging constructs. The vector portion is what carries the transgene of interest and the packaging portion carries the viral proteins needed for packaging (Robl et al., 2007).

#### Sperm-mediated Gene Transfer

Brackett and colleagues in 1971 were the first to show that mammalian spermatozoa have the capability to bind with exogenous DNA. Later in 1989, Spadafora and colleagues demonstrated that these spermatozoa could be used to introduce foreign DNA into oocytes at fertilization for the production of transgenic animals. This technique has come to be referred to as sperm mediated DNA transfer (SMGT) and has proven successful in producing transgenic animals including livestock, poultry, and fish (Smith et al., 2005). The most common method is referred to as DNA incubation, which entails the incubation of seminal plasma-free sperm cells with exogenous DNA (Brackett et al., 1971). The resulting DNA-carrying sperm are then used to fertilize oocytes by either in vitro fertilization (IVF) (Bachiller et al., 1991) or artificial insemination (AI) (Gavoa et al., 1991), or in the case of aquatic animals through natural waterborne fertilization (Sarangi et al., 1999). Electroporation and liposomes have also been used to force

sperm to take up the transgenes. More recently, studies have introduced transgenes directly into the reproductive tract of male animals, either as naked DNA or encapsulated in liposomes (Bachiller et al., 1991; Gagne et al., 1991). This type of transfer is known as testis-mediated gene transfer (TMGT). The sperm take up the exogenous DNA *in vivo* and then release it during mating or AI. Another recent innovation in SGMGT has been the use of intracytoplasmic sperm injection (ICSI) to deliver transgene-containing sperm cells directly into the oocyte, a process known as transgenICSI (Perry et al., 1999). The mechanism governing foreign DNA integration during SGMGT is not well understood. It has been proposed that chromatin sites, enriched with long interspersed nuclear elements, are potential sites for foreign DNA integration (Pittoggi et al., 1999).

Despite a large amount of success, this technology is still not considered to be a reliable form of genetic manipulation. Reports of stable integration of the transgene into the genome using SGMGT are rare (Smith and Spadafora, 2005). There are two barriers that prevent SGMGT from occurring spontaneously in nature. The first barrier is an inhibitory factor (IF-1), which is abundant in the seminal fluid or can be bound to the spermatozoa membrane in marine animals. It prevents the binding of foreign molecules to the spermatozoa (Malone et al., 1997). The second barrier is a sperm endogenous nuclease activity that is turned on in a dose-dependent manner upon interaction of spermatozoa with foreign molecules (Bachiller et al., 1991). This activity causes the degradation of exogenous sequences, or when a specific DNA threshold is reached it will induce an apoptotic-like suppression of the DNA-loaded spermatozoa. The plasma membrane is believed to play a critical role in the uptake of exogenous DNA. When the plasma membrane is intact, the binding of DNA molecules to the cell surface is likely to trigger the internalization and reverse transcription of the DNA, creating episomal cDNA molecules (Smith and Spadafora, 2005). On the other hand, the insertion of DNA without contact with the plasma membrane by ICSI or liposomes, facilitating interaction with the sperm chromatin, can increase the probability of integration. Integration is believed to occur early in the sperm nucleus

or later with the formation of the male pronuclei after fertilization of the oocyte (Smith and Spadafora, 2005).

There are high variations among livestock species in success of SGM (Robl et al., 2007). Swine have the highest efficiency with nearly 80% of piglets produced carrying the transgene (Lavitrano et al., 2002), while there is limited success in producing transgenic cattle (Schellander et al., 1995). There is also insufficient success in the transmission of the transgene past the  $F_0$  generation because the transgenes may be present as episomes in the cells of the  $F_0$  animals, being subsequently lost in the next generation (Rusconi, 1990).

The male germ line has several possible positive attributes for use in generating transgenic animals. Spermatogonial stem cells have the capability to self renew and to contribute genes to the next generation. The process of spermatogenesis occurs throughout the lifetime of the adult male, theoretically allowing the male to produce more offspring than a female (Nagano et al., 2001). Nagano and colleagues in 2001 showed that spermatogonial stem cells from adult and immature mice transfected *in vitro* with a retroviral vector resulted in stable integration and expression of a transgene in 2 to 20% of stem cells. After transplantation of the transformed stem cells into the testes of infertile mice, ~ 4.5% of the offspring were transgenic, and the transgene was transferred to and expressed in later generations (Nagano et al., 2001).

### Somatic Cell Nuclear Transfer

The technique of nuclear transfer was originally proposed 70 years ago as a method to study cellular differentiation and was mostly limited to amphibians (Spemann, 1938). It was not until the early 1980s that mammalian cloning was even demonstrated (McGrath and Solter, 1983). In 1986, Willadsen was able to show that blastomere nuclei of sheep embryos at the 8-16-cell stage were competent to support full development after transfer in to enucleated metaphase II oocytes. Somatic cell nuclear transfer (SCNT) is a technique in which the nucleus

of a somatic cell is transferred into an enucleated metaphase-II oocyte for the generation of a new individual, who is genetically identical to the somatic cell donor.

Dolly was the first successful attempt to clone an entire animal from undifferentiated adult mammary epithelial cells (Wilmut et al., 1997). Dolly revealed that genes inactivated during tissue differentiation can be completely re-activated by a process called nuclear reprogramming, which is the ability of a differentiated nucleus to return to a totipotent status (Tian et al., 2003). The morphological and temporal developmental changes that occur in the oocyte include reduction or cessation of transcription, changes in the nuclear structure (nuclear lamins), chromatin structure, nucleolar morphology and stage specific protein synthesis (Fulka et al., 1996; Campbell and Wilmut, 1997).

SCNT may be used to produce genetically superior livestock, transgenic animals for pharmaceutical protein production, xenotransplantation, or to preserve endangered species. SCNT has also become an essential tool for studying gene function (Capecchi et al., 2000), genomic imprinting (Solter, 1998), genomic reprogramming (De Sousa et al., 1999; Munsie et al., 2000; Surani, 2001; Winger et al., 2002), regulation of development, and genetic diseases (Tian et al., 2003).

One of the most difficult challenges facing SCNT is the low efficiencies and high incidence of developmental abnormalities. There are many factors that contribute to the development of reconstructed embryos, including the quality of the recipient oocyte, method and time of activation, and culture methods (Polejaeva and Campbell, 2000). Also, the induction and maintenance of pregnancy is dependent upon a range of factors influenced both by the quality of the transferred embryos and the age, seasonality, and hormonal status of the recipient (Polejaeva and Campbell, 2000). All of these factors may play a role in the ability of a cloned embryo to develop to term. The most common developmental defects include abnormalities in the fetus and placenta, high pregnancy losses, increased birth rates, and, in general, low survivability (Wolf et al., 1998). Increased birth weights of transgenic and cloned offspring has

been reported after nuclear transfer protocols that utilized both cultured adult or fetal cells (Wilmut et al., 1997; Schnieke et al., 1997) and blastomeres cells from 16 to 32 cell embryos (Wilson et al., 1995; Kruij and den Daas, 1997) as the donor nuclei. It is unknown whether the occurrence of large offspring syndrome is caused directly from nuclear transfer or from the culture system in which these embryos exist after manipulation. Most nuclear transfer embryos are cultured with *in vitro* protocols that are known to stunt the growth and development (Yazawa et al., 1997) or the asynchrony transfer of a manipulated embryo into a ligated oviduct (Young et al., 1998). The ligation of the oviduct may expose the embryo to unusual secretions affecting the development of the embryo (Young et al., 1998).

Large offspring syndrome has also been reported to occur after manipulation of the maternal diet (Young et al., 1998). In a study by McEvoy and colleagues in 1997, it was shown that when ewes are fed excess amounts of non-protein nitrogen in the form of urea from 21 days before mating to day 63 of gestation resulted in oversized lambs at birth. It is not known whether the oocyte or embryo (or both) was affected because of the long period exposure to urea (Young et al., 1998).

The exact cause of large offspring is still unknown; there has been no direct correlation between the different embryo manipulations that result in this syndrome. Pregnancies resulting from NT embryos have been reported to have placental abnormalities ranging from a decreased number of placentomes to chorioallantonic hypoplasia through partial placental development (Hill et al., 2000). The mean birth weights for NT or *in vitro* produced calves tend to be higher and have a greater variation than naturally conceived calves (Behboodi et al., 1995, Garry et al., 1996, Wilson et al., 1995). Hill and colleagues in 2001 examined the placenta of a NT born Holstein calf with a normal birth weight. It was theorized that the normal birth weight of this calf was due to a reduction in placental capacity and the birth weight would have increased significantly if she possessed a normal placenta. Although, placentome numbers were only decreased 10 to 20%, the increased size of the remaining placentomes probably compensated

for the decrease in their numbers (Hill et al., 2001). If the calf had developed large calf syndrome, it is not known if the under developed placenta would have supported this pregnancy, or if the calf had normal placental development would have developed large offspring syndrome (Hill et al., 2001). The biggest mystery surrounding large offspring syndrome is if all or some manipulated embryos are affected at different degrees (Young et al., 1998).

### Microchromosome Transfer

Artificial chromosomes have been regarded as the ideal vector for gene therapy and biotechnology purposes because they mimic the natural state of DNA in the cell (Cooke, 2001). Artificial chromosomes have the ability to carry large amounts of DNA in the range of megabases. They are also maintained in the cells as autonomous, replicating chromosomes when they contain an origin of replication, centromere and two telomeres in bacterial cells. Origin of replication provides a sequence at which replication can be initiated. After replication, it is essential that the daughter cells separate evenly at the centromere. The centromere is located in the middle of the chromosome and is where kinetochore assembles during mitosis and meiosis. The kinetochore is the protein structure located on the chromosomes where the spindle fibers attach during division to pull the chromosomes apart. The telomeres provide protection from degradation, recombination, and misrecognition as double stranded breaks by the checkpoint mechanisms that monitor genomic integrity.

There are several different types of artificial chromosomes including yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), P1-based artificial chromosomes (PACs), and human artificial chromosomes (HACs). Artificial chromosomes have been found to have gene expression levels and tissue specific expression levels that are similar to the endogenous gene (Huxley, 1998). The yeast chromosome was first described by Murray and Szostak in 1983, and was constructed by linearizing a plasmid with restriction enzymes and the gene of interest was added with DNA ligase. The use of YACs in transgenesis is likely to ensure position-independent, copy-number independent, and optimal levels of expression for

transgenes, as long as all the regulatory elements are located within the vector (Giraldo and Montoliu, 2001). YACs have been used to produce transgenic mice, pigs (Yannoutsos et al., 1995), rabbits (Brem et al., 1996; Rouy et al., 1998), and rats (Fujiwara et al., 1997, 1999). In livestock, the benefits of YACs are mainly focused on xenotransplantation and the efficient production of recombinant proteins (Giraldo and Montoliu, 2001). However, YACs have several disadvantages including insert chimaerism, insert instability, rearrangements, and potential contamination with endogenous yeast chromosomes, which can make it difficult for their efficient use (Monaco et al., 1994; Green et al., 1999). BACs and PACs are becoming increasingly more popular in the production of transgenic animals because they are large enough to contain many mammalian intact genes with all of their controlling elements and can be modified *in vivo* by homologous recombination (Huxley, 1998).

There are two main advantages to using BACs and PACs over YACs. First, BACs and PACs are generally more stable than YACs, which are frequently rearranged *in vivo* (Huxley, 1998). BACs are also much easier to prepare in large quantities and this can be done using conventional plasmid protocols (Huxley, 1998). PACs are usually derived from the DNA of P1 bacteriophages and are normally obtained by generating a linear DNA molecule, which is packaged together with viral particles (Ioannou et al., 1994). PACs are maintained as single copy circular plasmids and have a high copy number. BACs are also circular plasmid DNA molecules that are hosted in *E. coli*. BACs can hold up to 300 kb of foreign DNA and are derived from the F factor of *E. coli* (Shizuya et al., 1992). In contrast to PACs, BACs are maintained as low copy inserts, meaning they yield lower quantities of DNA when isolated (Giraldo and Monteliu, 2001).

PACs and BACs have both been successful in the production of transgenic mice (McCormick et al., 1997; Goodart et al., 1999; Chiu et al., 2000; Duff et al., 2000) and zebrafish (Jessen et al., 1999). BACs have also been evaluated for their ability to improve mammary



gland transgenesis and for the production of recombinant proteins in the milk of transgenic animals (Stinnakre et al., 1999; Zuelke, 1999).

HACs were developed by Ischida and colleagues in 2002 from randomly generated chromosomes fragments. Their aim was to produce transgenic calves that secreted human polyclonal antibodies for therapeutic use because they are only available from human donors, and their supply and application is limited (Kuroiwa et al., 2002). Twenty-one transchromosomal calves were produced by transferring a HAC into primary fetal fibroblasts, followed by nuclear transfer (Kuroiwa et al., 2002). The HAC was selected with Geneticin<sup>®</sup> and was shown to be retained as an independent chromosome. The proportion of the cells retaining the HAC ranged from 78 to 100% (Kuroiwa et al., 2002).

### Blood

Serum is thought to be a good source for recombinant proteins because it collects secretion from many tissues. Human  $\alpha$ 1 antitrypsin, an important protein for the treatment of pulmonary disease, only synthesized in the liver, and is produced at high levels in the serum of transgenic rabbits (Massoud et al., 1991). A major limitation is the ability to separate the recombinant proteins from the endogenous proteins. However, this can be solved by replacing the endogenous genes with human genes by homologous recombination (Houdebine, 2000). Recombinant antibodies have also been found in the blood of transgenic pigs and rabbits (Lo et al., 1991; Weidle et al., 1991; Limonta et al., 1995), however, the recombinant proteins were only available at low concentrations and were hybrid molecules containing chains from the endogenous genes (Houdebine, 2000). It has been proposed that the replacement of the loci that contains the antibody genes would allow for the production of human antibodies (Mendes et al., 1997). Even though serum is abundant, it does not appear to be a potential source for recombinant proteins because many proteins are unstable in serum and can potentially have negative effects on the animal's health (Houdebine, 2000).

The production of human hemoglobin in transgenic pigs is another potential source of recombinant proteins (Sharma et al., 1994). Human hemoglobin was functional and produced at high levels; however, it formed hybrids with the endogenous genes, similar to the production of human  $\alpha 1$  antitrypsin, but this problem may be solved in a similar way.

Reticulocytes have also been suggested as source of recombinant non-secreted proteins. Enzymes and peptides could be stored in reticulocytes of transgenic animals and extracted after having been collected from the abattoir (Houdebine, 2000).

### Urine

Urine is an abundant biological fluid that prepares proteins like gonadotropins for pharmaceutical use. Recent work has shown that the human growth hormone gene, when driven by the promoter for the mouse uroplakin II gene, was expressed specifically in urothelium and could produce 100 to 599 ng/ml of human growth hormone (Houdebine, 2000). The urothelium is the tissue layer that lines a majority of the urinary tract, including the renal pelvis, the ureters, the bladder, and parts of the urethra. This method of producing recombinant proteins could prove to be beneficial if the foreign proteins are matured correctly in the urothelium, or if the side effects are less harmful to the animal (Kerr et al., 1998).

### Seminal Plasma

Depending on the species, seminal plasma can be a relatively abundant biological fluid and easily collected; for example, swine. The promoter of the mouse P12 gene that promotes expression specifically in the male accessory sex glands can be used for the production of human growth hormone (Dyck et al., 1999). It was produced at a concentration as high as 0.5 mg/ml in the seminal plasma of the mouse (Dyck et al., 1999).

