

2013

Response of bovine fetal fibroblasts to pluripotency induction with in vitro transcribed mRNA

Tricia Adams

Louisiana State University and Agricultural and Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_theses



Part of the [Animal Sciences Commons](#)

Recommended Citation

Adams, Tricia, "Response of bovine fetal fibroblasts to pluripotency induction with in vitro transcribed mRNA" (2013). *LSU Master's Theses*. 3280.

https://digitalcommons.lsu.edu/gradschool_theses/3280

This Thesis is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Master's Theses by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.

RESPONSE OF BOVINE FETAL FIBROBLASTS TO PLURIPOTENCY
INDUCTION WITH *IN VITRO* TRANSCRIBED MRNA

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 of
Animal and Dairy Sciences

by
Tricia L. Adams
B.S., Southeastern Louisiana University, 2010
December 2013

ACKNOWLEDGEMENTS

First and foremost, the author would like to thank Dr. Kenneth Bondioli for all of his time and dedication to her education and overall learning experience. Graduate School was made possible by the opportunity he provided. His commitment to challenge, encourage, and mentor the author is unparalleled and she would never have achieved this great milestone without him. He was not only a mentor, but a great friend and confidant. A special thanks is given to the author's graduate committee, Dr. Kenneth Eilertsen, Dr. John Lynn, and Dr. Richard Cooper, for all of their advice, academic instruction, and thesis development.

Great appreciation goes to Dr. Eilertsen and all of the staff at NuPotential. The time and experience spent in the laboratory was beneficial to experiments carried out in this manuscript. A special thanks to Dr. Jaroslaw Staszkievicz for allowing the author to shadow him in the laboratory. It was a great learning experience.

The author would like to thank all of fellow graduate students for camaraderie, late study nights, and overall support through her time spent at the RBC. Sarah Farmer and Jairo Sarmiento greatly aided through critique and study comments during graduate school, and both became great friends. Javier Jarazo provided not only great discussion sessions, but also genuine friendship. Sincere thanks to Fabian Diaz, Cody Bailey, Whitney Gaspard, Meredith Addison, Michael Stout, and Paige Hardin.

The author is eternally grateful to her husband, John Eric Adams, for all of his unconditional support and love. The completion of this work would not have been possible without all of his dedication to marriage, fatherhood, and education. Special thanks are given to her children, Madelyn and Holden Adams. They were always (well, mostly) good sports during

the repeated trips to the lab on the weekend or school nights. The movie nights in the conference room provided long-lasting memories.

The author is grateful for the support provided by her family. Sisters, Lynne Cochran, Catherine Bihm, and Elizabeth Font listened and supplied feedback on endless situations. Mother, Karen James, was willing to babysit at a moment's notice, and participate in the author's complaining phone calls. A special thanks to Penny Font for editing this thesis, and encouraging remarks from father, Bobby Font. This work would not have been possible without the help from the author's husband's family. Long study hours and endless thesis editing was made easier with the endless babysitting offers. This work could not have been accomplished without the phenomenal support provided by the author's family.

A bittersweet appreciation goes to the late Eryk Richards. He provided relentless positive encouragement during moments of doubt. His passing during the evolution of this manuscript proved to be one of the most difficult experiences to overcome.

TABLE OF CONTENTS

ACKNOWLEDGMENTS.....	ii
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
LIST OF ABBREVIATIONS.....	ix
ABSTRACT.....	xi
CHAPTER ONE: INTRODUCTION.....	1
CHAPTER TWO: LITERATURE REVIEW.....	4
Embryonic Stem Cells.....	4
Distinctive Properties of Embryonic Stem Cells.....	4
Adult Stem Cell.....	6
Induced Pluripotent Stem Cells.....	6
RNA Induced Pluripotent Stem Cells.....	7
Benefits of Stem Cells in Research.....	8
Gene Expression of Embryonic Stem Cells.....	8
Somatic Cell Nuclear Transfer.....	12
Reprogramming by Viral Transfection.....	14
Problems Associated with Viral Transfection.....	15
DNA-Free Transfection Methods; Cell-Penetrating Peptide Moieties and RNA-Based Viral Systems.....	15
In Vitro Transcribed Messenger RNA Delivery for Reprogramming.....	16
Enhanced Reprogramming Using Small Molecules.....	17
Safety and Efficiency of Reprogramming to Pluripotency: Future Outlook.....	19
CHAPTER THREE: RESPONSE OF BOVINE FETAL FIBROBLASTS TO PLURIPOTENCY INDUCTION WITH IN VITRO TRANSCRIBED MRNA.....	21
Introduction.....	21
Materials and Methods.....	24
Experimental Design.....	28
Results.....	31
Discussion.....	44
CHAPTER FOUR: SUMMARY AND CONCLUSIONS.....	46
LITERATURE CITED.....	50
APPENDIX A: PROTOCOLS.....	58
APPENDIX B: MEDIA FORMULATIONS AND STOCK SOLUTIONS.....	77