### Egg White

Egg white is an abundant fluid that contains large amounts of proteins which are excreted out of the body, therefore making it an excellent system for the production of recombinant proteins. The technology, however, has been limited by the difficulty of generating

transgenic birds. Recently, this changed through the use of lentiviral vectors (Lillico et al., 2007; Pfeifer et al., 2006). More impressively, chickens have been shown to have pluripotent cell lines from primordial germ cells. These cells can be transfected with foreign genes and reintroduced into early embryos to produce a chimeric transgenic chicken (van de Lavoie et al., 2005). They were also able to show that a chimeric transgenic chicken generated by using non-pluripotent cells was able to secrete a monoclonal antibody in their egg whites (Zhu et al., 2005).

### Milk

Milk is currently the best system to produce recombinant proteins and complex recombinant proteins (Houdebine, 1994; Colman, 1996; Clark, 1998; Wall, 1999; Ruldoph, 1999). Several mammalian species including rabbits, pigs, sheep, goats and cows have been studied or used to produce recombinant proteins in their milk (Houdebine, 2008). Rabbits offer a number of advantages including the easy generation of transgenic founders and offspring, high fertility, relatively high milk production, insensitivity to prion diseases, and no transmission of severe diseases to humans. Pigs, while more costly, produce higher amounts of milk than rabbits. Ruminants have the most potential to produce large amount of proteins in their milk but in order for them to integrate foreign genes they must under go nuclear transfer, their reproduction is relatively slow, they do not glycosylate proteins as well as rabbits and pigs, and they are sensitive to prion diseases (Houdebine, 2008). Human IGF1 (Zinovieva et al., 1998), human NGF- $\beta$  (Coulibaly et al., 1999), hGH (Devinoy et al., 1994), human lysozyme (Lee et al., 1998), human lactoferrin (Platenburg et al., 1994), human erythropoietin (Masoud et al., 1996), human thrombopoietin (Sohn et al., 1999), and human parathyroid hormone (Rokkones et al., 1995) have all been successfully produced as recombinant proteins in the milk.

Milk has also been shown to be able to produce complex recombinant proteins like human fibrinogen in their fully functional form, which is composed of three subunits (Prunkard et al., 1996). This was also the case in the production of human collagen, which was obtained as a mature molecule after the two subunits joined and underwent posttranscriptional modification

(John et al., 1999). Other complex recombinant proteins have been successfully produced in milk, such as human extracellular superoxide dismutase (Strömqvist et al., 1997) and immunoglobulins (Castilla et al., 1998). These examples highlight the ability of the mammary gland to produce and secrete mature foreign proteins.

### **Effects of Transfection on Gene Expression Levels**

Transgene expression levels in stably transgenic organisms are affected by many factors; in particular, by the promoter driving the transgene, the copy number of the transgene in the genome, and by interaction between transgene and flanking sequence DNA (Rahman et al., 2000). The promoter sequence is essential for expression, and it is vital that these are not rearranged on integration of the exogenous DNA (Mellon et al., 1988). When a transgene construct contains enhancers, it inserts into the genome in unexpected regions where the gene can come under similar transcriptional control (Mellon et al., 1988). If integration of exogenous DNA is to be effective then it must not inactivate genes required for normal growth. Integration at sites where genes are not being expressed has no immediate mutagenic effect and there would not be expression of the transgene (Mellon et al., 1988). Transgene copy number has been shown to be important in genetic stability of the target gene in transgenic plants (Litao et al., 2005). Transgene copy number is referred to as the number of copies of a transgene that are inserted into the genome and has been shown to greatly influence the expression level and genetic stability of the target gene, particularly in transgenic plants (Litao et al., 2005). It has also been shown that there is a high correlation between gene copy number and expression in simpler ascomycete systems (Kelly et al., 1987; Turcq et al., 1987). Accurate determination of transgene copy number and levels of mRNA are necessary to understand the phenotypic changes (Ringel et al., 1998).

There are two main methods used for transfecting cells: liposome-mediated transfection and electroporation. Liposome-mediated transfection is the most widely used synthetic DNA delivery systems and is made up of an expression cassette that is inserted into a plasmid and

complexed with cationic polymer (polyplex), cationic lipid (lipoplex) or a mixture of these called a lipopolyplex (Lechardeur et al., 2002) The positively charged DNA complex is taken up from the extracellular compartment by endocytosis and transferred into the nucleus, a vital step for successful gene expression. The advantages of using liposome-mediated transfection are relatively high efficiency of gene transfer, the ability to transfect certain cell types that are resistant to calcium phosphate or DEAE-dextran both *in vivo* and *in vitro*, successful delivery of DNA of all sizes of oligonucleotides to yeast artificial chromosomes (Felgner et al., 1987), the ability to delivery RNA (Wilson et al., 1979; Malone et al., 1989), the delivery of proteins (Debs et al., 1990), the transient expression and stable integration, and can be used for *in vivo* transfer of DNA and RNA to animals and humans (Felgner et al., 1995). Clathrin-dependant endocytosis is predominantly responsible for the cellular uptake of the DNA complex, when a positively charged lipoplex or polyplex interacts with the negatively charged plasma membrane (Lechardeur et al. 2002). The size as well as composition of the complex may determine the mechanism of internalization. For example, large lipoplexes (up to 500 nm) enter the cell by receptor and clatherin-independent endocytosis while endocytosis could be used to internalize the smaller complex (<200 nm) via coated pits through a non-specific clathrin-dependent mechanism (Lechardeur et al., 2002). There is little knowledge regarding the mechanism of the exogenous DNA in the early endosome. It is hypothesized that the early endosome could be routed to the extracellular compartment for recycling, targeted to lysosomes and the exogenous DNA released into the cytoplasm (Lechardeur et al., 2002).

The release of the exogenous DNA into the cytoplasm occurs when the endosomal membrane is disturbed by interaction of the cationic lipid of the lipoplex (Lechardeur et al., 2002). It has also been suggested that the mechanism for the release of exogenous DNA into the cytoplasm involves the neutralization of the charge on the cationic complexing agent with anionic macromolecules like anionic lipids and proteoglycans, cationic lipid fusion, and membrane destabilization by pH-sensitive lipids (Lechardeur et al., 2002). After disruption of the

membrane, only a small portion of exogenous DNA is released into the cytoplasm, while the majority is eventually degraded.

Electroporation is a simple, reproducible, and highly efficient procedure, which disrupts the cell's membrane by a significant increase in electrical current with an externally applied electrical field (Toneguzzo et al. 1988). It has been used to introduce exogenous DNA into both plant and animal cells, and has been successfully applied to a wide range of cell types, which have not been accessible by other methods (Chu et al., 1987). A study performed by Gilbert Chu and colleagues (1987) showed that transfection efficiencies improved with an increase in plasmid DNA concentration even up to 80 µg/ml of DNA. They were also able to show that the linearization of the plasmid resulted in a moderate decrease in transient integration. However, there was increase in stable integration by four-fold when compared to super coiled plasmid.

Voltage is a critical parameter for electroporation. For a given capacitance and buffer there was sharply defined voltage for optimal transfection efficiency (Chu et al., 1987). The local potential difference across the cell is the driving force for pore formation, and is proportional to the product of the capacitor voltage and the cell diameter (Chu et al., 1987). Therefore, the voltage optimum is inversely related to cell size.

It is believed that the majority of cells integrate exogenous DNA at a low copy number even when electroporated in the presence of high DNA concentrations. Toneguzzo and colleagues believe there are subpopulations of cells, which can take up and integrate multiple DNA copies. The difference between these cells and the majority of cells could possibly be physical or biological factors, or a combination of both. The biological factors are believed to reflect periods in the cell cycle when the cells are more inclined to integrate exogenous DNA into their genome, and only a proportion of cells are in this phase at one time (Toneguzzo et al., 1988). Conversely, there may also be periods where the cells are more receptive to permeabilization by electroporation. This may be due to the changes in cell shape or in the plasma membrane properties (Toneguzzo et al., 1988).

Electroporation in the presence of increasing DNA concentrations results in higher levels of transient gene expression as well as in an increase in stable integration frequency.

Toneguzzo and colleagues showed in 1988 that even with increasing concentrations of exogenous DNA the majority of cells integrate DNA in low copy number, suggesting that the increase in gene expression is typically due to a greater number of cells being transformed.

The cytoplasm is believed to impede the mobility of exogenous DNA to the nucleus. After microinjection of exogenous DNA into the cytoplasm, small oligonucleotides diffused quickly in the nucleus where they became hindered. A 100 bps DNA fragment was fully mobile in the cytoplasm with a diffusion rate approximately five times slower than in water (Lukacs et al., 2002). Larger DNA fragments have significantly slower diffusion rates, and exogenous DNA with 2000 or greater bps had little to no movement in the cytosol. The limited cytoplasmic diffusion of plasmids or double stranded DNA fragments larger than 1 kb has been visualized by injecting fluorescein isothiocyanate (FITC)-conjugated DNA into the cytosol of HeLa cells (Leonetti et al., 1991; Lukacs et al., 2002).

In contrast, oligonucleotides or DNA fragments of 250 bps or smaller were able to enter into the nucleus. However, after 45 min incubation nucleic acids larger than 250 bp were excluded. When exogenous DNA is microinjected into the cytoplasm in close proximity to the nucleus, or when the size of the transgene is decreased, the transfection efficiency is increased, showing that the size of DNA is inversely related to its ability to move through the cytoplasm of the cell. The microinjection of exogenous DNA also showed the DNA disappears in a time dependant manner from the cytosolic compartment, demonstrating that the metabolic instability of exogenous DNA may contribute to the low efficiency of transfection (Lechardeur et al., 1999). In 1999, Lechardeur and colleagues did a study where they demonstrated that 50% of microinjected plasmid DNA is eliminated in one to two hours in HeLa and COS-1 cells (African Green monkey kidney cells). The fast turnover rate was independent of the copy number and the conformation of the plasmid delivered. DNA condensing agents act as barriers against

cytosolic nucleases by prolonging their half-life and enhancing the transfection efficiency (Lechardeur et al., 1999).

Recent evidence indicates the DNA transferred by a number of chemical methods is subject to mutations and rearrangements possibly as a result of passage through the lysosomal compartment (Toneguzzo et al. 1988). The gene of interest must be transferred in an intact and unmutated form to be successful and can be adversely affected at many levels. Accurate determination of transgene copy number and levels of mRNA are necessary to understand the phenotypic changes noted in these models (Ringel et al., 1998).

### **Delivery of Transgene to the Nucleus**

Pollard and colleagues in 1998 suggested that the nuclear membrane is a critical barrier in the nuclear delivery of plasmid DNA. Diffusion is the principle transport mechanism in nuclear delivery of small molecular weight compounds ranging from <20,000-40,000 kDa and is essential for the metabolism of eukaryotes. Movement of macromolecules between the cytoplasm and the nucleus is mediated by a nuclear pore complex (NPC), which is the formation of an aqueous channel through the nuclear envelope (Laskey, 1998). The transport of larger molecules through the NPC is signal mediated using shuttle molecules. The NPC, perhaps the largest protein complex in the cell, is responsible for the protected exchange of components between the nucleus and cytoplasm and for preventing the transport of material not destined to cross the nuclear envelope. The significant size of plasmid DNA makes it unlikely that nuclear entry occurs by passive transport. The diameter of the NPC channel during passive transport is 9 nm but can expand to a maximum of ~ 25 nm during active transport. The conformational change in the NPC shows that there are specific transport signals that trigger this transformation (Lechardeur et al., 2002). The addition of a nuclear localization signal (NLS) peptide to linearized exogenous DNA has been shown to increase the amount of DNA able to pass through the NPC (Zanta et al., 1999). The enhancement of the reporter gene expression was 10 to 1000 fold (Zanta et al., 1999). The NLS is a specific peptide sequence that acts as a signal to



localize the protein within the nucleus. Linearization of the exogenous DNA should increase the gene copy number, thereby increasing gene expression levels.

The close correlation between the onset of transgene expression and mitosis suggests that an event or events during mitosis somehow enhance transfection, possibly aiding the delivery of the plasmid to the nucleus (Mortimer et al., 1999). Mitotic cells, when compared to quiescent cells, had higher transfection rates, suggesting that plasmid DNA enter the nucleus preferentially upon disassembly of the nuclear envelope during mitotic cell division (Brunner et al., 2000). Blocking the cell cycle in the G1 phase by aphidicolin did not have an effect on the rate at which the lipoplex entered the nucleus or on the level of transgene expression in stably transfected cells (Mortimer et al., 1999). In addition, when cells were exposed to lipoplexes just before or during mitosis there was an increase in the level gene expression (Brunner et al., 2002). Wilke and colleagues in 1996 demonstrated that cells in which naked plasmid DNA was injected directly into the nucleus produced approximately five-fold greater transgene expression when compared to cells where the plasmid was injected into the cytoplasm.

Another attempt to overcome the barrier of the nuclear membrane with the sequence specific import of DNA into the nucleus succeeded by introducing DNA elements containing binding sites for transcription factors (Längle-Rouault et al., 1998, Dean, 1997). These transcription factors then surround the transfected DNA, and due to their NLS signal, transport it through the NPC into the nucleus of all cells or in specific cell types.

### **Mechanisms of Transgene Integration into the Genome**

Approximately one cell per thousand, depending on the cell type, will integrate exogenous DNA into their chromosomal DNA (Gorman et al., 1983; Staunton et al., 1994). In *in vitro* conditions, exogenous DNA is integrated by apoptotic body DNA integration, repair of chromosomal lesion by insertion of mitochondrial DNA, or retrotransposition event (Willet-Brozick et al., 1989; Berg et al., 1989; Esnault et al., 2000; Bergsmedh et al., 2001). There are two main mechanisms of integration of DNA into chromosomes: homology-dependent and non-

homology-dependent integration, also known as illegitimate integration (Würtele et al., 2003). Homologous recombination involves the alignment of similar sequences, a crossover between the aligned DNA strands, and breaking and repair of the DNA to produce an exchange of material between the strands (Würtele et al., 2003). Homologous dependent usually gives rise to predictable results, meaning that the position of the modified loci should be easily located. The process of homologous recombination naturally occurs in organisms, and is also utilized as a molecular biology technique for introducing genetic changes into an organism

Recombination events that show little or no dependence on nucleotide sequence homology represent important pathways for DNA rearrangement in animal cells (Roth et al., 1985). Some examples of nonhomologous recombination include chromosome translocation, gene amplification events, movement of retroviruses, transposable elements, developmental rearrangements of antibody and T-cell receptor genes, formation of processed pseudogenes, and programmed rearrangements in ciliates (Roth et al., 1985). Illegitimate integration events are usually more frequent than homology-dependant at a ratio ranging from 1:4 to 1:1,000,000, depending on experimental conditions and cell types, but illegitimate integration is typically 1,000-10,000 times more frequent than targeted integration (Smith et al., 2001). Since, non-homologous recombination is more frequent, it is difficult to target exogenous DNA into homologous chromosomal loci. To increase homologous integration it is necessary to inhibit non-homologous recombination (Roth et al., 1985). Illegitimate recombination has two different mechanisms: (i) end-joining, mediated by enzymes which cut and join DNA, such as topoisomerases, site-specific DNases and involve sequences which include or resemble those on which such enzymes normally act, and (ii) strand slippage, where, after pausing at the replication fork, the nascent strand is able to dissociate from one template and pair with another (Wang et al., 1997).

A majority of transfected DNA is rapidly degraded after entering cells (Würtele et al., 2003). DNA molecules that are not degraded are usually modified extrachromosomally by

several different mechanisms. Homologous recombination can modify extra chromosomal DNA very efficiently by an inter- or intramolecular sequence of homology and can lead to multiple products depending on the organization of the sequence sharing the identity (Folger et al., 1985; Subramani et al., 1985; Lin et al., 1987; Lin et al., 1990; Belmaaza et al., 1994; van dem Bosch et al., 2002). The results of this modification are very similar to chromosomal homologous recombination, suggesting that the two mechanisms may share similar processes (Wong et al., 1986; Wong et al., 1987; Dellaire et al., 2002; Richardson et al., 2000; Tremblay et al., 2000). Homology independent recombination also has the ability to mutate transfected DNA at high frequency by point mutations, deletions, and more complex rearrangements, such as insertion of genomic DNA. Concatomers can be formed by nonhomologous end joining from linear exogenous DNA that is circularized in the nucleus. Nonhomologous end joining usually involves short sequence homology between the joined ends, and additions or deletions of approximately 25 nucleotides or less at the junctions (Wake et al., 1984; Roth et al., 1986; Nicolas et al., 1994).

A single transgene is believed to be integrated into the genome by homologous recombination through a double stranded break repair. However, less is known about how multiple transgene copies are integrated at the homologous chromosomal locus. There are three mechanisms that are believed to be the product of multiple transgene integration due to the structure of the recombinants after integration: (i) targeted integration of a transgene concatomer, (ii) targeted transgene insertion followed by a second homologous recombination event between two replicated, unequally paired sister chromatids creating tandem copies of the transgene, and (iii) multiple homologous recombination events each involving the target insertion of a single transgene copy (Philip et al., 1999).

### **Circular vs. Linearized DNA**

The majority of transfection protocols utilize circular DNA; however, they are usually examining transient expression, not stable integration. The physical form of the transgene is

another factor that influences transformation. Folger and colleagues in 1982 performed a study where they compared the ability of a supercoiled plasmid construct with that of a linearized one in the microinjection of foreign DNA into the cytoplasm of a cell. Similar frequencies were observed when more than 50 molecules of either linear or supercoiled per cell were injected, however, as the number of plasmid DNA molecules injected per cell were decreased to less than 50, there was a noticeable decrease in the transformation efficiency. High transformation efficiency (approximately 20%) was retained by injecting an average of as few as five linear molecules per cell, whereas the transformation frequency dropped to less than 1 in 200 when 5 to 10 supercoiled molecules were injected per cell (Folger et al., 1982). It is theorized that when delivering DNA to the cytoplasm, circular DNA is better protected because there are no free ends and gives higher initial transient transfection rates. Although, circular DNA is better protected from endonucleases, there did not appear to be a preferred site for integration (Folger et al., 1982). Alternatively, single linear molecules appeared to be inserted into the host genome through their ends. The restriction sites at the ends of the plasmid molecule were degraded during the process of insertion.

### **Determining Gene Copy Number**

Transgene copy number has been traditionally estimated by Southern Blot analysis, although recently other methods have become available to determine gene copy number, including comparative genomic hybridization (Larramendy et al., 1998), fluorescence in situ hybridization (Kallioniemi et al., 1996), multiplex amplifiable probe hybridization (Armour et al., 2000; Hollox et al., 2002) and microarray analysis (Lucito et al., 2000; Li et al., 2002). Unfortunately, all of these methods are laborious and time consuming, and require considerable amounts of DNA from fresh or frozen samples (De Preter et al., 2002). In addition, estimations tend to be inaccurate when transgenes have mutated and lost their restriction sites (Mason et al., 2002). More recently, quantitative polymerase chain reaction (Q-PCR) has shown to be a fast, sensitive, effective, and a less expensive method for determining transgene copy number.

## Southern Blot Analysis

A Southern blot is a technique named after its inventor, Edwin Southern, and is routinely used in molecular biology to check for the presence of a DNA sequence in a DNA sample. Southern blotting combines agarose gel electrophoresis for size separation of DNA with methods to transfer the size-separated DNA to a filter membrane for probe hybridization. Restriction fragment size alteration may be generated by the rearrangement and appear as novel bands on the membrane (Sellnar and Taylor, 2004). The introduction of pulsed field gel electrophoresis, increases the range of resolution, allows for better identification of detection of deletions and duplications. On the other hand, a semi-quantitative approach is taken where intensity of probe hybridization to a specific target is compared to a control locus and a control sample. The main disadvantage to the Southern blot technique is the low throughput, meaning only a few samples can be run per gel, a limited number of loci can be identified per blot and the tests can take several days to complete (Sellnar and Taylor, 2004).

## Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) was the first efficient approach to scanning the entire genome for variations in DNA copy number (Pinkel and Albertson, 2005). CGH also known as chromosomal microarray analysis, is a molecular cytogenic method used to determine changes in the copy number in the DNA content. This technique is often used to identify crucial genes and pathways that are involved in biological processes and diseases such as cancer. CGH is able to produce a map of DNA sequence copy number as a function of chromosomal location throughout the entire genome (Kallioniemi et al., 1992). A gene copy number karyotype can be generated for a tumor by the comparison of DNAs from malignant and normal cells, thereby identifying regions of gain or loss of DNA (Kallioniemi et al., 1992). Typically, CGH is measured by isolating genomic DNA from a sample and control cell populations. The cells are labeled and hybridized to metaphase chromosomes or more recently, microarrays. The relative hybridization intensity of the sample and control signals is proportional to the relative copy

number of those sequences in the sample and reference genomes (Pinkel and Albertson, 2005). If the control genome is normal, then increases and decreases in the intensity ratio directly indicate DNA copy number variation in the genome of the sample cells (Pinkel and Albertson, 2005).

The quality of the genomic DNA isolated can have a considerable effect on the resulting data. Although genomic DNA is isolated from frozen and fresh samples on a routine basis through the use of numerous published protocols and commercial kits, there is an unknown class of contaminants that occasionally co-purify with the DNA and produce abnormally high noise in the ratios (Pinkel and Albertson, 2005). This noise is typically not random because if a different aliquot of the same DNA is labeled it would produce similar results (Pinkel and Albertson, 2005). The amount of genomic DNA is a major limitation on CGH analysis. Typically, CGH procedures need between 300 ng and 3  $\mu$ g of DNA in the labeling reaction; which is approximately equivalent to 50,000 to 500,000 cells (Pinkel and Albertson, 2005).

#### Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) is a cytogenic technique that can be used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH is ideal for visualization and analysis of genetic changes on specific DNA or RNA sequences on mitotic chromosome preparations or in interphase cells; however, it is not applicable to screen unknown genetic aberrations (Kallioniemi et al., 1996). DNA or RNA sequences are labeled in one color probe while the control probe is labeled in another color to detect deletions and amplifications of specific sequences in interphase cells (Kallioniemi et al., 1992; Matsumura et al., 1992). These copy number changes can be distinguished from those caused by chromosome trisomies, monosomies, and other ploidy aberrations with the selection of the correct probe. This type of analysis can determine the absolute gene copy numbers of the target sequence in individual interphase nuclei. It allows for the evaluation of cell-to-cell heterogeneity in gene copy number, and is able to detect small subpopulations of genetically

aberrant cells (Kallioniemi, et al., 1996). This is in contrast to traditional molecular genetic methods based on isolated DNA, such as Southern blotting and PCR, which can provide only an estimate of the average copy number relative to a reference locus (Kallioniemi et al., 1996).

#### Multiplex Amplifiable Probe Hybridization

In multiplex amplifiable probe hybridization (MAPH), specific hybridization to a sample is detected by recovery and amplification of the probe itself (Armour et al., 2000). In this technique, genomic DNA is fixed to a membrane and hybridized with a set of probes corresponding to the target sequences to be detected (Sellnar and Taylor, 2004). MAPH only requires 1 µg of DNA to be successful. The probes are produced by cloning the target sequences in a plasmid, then amplifying the cloned sequence using primers directed to the vector with the result that all probes are then flanked with the same sequence (Sellnar and Taylor, 2004). There is constraint on probe design when using the MAPH technique; probes that intended to be multiplexed must have a large enough size difference to be resolved by electrophoresis (Sellnar and Taylor, 2004). After the removal of the unbound probe by washing the membrane, the specifically bound probe will be present in an amount that is proportional to the gene copy number. The probes are then removed from the membrane and amplified using a universal primer pair probe. Products are then separated by electrophoresis and a relative comparison can be made between the band intensities (Sellnar and Taylor, 2004). Reduced band intensities compared with the internal control refer to a reduction in gene copy number and an increase in gene copy number results in increase band intensities (Sellnar and Taylor, 2004). MAPH has been used to multiplex 40 probes and ran on gel electrophoresis simultaneously (Armour et al., 2000).

MAPH probes can be contaminated quite easily, because they are naturally amplified. The washing step, which is necessary to remove the unbound probe, can introduce containments and this significantly increases the labor intensity as does the separation and identification of the washed membranes (Sellner and Taylor, 2004).

The polymorphisms, or single base mutations in the probe binding, do not affect MAPH results. MAPH probes being 100 to 200 bp are unlikely to be affected by base changes. However, if part of the region targeted by a MAPH probe is deleted, the probe may still hybridize and the target will be scored as being present (Sellner and Taylor, 2004).

### Microarray Analysis

A DNA microarray is a high throughput technology used in the molecular biology and medicine industries. Microarrays can be used to determine gene expression, DNA copy number, status of methylation at the promoter region of genes and histone acetylation. Microarrays are glass microscope slides with a variety of target DNA oligonucleotides containing specific sequences that are synthesized on to the slide. The microarray is incubated with the sample and control labeled DNA, allowing hybridization to occur. The array is then washed to remove any unbound DNA and then analyzed using software that measures the intensities for each hybridization spot and ratio values between the control and the sample.

Pollack and colleagues (1999) compared genomic DNA from an XO cell line with that from normal female cells, which models single-copy DNA deletion for X-chromosomal genes, and estimated that each individual array element provided ~85% specificity for detection of single-copy gene deletion. Another important advantage of microarray is that DNA copy number and gene expression patterns can be characterized in parallel in the same sample (Pollack et al., 1999).

### Quantitative Polymerase Chain Reaction

Real-time or quantitative polymerase chain reaction (Q-PCR) is widely used to detect and quantify DNA and cDNA in very diverse applications ranging from transgenic contamination in food to gene expression studies (Bubner et al., 2004). The first reported method of Q-PCR was by Higuchi (1993). They used ethidium bromide intercalation during the PCR process and a modified thermocycler to irradiate the samples with ultraviolet light, and then detected the resulting fluorescent signal with a charged coupled device camera (Ginzinger, 2002). The



fluorescent signal was plotted as a function of cycle number, and is very similar to the amplification plot that is used today. The plot gave a good indication of the amount of PCR product that was generated during each cycle of PCR (Ginzinger, 2002). The major disadvantage to this technique, other than the use of a carcinogen, is the nonspecific PCR products are equally detected and are included in the total amount of fluorescent signal measured (Ginzinger, 2002). Q-PCR is used to amplify and simultaneously quantify a targeted DNA molecule. It allows for the detection and quantification in either absolute or relative number of copies of a specific sequence in a DNA sample.

Q-PCR has made it possible to accurately quantify initial amounts of nucleic acids during the PCR reaction without the need for post-PCR analyses (Mason et al., 2002). A fluorescent reporter, specific or nonspecific in nature, is used to monitor the PCR reaction as it occurs. The use of fluorescent probe is the more accurate method, but at the same time is very expensive. It uses a sequence-specific RNA or DNA-based probe to quantify only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and allows quantification even in the presence of some non-specific DNA amplification. Q-PCR offers several advantages over the conventional methods such as large dynamic range of quantification, no requirement for post-PCR sample handling, the need for very small amounts of starting material and the high throughput capacity. (De Preter et al., 2002).

The disadvantages of Q-PCR are that the multiplexing needed to inquire a useful number of loci can be difficult to optimize, and the cost of buying fluorescently labeled probes for every intended target can be prohibitive (Sellner and Taylor, 2004).

### **Caprine as a Suitable Model**

The improvement of gene expression is an essential task to meet the increasing demand for recombinant DNA for the use in therapeutic drugs. The caprine species serves as a good platform for optimizing the procedures and the protocols for improving gene expression for transgenic animals because they are smaller, easier to maintain, and require less space than

their bovine counterparts. Goats are not considered to be the best model for the production of the proteins needed for the production of therapeutic drugs, since most are required in large amounts; however, protocols could be transferred to the bovine species once they have been optimized and been determined to be successful.

## CHAPTER III

### DETERMING GENE COPY NUMBER IN CAPRINE FIBROBLAST CELLS TRANSFECTED WITH LINEAR AND CIRCULAR DNA BY A LIPOSOME MEDIATED TRANSFECTION AND ELECTROPORATION

#### Introduction

Transgenesis allows for the study of models for single gene function, human diseases, production of pharmaceutical proteins, such as humanized recombinant antibodies, hormones, enzymes and vaccines, improvement of animal production and health, and xenotransplantation (Bacci, 2007). Transgenesis also provides a method by which economically important traits can be attained more rapidly. Genes can be transferred across species boundaries and can be modified to function very differently than they do in their native form or environment (Wall, 1996). There are several different methods that can be used to produce transgenic animals; microinjection, retroviral vectors, somatic cell nuclear transfer and microchromosome transfer. All of these techniques are used to produce recombinant proteins in different animal systems including blood, urine, seminal plasma, egg whites, milk and even in the silk worm cocoon (Houdebine, 2000). Milk is currently the most developed system to produce recombinant proteins and complex recombinant proteins (Houdebine, 1994; Clark, 1998; Wall, 1999; Ruldoph, 1999).

Numerous experiments have shown that the level and the specificity of the expression of a transgene cannot be easily predicted. However, gene copy number is believed to be correlated to gene expression. The efficiency of transgene integration is low and ranges from about 1% in farm animals to about 3% in laboratory animals (Wall, 1996). The number of times a transgene is integrated into the genome may affect the level of transgene expression observed in transgenic animals. Transgenes are expressed in only about half of cells lines that are transformed and may be aberrantly expressed (Wall, 1996). Aberrant expression patterns are attributed to position effects, meaning if a transgene is integrated near a highly active genes, the transgenes behavior maybe influenced by the endogenous genes (Wall, 1996).

Transgenes may also insert in heterochromatin regions, which is an area that is transcriptionally inactive. The transgene may function normally or be completely silenced by the heterochromatin (Wall, 1996). Expression may also be affected by the integration of multiple copies in concatomeric arrays (Heinkoff, 1998). The reduction of gene expression by concatomers has been attributed to DNA methylation (Collas, 1998), or formation of heterochromatin (Manuelidis, 1998) or both (Dobie et al., 1997). The degree of silencing has been found to be increased in inverted repeat transgene arrays, perhaps since inverted repeats are able to form hairpin structures which pair more easily than looped structures (Dobie et al., 1997). Reducing the copy number resulted in a marked increase in expression of the transgene and was accompanied by decreased chromatin compaction and decreased methylation at the transgene integration site (Garrick et al., 1998).

In contrast, Rahman and colleagues in 2000 observed gene expression levels in tilapia where transgenes inserted as concatomers can be effectively insulated and can function as an independent regulatory unit. This led to the belief that copy number dependent gene expression is the exception rather than the rule in transgenic organisms (Rahman et al., 2000). It is important to note that concatomers are usually formed by nonhomologous end joining from linear exogenous DNA that is circularized in the nucleus (Würtele et al., 2003).

Approximately one cell per thousand depending on cell type will integrate exogenous DNA in their chromosomal DNA (Würtele et al., 2003). There are two main mechanisms of integration of DNA into chromosomes; homology-dependent and non-homology integration (Würtele et al., 2003). Homologous recombination involves the alignment of similar sequences, a crossover between the aligned DNA strands, and the breaking and repair of the DNA to produce an exchange of material between strands (Würtele et al., 2003). Homologous recombination usually produces predictable results allowing for the modified loci to be easily located. Non-homologous recombination events are more frequent than homologous recombination, and it is difficult to target exogenous DNA into a homologous loci. Non-

homologous recombination can integrate exogenous DNA by two different mechanisms. The first mechanism for integration involves end joining, where topoisomerases or site-specific DNases, cut and join DNA, and usually involves sequences that include or resemble those on which such enzymes normally act. The second mechanism for nonhomologous recombination is a strand slippage where after pausing at the replication fork, the emerging strand is able to dissociate from one template and pair with another (Würtele et al., 2003).

The mechanism for multiple transgene integration is not well understood, however, there are three proposed mechanisms: (i) targeted integration of a transgene concatomer, (ii) targeted transgene insertion followed by a second homologous recombination event between two replicated, unequally paired sister chromatids creating tandem copies of the transgene, and (iii) multiple homologous recombination events each involving the target insertion of a single transgene copy (Philip et al., 1999). The mechanisms were determined by the structure of the recombinants after integration and may play an important role in the gene expression or the silencing of the transgene.

Pronuclear injection usually integrates transgenes at one only one site, or a very limited number of different sites. The number of copies integrated into a cell after injection can be highly variable, ranging from one copy to as many as 200 arranging in a tandem head-to-tail array at the site of integration (Pinkert, 1993; Brinster et al., 1981). The variability is believed to be attributed to the influences of the chromosomal sequences flanking the different sites of integration (Pinkert, 1993).

Recently, Graham et al., (2009) examined site-specific modification of the bovine genome using Cre recombinase-mediated gene targeting with nucleofection and liposome-mediated transfections, however, they only reported the gene copy number for the nucleofection procedure. Nucleofection is based on the physical method of electroporation, combining optimized electrical parameters, generated by a Nucleofector, with cell-type specific reagents.

Nucleofection generally produced low gene copy numbers ranging from 1 to 3 copies; however, one cell line had elevated copy numbers of 8 to 13.

Gene copy number has been traditionally estimated by Southern Blot analysis, although recently other methods have become available, including comparative genomic hybridization, fluorescence in situ hybridization, multiplex amplifiable probe hybridization and microarray analysis. All of these methods are laborious and time consuming, require considerable amount of DNA and tend to be inaccurate when transgenes have mutated or lost restriction sites. Q-PCR has been shown to be a fast, sensitive, effective, and less expensive method for determining gene copy number. Some limitations for using Q-PCR to determine gene copy number are the need for a standard curve, which takes up a lot of space on the standard 96-well plate. The need for a plasmid, oligonucleotide, or other source for the standard curve is an extra requirement and can lead to variation, making it difficult to compare data from different plates (Ginzinger, 2002).

## **Materials and Methods**

### Experimental Design

One caprine fetal fibroblast cell line was transfected 6 different times with circular or linear plasmid DNA by either electroporation or liposome mediated transfection (4 treatments). The treatments were electroporation with a circular plasmid construct, electroporation with a linear plasmid construct, liposome mediated transfection with a circular plasmid construct, and liposome mediated transfection with a linear plasmid construct. One cell line was used to reduce variability seen in the experiment. Cells were plated at low concentrations to form single cell colonies. Numbers of colonies formed after 10 days of culture was recorded, isolated and expanded in a 35 mm dish. Genomic DNA was isolated, quantified and amplified via Quantitative (Real-Time) PCR (Q-PCR). Amplified DNA from individual colonies was compared to a standard curve constructed from the same plasmid used to determine transgene copy number.

### Isolation and Linearization of Plasmid

The pHEGFP plasmid DNA contains an origin of replication, an intron, two promoters, ColE1 origin, and two reporter genes: kanamycin resistance and green fluorescent protein. Exogenous DNA or pHEGFP plasmid DNA (Figure 3.1) was isolated from *E. coli* using QIAfilter Plasmid Midi Purification Kit per the manufacturer's recommended procedure. *E. coli* cells were lysed, proteins were precipitated with salt and separated by filtration. Plasmid DNA was isolated by isopropanol precipitation.

Isolated plasmids were linearized using a digestive enzyme that was specific to the restriction site BamH1 and purified using Invitrogen™ PureLink™ PCR Purification Kit according to the manufacturer's protocol. Linearization was verified by running electrophoresis and DNA concentration was determined using BioRad SmartSpec™ Plus Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a dilution factor of 20.

### Electroporation

Caprine fibroblasts cells were cultured to 80 to 90% confluency in Dulbecco's Modified Eagle Medium with high glucose (DMEM) containing 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) or complete media in 60 mm culture dish at 39°C incubator containing CO<sub>2</sub>. Cells were then washed once with Dulbecco's Phosphate-Buffered Saline (PBS) without calcium and magnesium and released with trypsin-EDTA (0.25%). After dissociation of cells, equilibrated complete media was added to deactivate the enzyme. Cell suspension was centrifuged at 350 x *g* for 5 min. Supernatant was removed and cell pellet was resuspended in OptiMEM I® Reduced Serum medium modification of MEM (Eagle's) before being centrifuged at 350 x *g* for 5 min. Supernatant was removed; 20 µg of either circular or linearized DNA and OptiMEM were added to the cell pellet, giving a final volume of 800 µl. The cell pellet was resuspended and incubated at room temperature for 10 min before being transferred to the electroporation cuvette. Cells were pulsed in the BioRad Gene Pulser™

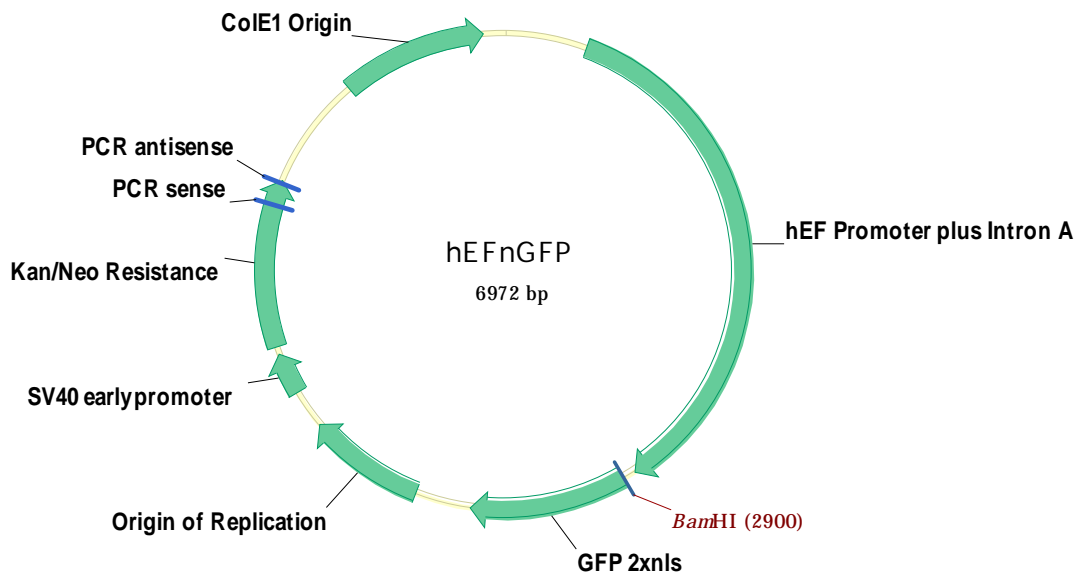


Figure 3.1 Circular pHEFGFP plasmid construct used to transfect caprine fibroblast cells. Plasmid was linearized at the BamH1 restriction site.



(Bio- Rad Laboratories, Inc., Hercules, CA, USA) at 350 volts and 500  $\mu$ F and transferred to a 35 mm dish containing complete media. Cells were incubated at 39°C in CO<sub>2</sub> for 72 h.

#### Liposome Mediated Transfection

Liposome mediated transfection was performed using FuGENE<sup>®</sup> HD transfection reagent by Roche. Caprine fibroblast cells were cultured to 80 to 90% confluency in complete medium in a 39°C incubator containing CO<sub>2</sub>. The optimum ratio of FuGENE<sup>®</sup> HD reagent to DNA concentration and buffer was determined to be 6:2 in previous studies (data not shown). FuGENE<sup>®</sup> HD transfection was performed by manufactures recommended protocol.

#### Isolation of Single Cell Colonies

After 72 h, transfected cells were released with trypsin-EDTA (0.25%), counted using a hemacytometer and plated in 100 mm dishes with a complete medium containing 600  $\mu$ g/ml Geneticin<sup>®</sup> (G418) and 2 ng/ml fibroblast growth factor basic (bovine brain-derived) (bFGF) referred to as selective medium. The optimum cell seeding density to obtain single cell colonies for electroporation with and without bFGF was determined to be 10,000 cells for a circular DNA construct and 10,000 for linearized DNA construct. For FuGENE<sup>®</sup> HD, the optimum seeding density was determined to be 40,000 cells without bFGF, however, with bFGF the seeding density decreased to 20,000 cells with circular DNA and 25,000 cells with linearized DNA. Cells were incubated in 39°C with CO<sub>2</sub> for 10 d in selective medium to allow for the formation of single cell colonies.

After ten days under selection, colonies were selected, harvested with a cloning ring, and to a 24 well plate and incubated until confluent. Once confluency was reached, cells were passaged using trypsin-EDTA (0.25%) into 35 mm dish with selective media and allowed to reach confluency. Confluency in the 35 mm dishes was vital to obtain high concentration of genomic DNA after isolation.

### Isolation and Quantification of Genomic DNA

Genomic DNA was isolated from Caprine fibroblast cells once confluency occurred in the 35 mm dishes using Gentra<sup>®</sup> Puregene kit from Qiagen<sup>®</sup> according to the recommended protocol. The cells were lysed, proteins were precipitated by the addition of salt and genomic DNA was precipitated with isopropanol.

After the overnight incubation, the DNA sample concentration is initially checked using the BioRad SmartSpec<sup>™</sup> Plus Spectrophotometer with a dilution factor of 20. Sample with DNA concentration higher than 150 µg/ml and a 260 nm wavelength greater than 0.1 AU were kept for further analysis. The initial concentration readings were used to adjust DNA samples to be equal to  $1 \times 10^5$  cells or 600 ng in a 5 µl sample to be equal to the 6 pg of genomic DNA in a caprine fibroblast cell (Honaramooz et al., 2003). All samples concentrations were re-verified using a Cytoflour<sup>®</sup> 4000 Plate Reader (Applied Biosystems, Foster City, CA, USA) and a Quant-iT<sup>™</sup> dsDNA Broad-Range Assay Kit from Invitrogen<sup>™</sup>. First, a working solution was prepared by diluting Quant-iT<sup>™</sup> dsDNA BR reagent 1:200 in Quant-iT<sup>™</sup> and 200 µl was loaded into each microplate well that was going to be used. Ten µl of each λ DNA standard, eight total, were added in triplicates to the microplate well containing the working solution. The DNA samples were also run in triplicate by adding 5 µl to each microplate well containing the working solution. The samples were read on the microplate by the Cytoflour<sup>®</sup> 4000 Plate Reader with a maximum excitation/emission of 510/527 nm and standard excitation/emission of ~480/530. After fluorescence was read, a standard curve was developed to determine the DNA concentrations of each sample.

### Validation and Optimization of Q-PCR

Primers were designed to amplify position 5,542 (sense) with a sequence CCGTGATATTGCTGAAGAG (5' – 3') and 5,649 (anti-sense) annealing to the following sequence TCAAGAAGGCGATAGAAGG of the plasmid and were manufactured by Invitrogen<sup>™</sup> Illumina. Annealing temperature gradient and primer concentrations matrix were performed with

sense and antisense primers to determine optimal annealing temperature and primer concentration to allow for maximum amplification. The optimal annealing temperature was determined by amplifying plasmid DNA at varying temperatures and separating of PCR products by electrophoresis. The primer concentration matrix was run using the optimum annealing temperature of 55°C and the sense and antisense primers were utilized in varying pmol concentrations ranging from 6.25 pmols to 50 pmols. Q-PCR amplification was performed on the plasmid and the optimized primers concentration determined by the lowest  $C_T$ . Q-PCR was used to develop a standard curve by 10-fold dilution to determine if the primers were amplifying a single product in a quantitative manner, with amplification efficiency between 80 to 120% and a correlation coefficient close to 1.0 (Figure 3.2).

### Q-PCR

Genomic DNA isolated from transfected caprine fibroblast cells from all treatments were amplified using the iQ™ SYBER Green Supermix in the MyiQ Reverse Transcription PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The Q-PCR Reaction Mix was made up of 12.5 µl of iQ™ SYBER Green 2X Supermix, 5 µl of DNA or H<sub>2</sub>O, 5.5 µl of nuclease-free water, and 1 µl of each primer (sense and anti-sense at 25 pmol concentrations). All samples, including the no template negative control were run in triplicates and compared to a standard curve developed by a 10-fold serial dilution of the same linearized plasmid used to transfect cells equivalent to  $1 \times 10^3$  to  $1 \times 10^7$  copies (Figure 3.3). The Q-PCR program used for the amplification of all samples comprised of a denaturing cycle of 3 min at 95°C; 40 cycles of PCR (95°C for 10 sec and 55°C for 45 sec); a melting curve analysis, which was made up of 95°C for 1 min followed by 55°C for 1 min, a step cycle with 80 repeats starting at 55°C for 10 sec with a +0.5°C/sec transition rate; and a final holding temperature of 10°C. The 10-fold serial dilution ran with each Q-PCR session, produced an equation for a line, which was used to determine the gene copy number. The mean threshold cycle ( $C_T$ ) value of the triplicate produced by each sample is entered into the equation for the line and solved for Y, giving the

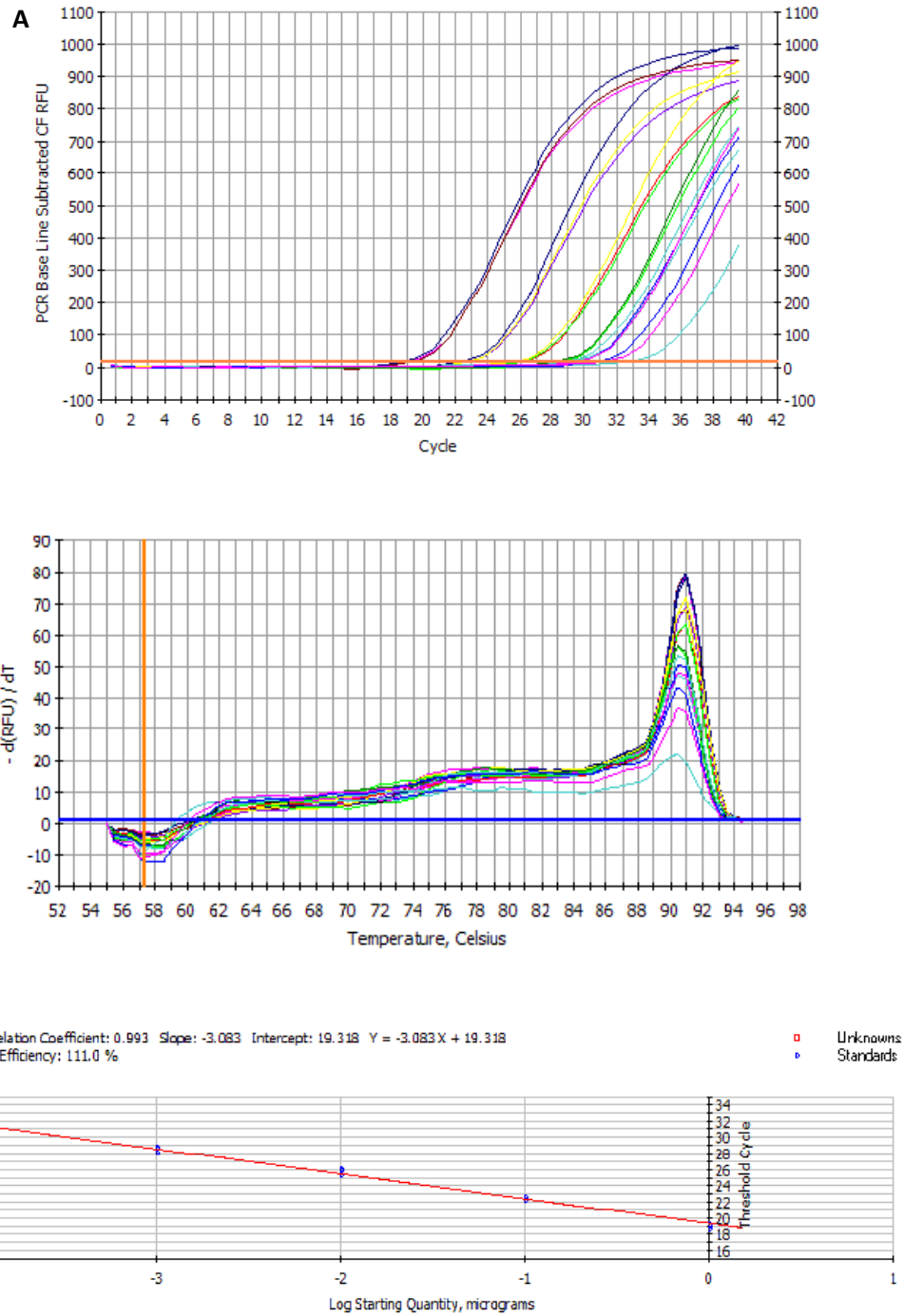


Figure 3.2 Dilution Ct values, melting curves, and standard curve obtained from the optimization of the forward and reverse primers. Five 10 fold dilutions (exogenous DNA equivalent to  $1 \times 10^3$  to  $1 \times 10^7$  cells) were used to generate the dilution curves: (A), melt curve data (B), and standard curve (C).

transgene copy number for the sample. The Cytoflour<sup>®</sup> 4000 reading allowed for the number of cells per 5 µl sample to be determined, and the number of times the exogenous DNA was integrated into the genome of an individual cell. Both individual gene copy numbers were calculated from each replicate as well as the mean gene copy number from mean C<sub>T</sub> value.

### Statistical Analysis

Data was analyzed using SigmaStat Statistical Software Version 3.5 (Systat Software, Richmond, CA, USA). Before any statistical analysis could be performed the natural log was taken to normalize the data. Mean gene copy numbers from all treatments were tested for normality and equal variance using Kolmogorov-Smirnov. One Way ANOVA, followed by multiple pair-wise comparisons using Tukey's method, were used to determine if there was significant difference in the number of times the exogenous DNA was integrated into the genome between the four treatments. Differences of  $P \leq 0.05$  were considered to have a significant difference. All samples with a gene copy number of 0.1 and below were omitted from the data due to the possibility the sample may have been a mixed colony and not all cells were transgenic.

## **Results**

### Caprine Fibroblast Cells Transfected by Electroporation with a Circular or Linear Plasmid

#### Construct

Transfecting caprine fibroblast cells by electroporation using a circular plasmid construct generated a mean gene copy number of  $2.8 \pm 0.75$  with 32 samples (n=32) (Table 3.1). A significant difference was detected between electroporation circular when compared with all other treatments (Figure 3.4). This treatment also produced the highest variability in gene copy number between isolated colonies of cells (Figure 3.5). Stable integration for electroporation with a circular plasmid construct was determined to be 246 cells per 1 million cells.

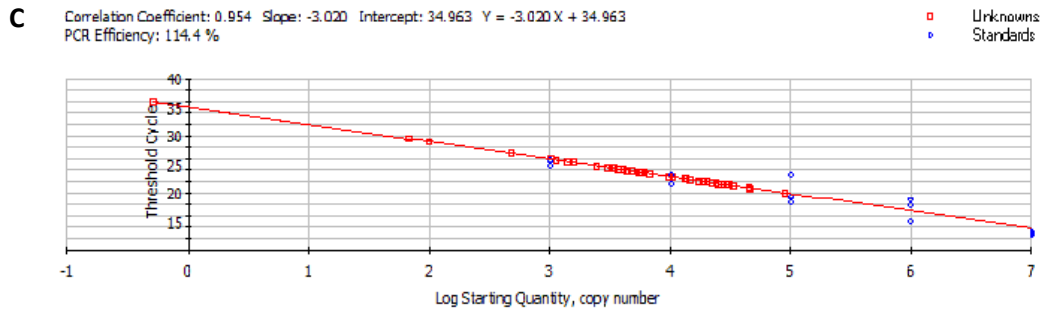
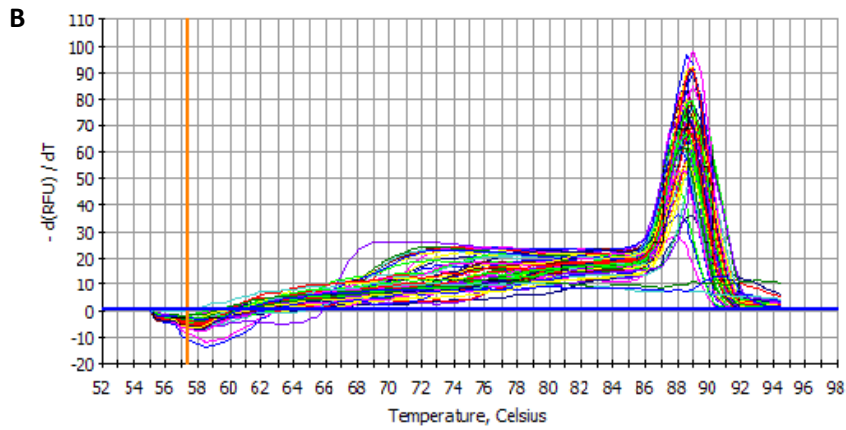
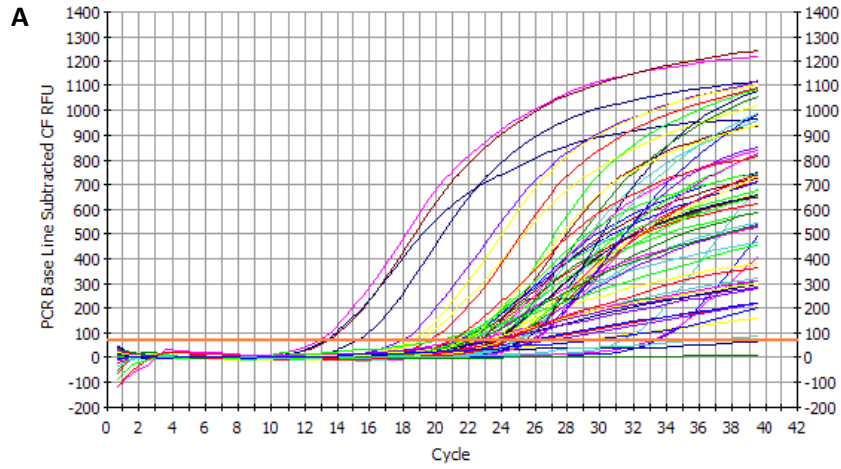


Figure 3.3 Ct values (A), melting curves (B), and standard curve (C) obtained when sample are amplified. All samples, including the no template negative control were run in triplicates and compared to a standard curve developed by a 10 fold serial dilution.

The mean gene copy number was determined to be  $1.32 \pm 0.64$  with 19 samples ( $n = 19$ ) when using a linear DNA construct with electroporation (Table 3.1). A significant difference was found when comparing electroporation linear to electroporation circular, however, there was no significant difference when comparing this treatment to the other two treatments (Figure 3.4). Electroporation with a linear plasmid construct stably integrated 150 cells per 1 million cells.

#### Caprine Fibroblast Cells Transfected by FuGENE<sup>®</sup> HD with a Circular or Linear Plasmid

##### Construct

The mean gene copy number for transfecting Caprine fibroblast cells by FuGENE<sup>®</sup> HD, using a circular plasmid construct, was determined to be  $0.5 \pm 0.11$  with 14 samples ( $n=14$ ) and was shown to be significantly lower than the mean gene copy number produced by the electroporation circular method (Table 3.1). However, there was no significant difference when comparing FuGENE<sup>®</sup> HD circular to electroporation and FuGENE<sup>®</sup> HD linear (Figure 3.4). Stable integration for FuGENE<sup>®</sup> HD with a circular plasmid construct was determined to be 51.6 cells per 1 million cells.

FuGENE<sup>®</sup> HD using a linear construct generated a mean gene copy number of  $0.64 \pm 0.13$  with 16 samples ( $n=16$ ) when transfecting caprine fibroblast cells (Table 3.1). Transfecting cells by FuGENE<sup>®</sup> HD with linear construct was not different than from any other treatments. However, FuGENE<sup>®</sup> HD both linear and circular plasmid constructs had less variability and were more consistent within samples (Figure 3.5). FuGENE<sup>®</sup> HD with a linear plasmid construct was the most consistent transfection treatment. FuGENE<sup>®</sup> HD with a linear plasmid construct stably integrated at 65.2 cells per 1 million cells.

#### **Discussion**

Little is known about the mean transgene copy number or variability of copy number when using different transfection methods, such as electroporation or liposome-mediated transfection. Transgene expression level in stably transgenic organisms is affected by many

Table 3.1 Mean Gene Copy Number for Isolated Single Cell Colonies

Treatment	n	Mean gene copy number
Electroporation Circular	32	2.8 ± 0.75 <sup>a</sup>
Electroporation Linear	19	1.32 ± 0.64 <sup>b</sup>
FuGENE <sup>®</sup> HD Circular	14	0.5 ± 0.11 <sup>b</sup>
FuGENE <sup>®</sup> HD Linear	16	0.64 ± 0.13 <sup>b</sup>

Statistical difference were determined by One-Way ANOVA (P<0.05).

<sup>a,b</sup> Different letters within columns indicate significant difference between treatments.



factors, such as the promoter driving the transgene, the copy number of the transgene in the genome, and by the interaction between the transgene and the flanking sequence DNA (Rahman et al., 2000). When a transgene is integrated into the genome of a cell it must not inactivate genes required for normal growth or be inserted where genes are not being expressed for it to be successful. Electroporation is a simple, reproducible and highly efficient method for introducing exogenous DNA into wide range of cells types. Electroporation uses an electrical field to create pores in the plasma membrane of the cell, which allows exogenous DNA to travel through the positively charged membrane. Liposome mediated transfection is the most widely used synthetic DNA delivery systems. This method provides a high efficiency of gene transfer, the ability to transfect certain cell types that are resistant to other methods, and the successful delivery of exogenous DNA of all sizes.

Transfection of caprine fibroblast cells with electroporation using a circular plasmid produced the highest mean gene copy numbers. However, it also produced the highest variability in gene copy number between isolated colonies of cells. It has been theorized that when delivering DNA into cytoplasm, circular DNA is better protected because there are no free ends that can be digested by endonucleases (Folgers et al., 1982). The lack of free ends may have had an important role in the high variability of the mean gene copy number seen in this treatment, because free ends provide a location for the exogenous DNA to be intergraded into the genome of the cell. Cells that were transfected using a linear plasmid construct with electroporation had a lower mean gene copy number and less variability between samples when compared with electroporation circular. The decrease in the gene copy number is most likely due to the exogenous DNA being degraded by endonucleases while traveling through the cytosol. The exogenous DNA that was not degraded in the cytosol was more likely to be integrated into the genome of the cell, therefore decreasing the variability seen between samples.

### Liposome Mediated vs. Electroporation

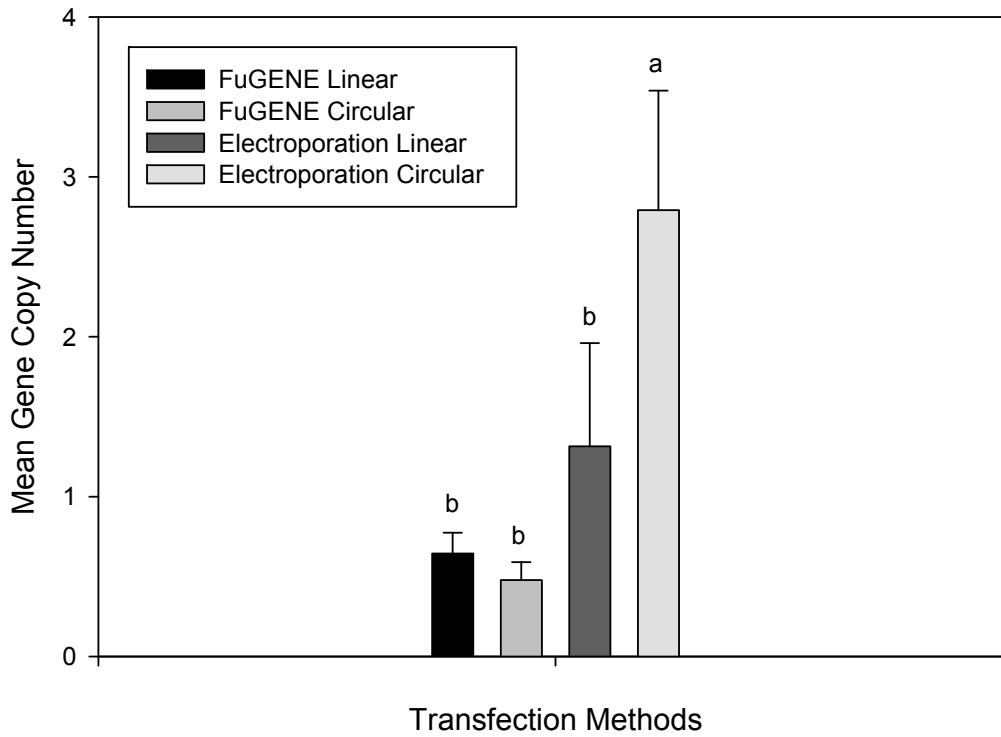


Figure 3.4 Mean gene copy number for all treatments. Bars indicated  $\pm$ SEM. Statistical difference were determined by One-Way ANOVA ( $P < 0.05$ ).

<sup>a,b</sup>Significant differences between treatments.

### Liposome Mediated vs. Electroporation

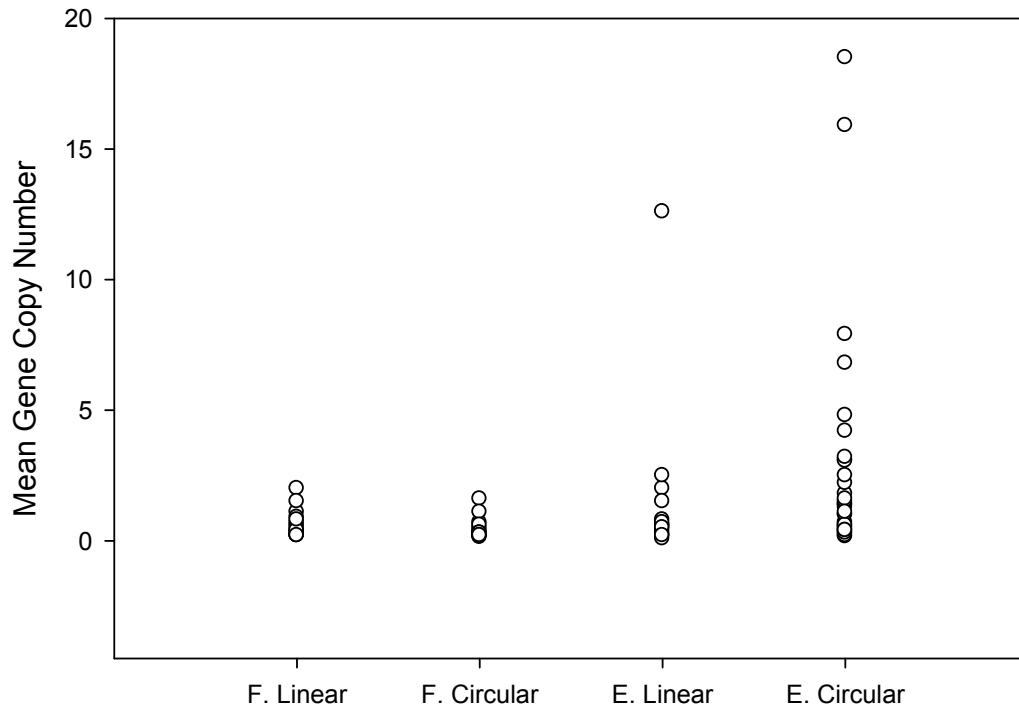


Figure 3.5 Mean gene copy number from technical replicates of isolated single cell colonies.

FuGENE<sup>®</sup> HD in contrast to electroporation treatments produced a lower gene copy number with a circular rather than a linear plasmid construct. Little is known about the fate of the of exogenous DNA after it has been internalized into the cell. It is hypothesized that the early endosomes could be sent to the extracellular compartment for recycling, targeted to lysosomes and only a small portion exogenous DNA is released into the cytoplasm, while a majority will be degraded (Lechardeur et al., 2002). Using a liposome-mediated reagent, such as FuGENE<sup>®</sup> HD appeared to decrease the variability of the mean gene copy number between samples with both the circular and linear construct. The decrease in variability when compared to electroporation may be due to the mechanism and the compartment exogenous DNA is carried in when traveling through the cytosol, protecting it from degradation. This may also play a role in the increase in the mean gene copy number when using a linear plasmid construct, protecting its free ends from the endonucleases and allowing more copies to integrate into the genome. Circular plasmid constructs are hindered in their ability to integrate into the genome of a cell because they do not have the free ends available.

A significant difference was found when comparing the electroporation circular treatment to electroporation linear, FuGENE<sup>®</sup> HD linear and FuGENE<sup>®</sup> HD circular treatments. Recent evidence indicates that exogenous DNA transferred by chemical methods, such as FuGENE<sup>®</sup> HD, is subject to mutations and rearrangements possibly as a result of passage through the lysosomal compartment (Toneguzzo et al., 1988). For integration of exogenous to be successful they need to be integrated into the genome completely intact and unmutated.

Both treatments of FuGENE<sup>®</sup> HD produced mean gene copy numbers that were less than one, but is interpreted as being equal to one. It is not feasible to have less than one gene copy number. Gene copy number is determined by the interpolation of the data from the standard curve produced with every run of Q-PCR. Since the data is run in three technical replicates and the mean is taken from the replicates, the data will never produce even integers such as 1 or 2. After Caprine fibroblast cells were transfected, they were put under antibiotic

selection until genomic DNA isolation making the chances the gene copy number is equal to zero very small. If the Caprine fibroblast cells did not integrate the exogenous DNA into their genome they would not survive the antibiotic selection, because the cells do not naturally carry the resistance.

The low gene copy numbers and variability seen between transfections produced by FuGENE<sup>®</sup> HD and electroporation were similar to the gene copy numbers reported in 2009 with nucleofection. (Graham et al., 2009). The gene copy numbers ranged from 1 to 3, with one cell line having an elevated gene count number of 8 to 13. Both FuGENE<sup>®</sup> HD and electroporation gave relatively low copy numbers, which is quite different from the reported results with pronuclear injection. Pronuclear injection can produce gene copy numbers varying from 1 to 200 (Pinkert, 1993). When transgenes are inserted into the genome by pronuclear injection, the transgenes completely avoids the cytoplasm and are inserted directly into the nucleus of the cell. By avoiding the cytoplasm transgenes are not degraded by endonucleases, which allow for more transgenes to be inserted into the genome at one time. These results also show a difference in gene copy number between electroporation, liposome mediated transfection, and pronuclear injection. The difference seen between transfection methods may play an important role because NT is used in almost all large animal transgenics. NT requires that exogenous DNA is transformed by either electroporation or liposome mediated transfection. FuGENE<sup>®</sup> HD on average produced gene copy number close to one; whereas; electroporation with a circular plasmid construct produced a mean gene copy number of 3. By inserting 3 transgenes, electroporation is insuring at least one transgene will be expressed after translation. It is required that all transgenes be inserted into the genome unmutated to be effective, meaning it is vital to have a transfection method that inserts more than one gene copy number to ensure expression. At the same time, the chosen transfection method must not insert too many transgenes, causing the gene to be silenced.

Our results indicate that the transfection method used to transform cells can affect the gene copy number. Electroporation, using a circular plasmid construct, was the most efficient method of transfecting Caprine fibroblast cells producing the highest gene copy number. However, this method also produced the highest variability between samples. Electroporation using a linear plasmid construct produced a lower gene copy number, but the variability between samples was also smaller. Similarly, FuGENE<sup>®</sup> HD with a linear plasmid produced lower gene copy numbers and had less variability between samples than both electroporation treatments.

Using FuGENE<sup>®</sup> HD with a linear construct will produce the most consistent results with a lower gene copy number. If high gene copy number is needed, then using electroporation with a circular construct would produce this result but it needs to be understood that this method will not be consistent between samples and transfections.

## CHAPTER IV

### SUMMARY AND CONCLUSION

Determining how gene copy number is affected by the two main methods of transfection will allow for the optimization of gene expression in transgenic animals. Due to the limited success of NT embryos to give rise to live offspring, it is essential to ensure that the transgene is being expressed at its highest potential. Transgenic animals can and have been used for disease models, production of pharmaceutical proteins, production of genetically superior animals, and xenotransplantation.

The present study focused on improving in vivo expression of transgenes introduced into the genome by determining how electroporation and liposome-mediated transfection affects gene copy number. All data was compared to a standard curve that was produced by 10-fold dilution of plasmid DNA with every run of Q-PCR. Treatment 1 examined how electroporation using a circular plasmid construct affected mean gene copy number. Treatment 2 used the same method of transfection with a linear plasmid construct. Results from these two treatments produced the highest mean gene copy numbers along with the most variability between samples. In Treatment 3 FuGENE<sup>®</sup> HD was used as the chemical reagent to execute liposome-mediated transfection using a circular plasmid construct. Treatment 4 used the same transfection as treatment three with a linear plasmid construct. The FuGENE<sup>®</sup> HD treatments produced lower mean gene copy numbers with less variability when compared with the electroporation treatments. In addition, there was a significant difference between the electroporation circular and all other treatments.

It is also important to note the difference in gene copy numbers produced by liposome mediated transfection, electroporation, and pronuclear injection. When using NT to produce transgenic animals, the transfection method needs to be capable to insert more than one transgene at a time, however, the transfection should not allow for highly elevated gene copy numbers, which could cause gene silencing.

In conclusion, these data obtained can be used to increase the transgene expression in cultured cells and in cloned animals. We hypothesized that gene expression levels could be increased when using a transfection method that does not alter or mutate the transgene. Our results indicate that the transfection method used to transform cells can affect the gene copy number, however, it is unknown if the exogenous DNA is mutated or altered during the delivery of the transgene to the nucleus or integration. Without better knowledge of the molecular mechanisms it is going to be difficult to devise approaches to make transgene expression efficient. Until these molecular mechanisms are better understood, the results obtained from this study will aid the increase *in vivo* transgene expression.



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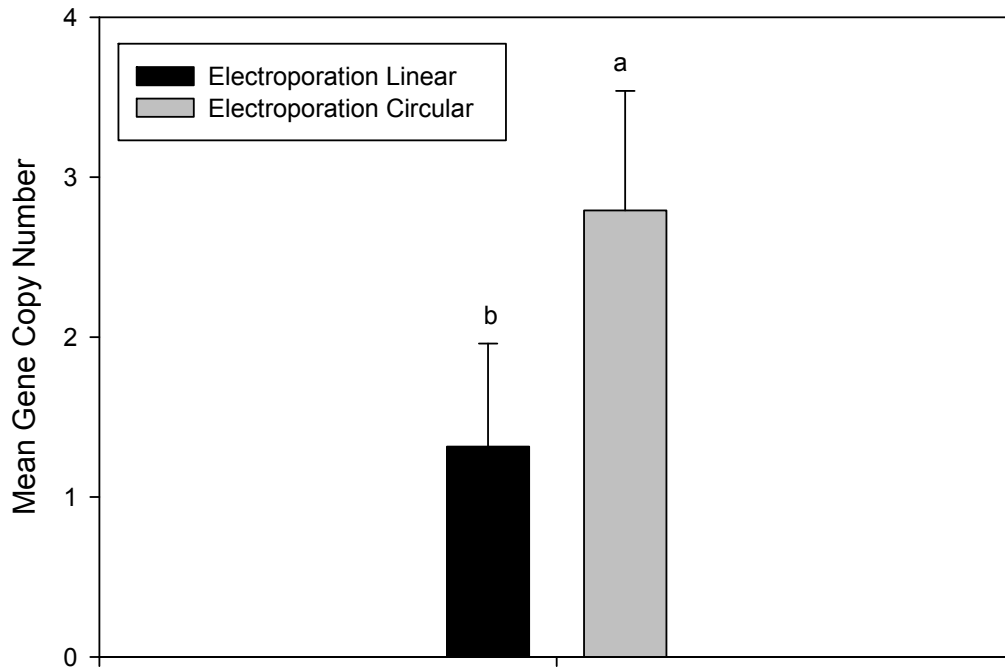
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## APPENDIX A: SUPPLEMENTAL FIGURES

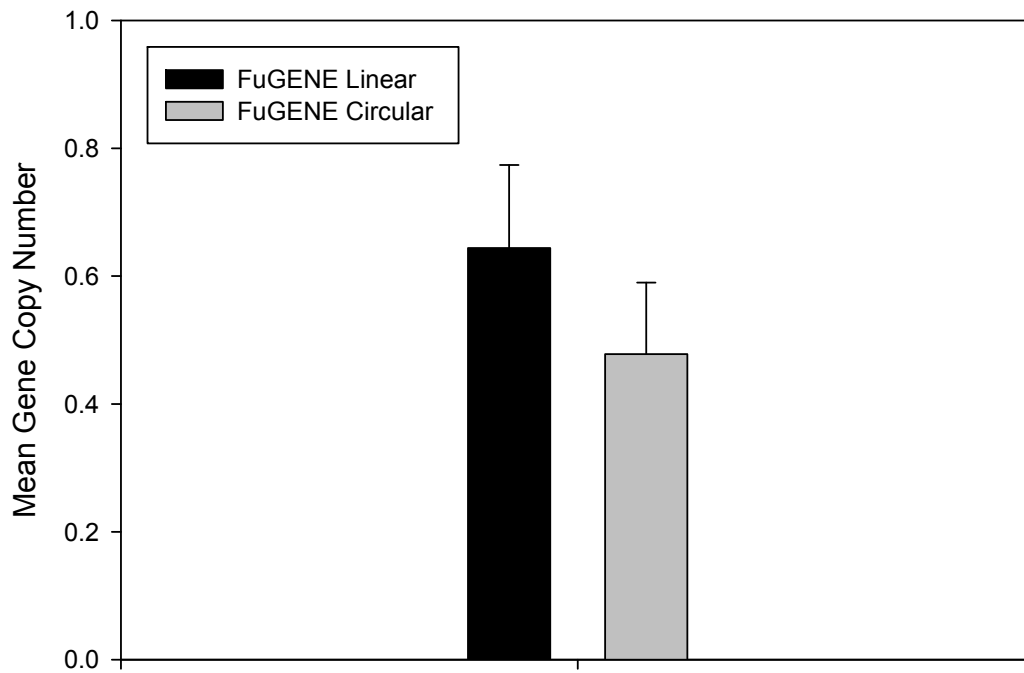
### Electroporation Linear vs. Circular



A1 Mean gene copy number for electroporation linear and electroporation circular. Bars indicated  $\pm$ SEM. Statistical difference were determined by One-Way ANOVA ( $P < 0.05$ ).

<sup>a,b</sup>Significant differences between treatments.

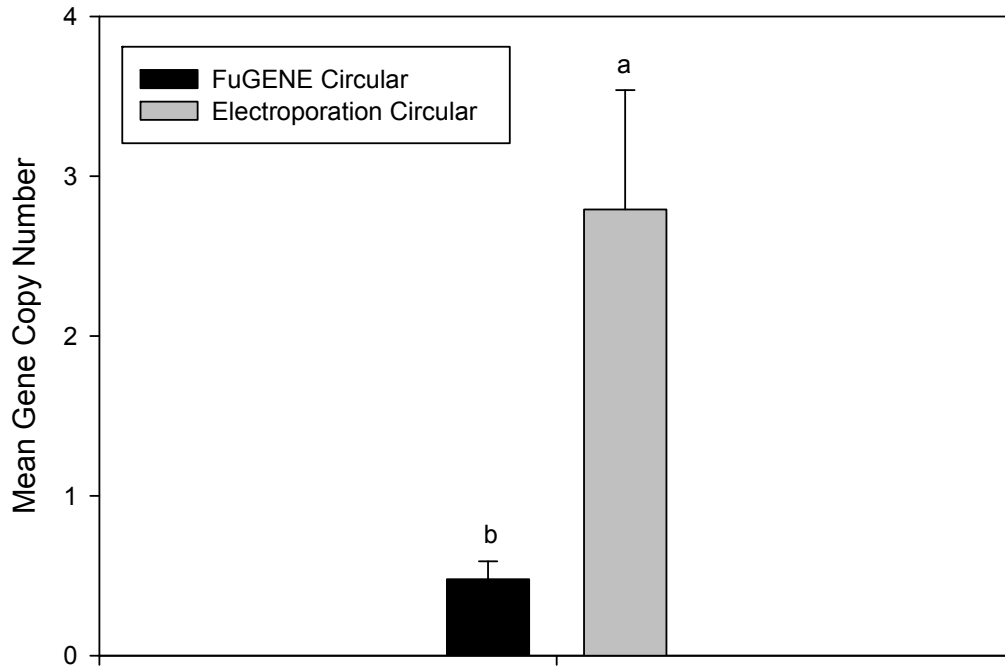
## Liposome Mediated Linear vs. Circular



A2 Mean gene copy number for FuGENE<sup>®</sup> HD linear and FuGENE<sup>®</sup> HD circular. Bars indicated  $\pm$ SEM. Statistical difference were determined by One-Way ANOVA ( $P < 0.05$ ).



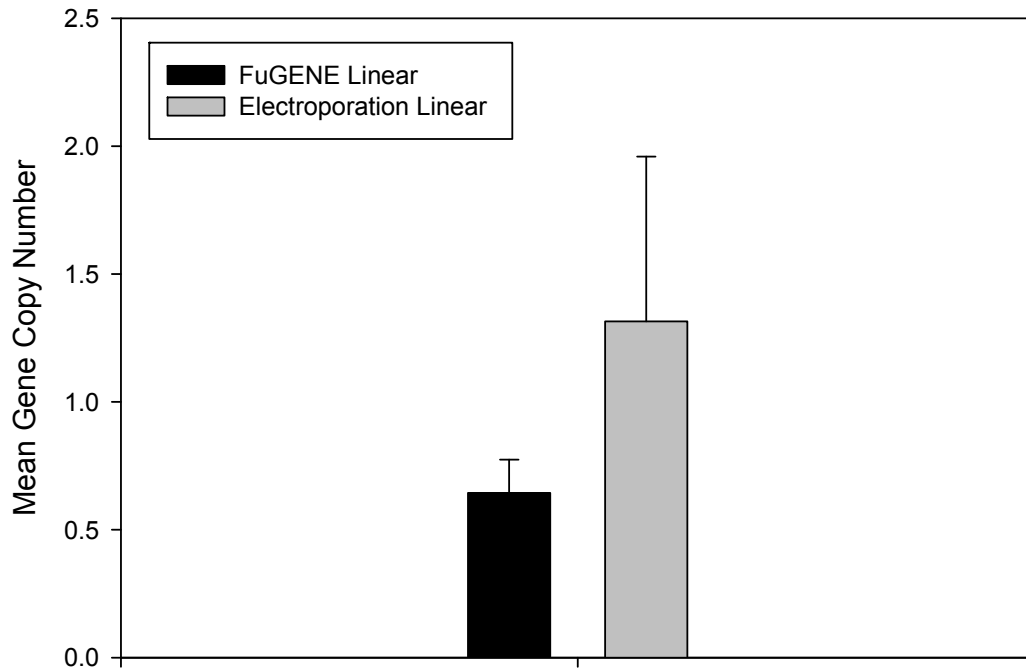
### Liposome Mediated vs. Electroporation Circular



A3 Mean gene copy number for FuGENE<sup>®</sup> HD and electroporation circular. Bars indicated  $\pm$ SEM. Statistical difference were determined by One-Way ANOVA ( $P < 0.05$ ).

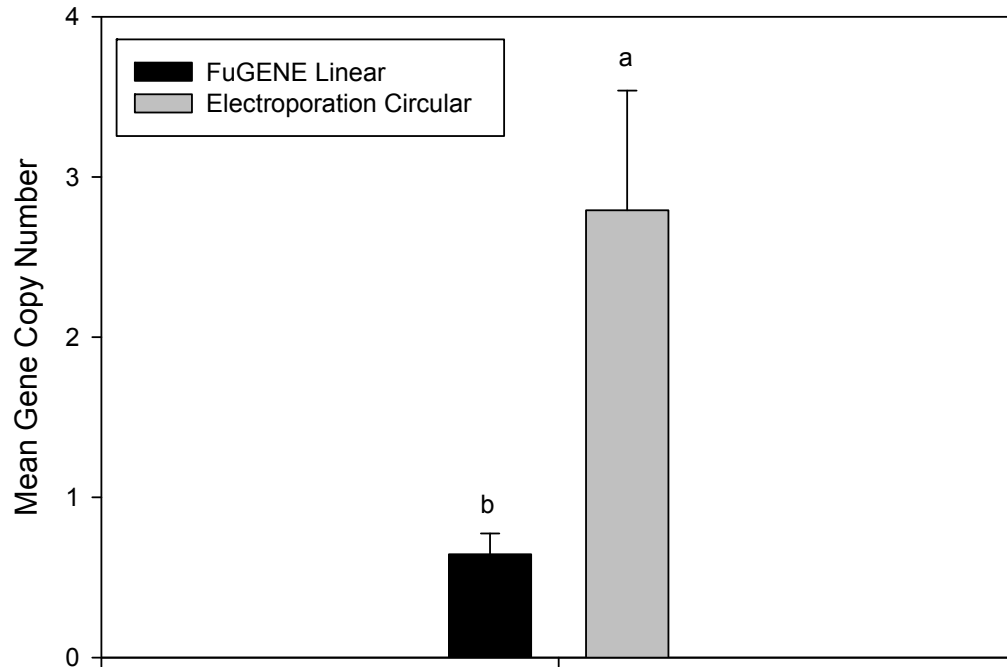
<sup>a,b</sup>Significant differences between treatments.

### Liposome Mediated vs. Electroporation Linear



A4 Mean gene copy number for FuGENE<sup>®</sup> HD and electroporation linear. Bars indicated  $\pm$ SEM. Statistical difference were determined by One-Way ANOVA ( $P < 0.05$ ).

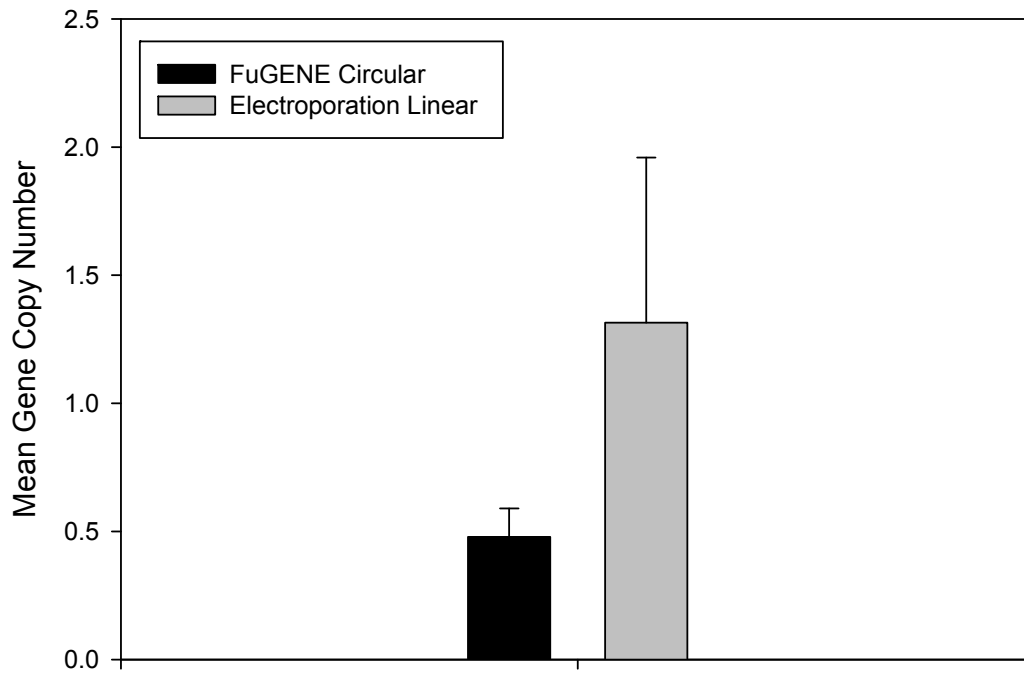
## Liposome Mediated Linear vs. Electroporation Circular



A5 Mean gene copy number for FuGENE<sup>®</sup> HD linear and electroporation circular. Bars indicated  $\pm$ SEM. Statistical difference were determined by One-Way ANOVA ( $P < 0.05$ ).

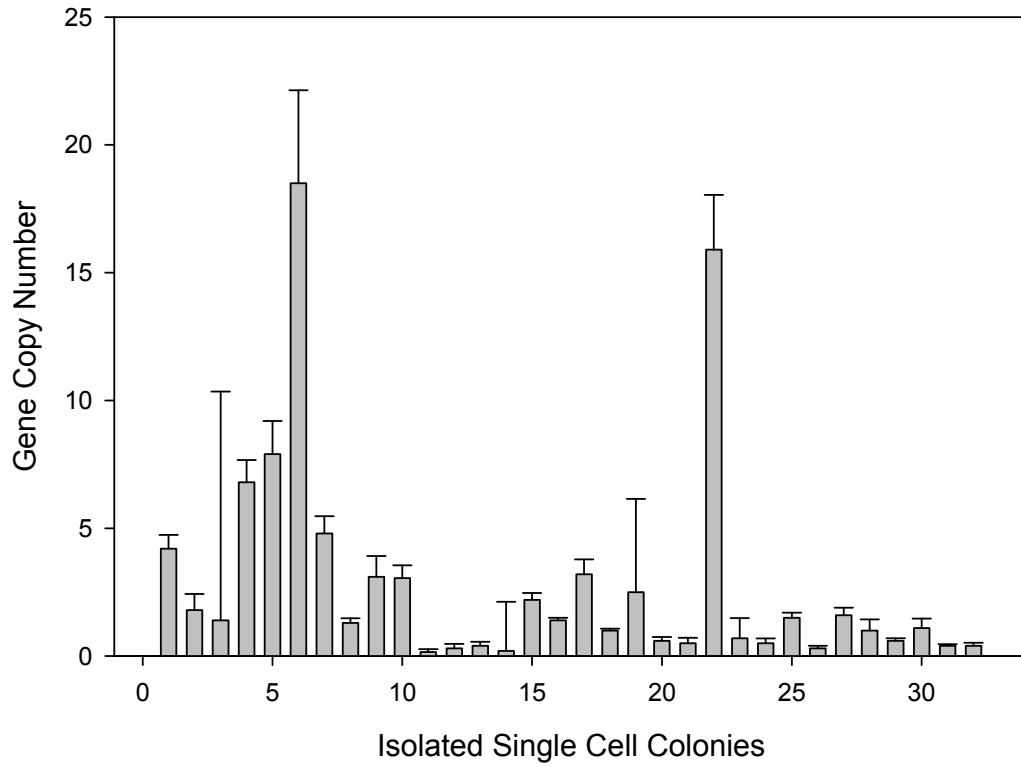
<sup>a,b</sup>Significant differences between treatments.

## Liposome Mediated Circular vs. Electroporation Linear



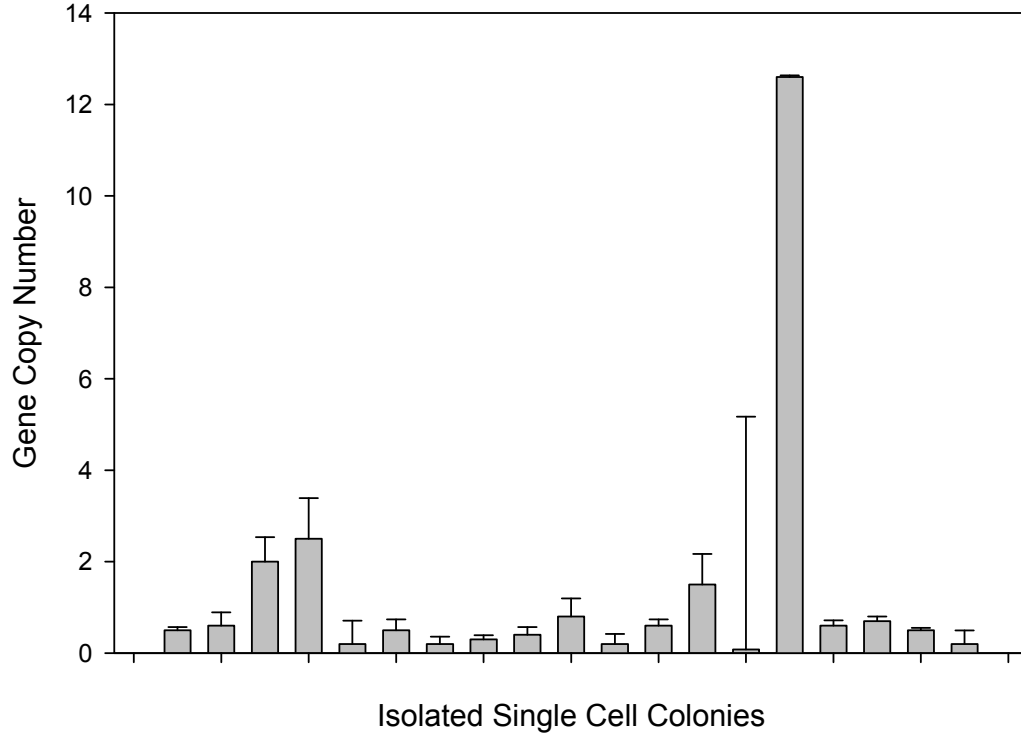
A6 Mean gene copy number for FuGENE<sup>®</sup> HD circular and electroporation linear. Bars indicated  $\pm$ SEM. Statistical difference were determined by One-Way ANOVA ( $P < 0.05$ ).

## Electroporation using a Circular Plasmid Construct



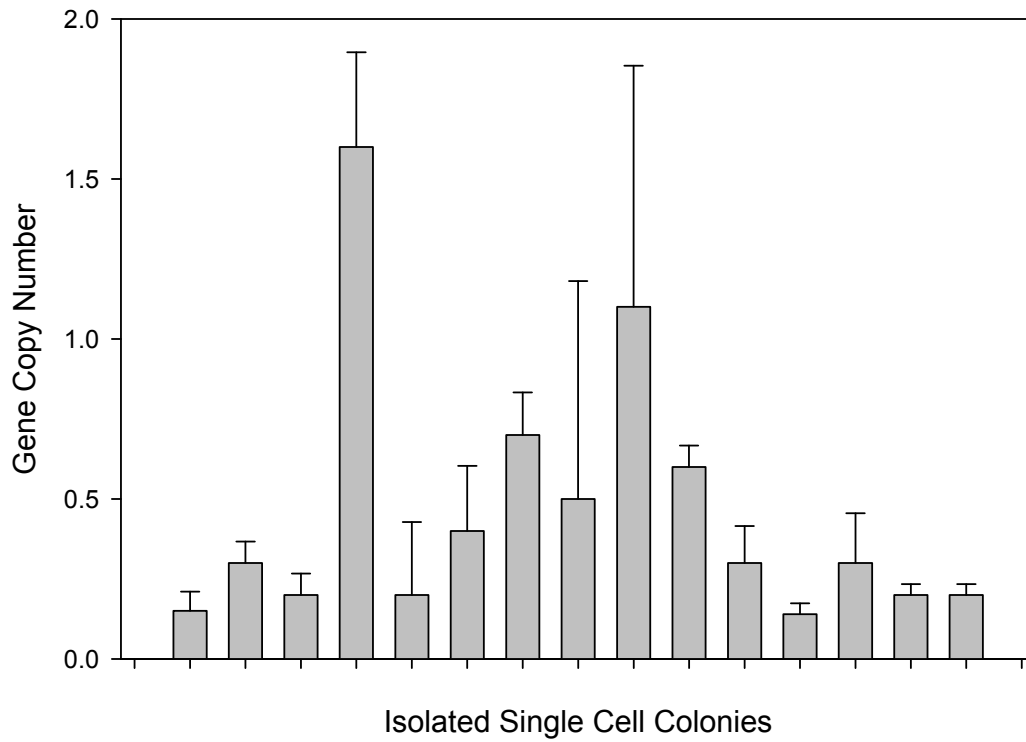
A7 Mean gene copy number for the technical replicates for isolated single cell colonies from electroporation circular. Bars indicate  $\pm$ SEM.

## Electroporation using a Linear Plasmid Construct



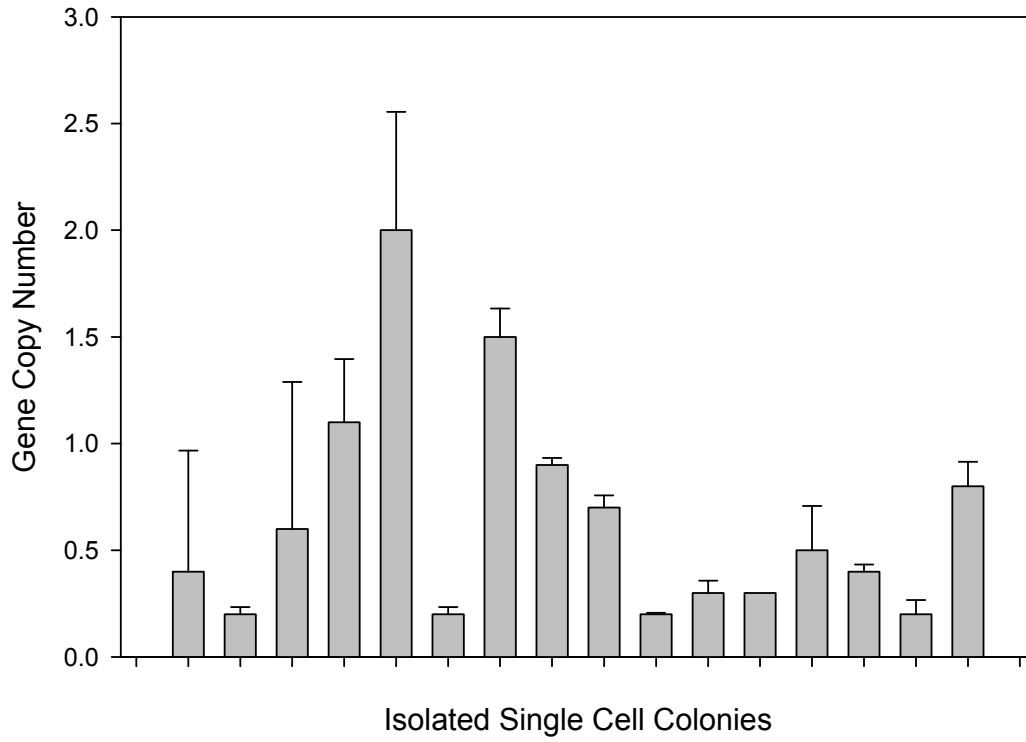
A8 Mean gene copy number for the technical replicates of isolated single cell colonies from electroporation linear. Bars indicate  $\pm$ SEM.

### FuGENE HD using a Circular Plasmid Construct



A9 Mean gene copy number for the technical replicates for isolated single cell colonies from FuGENE<sup>®</sup> HD circular. Bars indicate  $\pm$ SEM.

### FuGENE HD using a Linear Plasmid Construct



A10 Mean gene copy number for the technical replicates for isolated single cell colonies from FuGENE<sup>®</sup> HD linear. Bars indicate  $\pm$ SEM.



## APPENDIX B: PROTOCOLS

### Isolation and Linearization of Plasmid

Exogenous DNA or pHEGFP plasmid DNA (Figure 3.1) was isolated from *E. coli* using QIAfilter Plasmid Midi Purification Kit. *E. coli* initially cultured in 3 ml of terrific broth containing glycerol and 30  $\mu$ g Kanamycin for 3 h at 37°C with vigorous shaking. After first incubation, the *E. coli* culture is transferred into 100 ml of terrific broth with glycerol and 50  $\mu$ g of Kanamycin overnight under the previously stated conditions. The bacterial cells were harvested by centrifugation at 6000 x *g* for 15 m at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 4 ml of buffer P1. Four ml of buffer P2 was added and mixed by vigorously inverting the sealed tube 4 to 6 times before being incubated at room temperature (15 to 25°C) for 5 min.

During the incubation the QIAfilter cartridge was prepared by screwing the on the outlet nozzle of the QIAfilter Midi. After the 5 min incubation, 4 ml of chilled buffer P3 was added to the lysate, and was mixed immediately and thoroughly by vigorously inverting the tube 4 to 6 times. The lysate was then poured into the barrel of the QIAfilter cartridge, and was allowed incubate for 10 min at room temperature (15 to 25°C). While lysate was incubated the QIAGEN-tip 100 was equilibrated by adding for 4 ml of buffer QBT and emptied by gravity flow. The cap on the QIAfilter cartridge was then removed, the plunger was inserted into the cartridge, and the cell lysate was filtered into the previously equilibrated QIAGEN-tip. The cleared lysate was then allowed to drain the QIAGEN-tip by gravity flow before being washed twice with 10 ml of buffer QC. A 15 ml tube was then attached to the bottom of the QIAGEN-tip and the plasmid DNA was eluted by adding 5 ml of buffer QF. Plasmid DNA was precipitated by adding 3.5 ml of room temperature isopropanol and was immediately centrifuge at 15,500 x *g* for 30 min at 4°C. The supernatant is carefully discarded and the DNA pellet is washed with 2 ml of room temperature 70% ethanol before being centrifuged for 10 min at 15,500 x *g* at 4°C. After the last centrifugation, the supernatant was carefully discarded without disturbing the DNA pellet and

was allowed to air dry for 10 min before dissolving plasmid DNA in TE Buffer. The concentration of isolated plasmid DNA was determined by using the BioRad SmartSpec™ Plus Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). with a dilution factor of 20.

Isolated plasmids were linearized using a digestive enzyme that was specific to the restriction site BamH1 and purified using Invitrogen™ PureLink™ PCR Purification Kit. DNA was linearized in a mixture consisting of 1 to 2 µL of BamH1 enzyme, buffer, water and DNA and is incubated for 3 h in a 37°C water bath. Four volumes of PureLink™ binding buffer containing isopropanol was added to linearized DNA sample and mixed thoroughly before being added to a PureLink™ spin column. The sample was then centrifuged at room temperature at 10,000 x *g* for 1 min and the flow through was discarded. Six hundred and fifty µl of wash buffer containing ethanol was added to the sample and was centrifuge at 10,000 x *g* for 1 min. The flow was discarded and the sample was centrifuge again at maximum speed for 3 min. The spin column was placed in a clean 1.7 ml PureLink™ elution tube and 50 µl of elution buffer (10mM Tris-HCl, pH 8.5) to the center of the spin column. The sample is allowed to incubate for 1 min at room temperature before being centrifuged at maximum speed for 2 min. Linearization was verified by running electrophoresis and DNA concentration was determined using BioRad SmartSpec™ Plus Spectrophotometer with a dilution factor of 20.

### Electroporation

Caprine fibroblasts cells (CF1) were cultured to 80-90% confluency in Dulbecco's Modified Eagle Medium with high glucose (DMEM) containing 15% fetal bovine serum (FBS) and 1% P/S or complete media in 60 mm culture dish at 39°C incubator containing CO<sub>2</sub>. Cells were then washed once with Dulbecco's Phosphate-Buffered Saline (PBS) without calcium and magnesium and released with trypsin (0.25%)/EDTA (T/E) Trypsin (0.5%)/5.3 mM EDTA solution (Gibco–BRL). After dissociation of cells, equilibrated complete media was added to deactivate the enzyme. Cell suspension was centrifuged at 350 x *g* for 5 min. Supernatant was

removed and cell pellet was resuspended in OptiMEM I<sup>®</sup> Reduced Serum Medium modification of MEM (Eagle's) before being centrifuge at 350 x g for 5 min. Supernatant was removed; 20 µg of either circular or linearized DNA and OptiMEM were added to the cell pellet, giving a final volume of 800 µl. The cell pellet was resuspended and incubated at room temperature for 10 min before being transferred to the electroporation curvette. Cells were pulsed in the BioRad Gene Pulser<sup>™</sup> (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 350 volts and 500 µF and transferred to a 35 mm dish containing complete media. Cells were incubated at 39°C in CO<sub>2</sub> for 72 h.

### Liposome Mediated Transfection

Liposome mediated transfection was performed using FuGENE<sup>®</sup> HD transfection reagent by Roche. Caprine fibroblast cells were cultured to 80-90% confluency in complete medium in a 39°C incubator containing CO<sub>2</sub>. The optimum ratio of FuGENE<sup>®</sup> HD reagent to DNA concentration and buffer was determined to be 6:2 in previous studies (data not shown). FuGENE<sup>®</sup> HD reagent, DNA, and OptiMEM I<sup>®</sup> Reduced Serum Medium modification of MEM (Eagle's) were allowed adjust to room temperature before use. After all solutions reached room temperature, 2 µg of circular or linearized DNA was diluted into 100 µl of OptiMEM I<sup>®</sup> and thoroughly well. The FuGENE<sup>®</sup> HD vial was vortexed for one second before adding 6 µl of reagent directly to the diluted DNA mixture, being sure to avoid contact with the sides of the microcentrifuge tube. The DNA-FuGENE<sup>®</sup> HD complex or transfection complex was vortexed for 2 sec and was allowed to incubate for 15 min at room temperature. After incubation, the transfection complex was added directly to the cells under the existing media and the dish was swirled to ensure distribution over the entire surface. Cells were incubated at 39°C with CO<sub>2</sub> for 72 h.

### Isolation of Single Cell Colonies

After 72 h, transfected cells were released with trypsin (0.25%), counted using a hemacytometer and plated in 100 mm dishes with complete media containing 600 µg/ml

Geneticin<sup>®</sup> (G418) and 2 ng/ml fibroblast growth factor basic (bovine brain-derived) (bFGF) referred to as selective media. The optimum cell seeding density to obtain single celled colonies for electroporation with and without bFGF was determined to be 10,000 cells for a circular DNA construct and 15,000 for linearized DNA construct. For FuGENE<sup>®</sup> HD, the optimum seeding density was determined to be 40,000 cells without bFGF, however, with bFGF the seeding density decreased to 20,000 cell with circular DNA and 25,000 cells with linearized DNA. Cells were incubated in 39°C with CO<sub>2</sub> for 10 d in selective media to allow for the formation of single cell colonies.

After ten days under selection, colonies were selected, harvested with a cloning ring, and to a 24 well plate and incubated until confluent. Once confluency was reached, cells were passaged using trypsin (0.25%) into 35 mm dish with selective media and allowed to reach confluency. Confluency in the 35 mm dishes was vital to obtain high concentration of genomic DNA after isolation.

#### Isolation and Quantification of Genomic DNA

Genomic DNA was isolated from Caprine fibroblast cells once confluency occurred in the 35 mm dishes using Gentra<sup>®</sup> Puregene kit from Qiagen<sup>®</sup>. Cells were washed, trypsinized, and transferred to a 1.5 ml microcentrifuge tube. To pellet cells the microcentrifuge tube was centrifuged for 5 sec at 13,000 x *g* and the supernatant was carefully discarded leaving 20 µl of residual liquid. The microcentrifuge tube was then vortexed to resuspend the cells in the supernatant. A volume of 300 µl of cell lysis solution was added to the resuspended cells and vortexed on high speed for 10 sec to lyse the cells. RNA-free DNA was required, requiring 1.5 µl of RNase A Solution to be added and mixed by inverting tube 25 times. Cells were incubated at 37°C for 5 min and then transferred to ice for 1 min. After sample was cooled, 100 µl of precipitation solution was added and sample was vortexed on high 20 sec before being centrifuged for 1 min at 13,000 x *g*. While sample was centrifuging, 300 µl of isopropanol was pipetted into a clean microcentrifuge tube. The supernatant from the sample was carefully

added directly to the isopropanol and the sample was mixed gently by inverting the tube 50 times. After proper mixing, the sample was centrifuge again at 13,000 x g for 1 min and the supernatant was discarded be carefully draining the tube on a clean piece of absorbent tissue. Three hundred  $\mu\text{l}$  of 70% ethanol was added and the tube was inverted several times to wash the DNA pellet. The DNA pellet was centrifuged one last time at 13,000 x g for 1 min and the supernatant was carefully drained onto a clean piece of absorbent tissue for 5 sec. The DNA pellet was allowed to air dry for 15 min before adding 50  $\mu\text{l}$  of DNA hydration solution and vortexing to for 5 sec on medium speed to ensure sample is mixed. The sample is incubated at 65°C for 1 h to dissolve the DNA and then allowed to incubate overnight at room temperature.

After the overnight incubation, the DNA sample concentration is initially checked using the BioRad SmartSpec™ Plus Spectrophotometer with a dilution factor of 20. Sample with DNA concentration higher then 150  $\mu\text{g/ml}$  and a 260 nm wavelength greater then 0.1 AU were kept for further analysis. The initial concentration readings were used to adjust DNA samples to be equal to  $1 \times 10^5$  cells or 600 ng in a 5  $\mu\text{l}$  sample. All samples concentrations were re-verified using a Cytoflour® 4000 Plate Reader (Applied Biosystems, Foster City, CA, USA) and a Quant-iT™ dsDNA Broad-Range Assay Kit from Invitrogen™. First, a working solution was prepared by diluting Quant-iT™ dsDNA BR reagent 1:200 in Quant-iT™ and 200  $\mu\text{l}$  was loaded into each microplate well that was going to be used. Ten  $\mu\text{l}$  of each  $\lambda$  DNA standard, eight total, were added in triplicates to the microplate well containing the working solution. The DNA samples were also ran in triplicate by adding 5  $\mu\text{l}$  to each microplate well containing the working solution. The samples were read on the microplate by the Cytoflour® 4000 Plate Reader with a maximum excitation/emission of 510/527 nm and standard excitation/emission of approximately 480/530. After fluorescence was read, a standard curve was developed to determine the DNA concentrations of each sample.

### Validation and Optimization of Q-PCR

Primers were designed to amplify position 5,542 (sense) with a sequence CCGTGATATTGCTGAAGAG (5' – 3') and 5,649 (anti-sense) annealing to the following sequence TCAAGAAGGCGATAGAAGG of the plasmid and were manufactured by Invitrogen™ Illumina. Annealing temperature gradient and primer concentrations matrix were performed with sense and antisense primers to determine optimal annealing temperature and primer concentration to allow for maximum amplification. The optimal annealing temperature was determined by amplifying plasmid DNA at varying temperatures and separating of PCR products by electrophoresis. The primer concentration matrix was run using the optimum annealing temperature of 55°C and the sense and antisense primers were utilized in varying pmol concentrations ranging from 6.25 pmols to 50 pmols. Q-PCR amplification was performed on the plasmid and the optimized primers concentration determined by the lowest  $C_T$  Q-PCR was used to develop a standard curve by 10-fold dilution to determine if the primers were amplifying a single product in a quantitative manner, with amplification efficiency between 80% to 120% and a correlation coefficient close to 1.0 (Figure 3.2).

### Q-PCR

Genomic DNA isolated from transfected Caprine fibroblast cells from all treatments were amplified using the iQ™ SYBER Green Supermix in the MyiQ Reverse Transcription PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The Q-PCR Reaction Mix was made up of 12.5 µl of iQ™ SYBER Green 2X Supermix, 5 µl of DNA or H<sub>2</sub>O, 5.5 µl of nuclease-free water, and 1 µl of each primer (sense and antisense at 25 pmol concentrations). All samples, including the no template negative control were run in triplicates and compared to a standard curve developed by a 10-fold serial dilution (Figure 3.3) of the same linearized plasmid used to transfect cells equivalent to  $1 \times 10^7$  to  $1 \times 10^3$  copies. The Q-PCR program used for the amplification of all samples comprised of a denaturing cycle of 3 m at 95°C; 40 cycles of PCR (95°C for 10 sec and 55°C for 45 sec); a melting curve analysis which was made up of 95°C for

1 min followed by 55°C for 1 min, a step cycle with 80 repeats starting at 55°C for 10 sec with a +0.5°C/sec transition rate; and a final holding temperature of 10°C.

The 10-fold serial dilution ran with each Q-PCR session, produced an equation for a line, which was used to determine the gene copy number. The mean threshold cycle ( $C_T$ ) value of the triplicate produced by each sample is entered into the equation for the line and solved for Y, giving the transgene copy number for the sample. The Cytoflour<sup>®</sup> 4000 reading allowed for the number cells per 5  $\mu$ l sample to be determined, and the number of times the exogenous DNA was integrated into the genome of an individual cell. Both individual gene copy numbers were calculated from each replicate as well as the mean gene copy number from mean  $C_T$  value.

## APPENDIX C: PRODUCTS

<b>Product</b>	<b>Product Number</b>	<b>Company</b>
Kanamycin	11815-032	Gibco
Fetal Bovine Serum	SV30014.03	HyClone
Penicillin-streptomycin (P/S)	15140	Gibco
DMEM/HIGH GLUCOSE	SH30243.02	HyClone
dPBS w/o Ca <sup>2+</sup> & Mg <sup>2+</sup>	21600-010	Gibco
OptiMEM I <sup>®</sup>	31985	Gibco
.25% Trypsin-EDTA	25200	Gibco
FuGENE <sup>®</sup> HD	04 709 691 001	Roche
Bovine Fibroblast Growth Factor	133-FB	R&D Systems
Geneticin	11811-023	Gibco
iQ <sup>™</sup> SYBER Green Supermix	170-8882	Bio-Rad
BamH1 Restriction Enzyme	15201-023	Invitrogen
QIAFilter Plasmid Midi Kit	12243	Qiagen
PureLink PCR Purification Kit	K3100-01	Invitrogen
Gentra <sup>®</sup> PureGENE Kit	1042601	Qiagen
Quant-iT <sup>™</sup> dsDNA Broad-Range Assay Kit	Q33130	Invitrogen



## VITA

Jessica Ann Wilson is the only child born to Lisa Snyder in Tucson, Arizona. Jessica was raised in the Southwest before moving to Oregon in 1997. She attended James Madison High School in Portland, Oregon in 2002. While in school, she volunteered at the Oregon Zoo for 6 years, earning the President's Award for outstanding community service presented by President Bill Clinton.

After graduating in June 2002, she attended Oregon State University, where she earned a Bachelor of Science degree in animal, dairy and poultry science with a minor in chemistry in June, 2006. During her senior, she was a student teacher for Dr. Alfred R. Menino, Jr.

She entered Graduate School in August of 2006 under the direction of Dr. Kenneth R. Bondioli and is now a candidate for the degree of Master of Science in reproductive physiology in the School of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana. While pursuing her graduate degree, Jessica was married to Kiel Wilson in June, 2008.