VITA	90
------------	----

LIST OF TABLES

3.1	Optimal PCR conditions for binding bovine OCT4 sequence.....	37
3.2	Expression comparison to housekeeping gene.....	38
A.1	PCR primers.....	73
A.2	GFP ivtRNA quantification.....	74
A.3	ivtRNA concentrations.....	74
A.4	Plasmid DNA quantification.....	74
A.5	hOCT4 ivtRNA spectrophotometer quantification.....	74
A.6	ivtRNA concentrations.....	75
A.7	Plasmid DNA quantification.....	75
A.8	SOX2, KLF4, cMYC ivtRNA quantification.....	75
A.9	Purified PCR products.....	76
B.1	List of plasmids	77

LIST OF FIGURES

2.1	Oct4 expression during development.....	9
2.2	Efficiency and safety comparison of cellular reprogramming methods	20
3.1	GFP ivtRNA concentration fluorescence.....	32
3.2	GFP ivtRNA effect on cellular viability.....	32
3.3	GFP ivtRNA expression time course.....	33
3.4	GFP ivtRNA expression time course cellular viability.....	34
3.5	GFP ivtRNA expression extended time course.....	35
3.6	GFP ivtRNA extended time course cellular viability.....	35
3.7	PCR gradient with decreasing annealing temperature.....	37
3.8	Semi-Quantitative RT-PCR displaying endogenous Oct4 up-regulation containing PAP standardization.....	38
3.9	Relative fluorescence of modified GFP ivtRNA transfection.....	39
3.10	Measurement of cellular viability with modified GFP ivtRNA transfection.....	39
3.11	Measurement of cellular viability post modified 4F/3F ivtRNA transfection	40
3.12	Cell viability after 21 day transfection with 4F KMOS +/-3i culture media.....	41
3.13	Relative expression of Oct4 and Nanog normalized against housekeeping genes PAP and GAP.....	42
3.14:	BFF cells 15 days culture and daily transfections: (A) 4F -3i Day 15, (B) 4F +3i Day 15, (C) Control Day 15, photos obtained from EVO phase Contrast microscope.....	43
3.15:	BFF cells 21 days culture and daily transfections: (A) -3i Day 21, (B) +3i Day 21, (C) Control Day 21, photos obtained from EVO phase contrast microscope.....	43

B.1	Oct4 PCR amp/cycle graph of SYBR-490.....	80
B.2	Oct4 standard curve.....	80
B.3	Oct4 melting curve.....	81
B.4	Sox2 PCR amp/cycle graph for SYBR-490.....	82
B.5	SOX2 standard curve.....	82
B.6	SOX2 melting curve.....	83
B.7	Nanog PCR amp/cycle graph for SYBR-490.....	84
B.8	Nanog standard curve.....	84
B.9	Nanog celting curve.....	85
B.10	PAP PCR amp/cycle graph for SYBR-490.....	86
B.11	PAP standard curve.....	86
B.12	PAP melt curve.....	87
B.13	GAPDH PCR amp/cycle graph for SYBR-490.....	88
B.14	GAPDH standard curve.....	88
B.15	GAPDH melt curve.....	89

LIST OF ABBREVIATIONS

ADAS.....	Adipose-derived Adult Stem Cells
ASC.....	Adult Stem Cells
ATP.....	Adenosine 5'-triphosphate
BFF.....	Bovine Fetal Fibroblast
bOCT4.....	Bovine Oct4
bPAP.....	Bovine Poly-Adenylate Polymerase
CS.....	Calf Serum
CT.....	Threshold Cycle
CTR.....	Threshold Cycle for the Reference Gene
CTT	Threshold Cycle for the Target Gene
DMEM.....	Dulbecco's Modified Eagle Medium
DPBS.....	Dulbecco's Phosphate-Buffered Saline
ESC.....	Embryonic Stem Cells
FBS.....	Fetal Bovine Serum
GAPDH.....	Glyceraldehyde 3-phosphate dehydrogenase
GFP.....	Green Fluorescent Protein
GTP.....	Guanosine 5'-triphosphate
hESC.....	Human Embryonic Stem Cells
hASC.....	Human Adult Stem Cells
HSC.....	Hematopoietic Stem Cells
ICM.....	Inner Cell Mass
ivtRNA.....	<i>In Vitro</i> Transcribed RNA

iPSC.....	Induced Pluripotent Stem Cells
MEF.....	Mouse Embryonic Fibroblast
MSC.....	Mesenchymal Stem Cells
NPC.....	Neural Progenitor Cells
PAP.....	Polyadenylate Polymerase
P/S	Penicillin/Streptomycin
qRT-PCR.....	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RiPSC.....	RNA Induced Pluripotent Stem Cells
RT	Reverse Transcription
SCNT	Somatic Cell Nuclear Transfer

ABSTRACT

Numerous studies have contributed to the induction of pluripotency in an abundance of cell types; however, transfection techniques and efficiency have yielded undesirable outcomes. Traditionally, the use of viral vectors as a mode of transmission has proven to be efficient in the induction of pluripotency transcription factors in mammalian cells. The increasing concern is random insertion of viral components within the host genome due to the viral mode of replication. The delivery of messenger RNA by cationic lipid delivery vehicles circumvents the viral concerns and provides an efficient and safe mode of reprogramming. Synthetic mRNA can be used to initiate endogenous gene expression while maintaining cellular viability in bovine somatic cells. In this study, bovine fetal fibroblast cells were initially transfected with *In Vitro* Transcribed (IVT) RNA expressing Green Fluorescent Protein (GFP) to determine adequate transfection parameters. Mammalian expression vectors, encoded with either GFP or pluripotency associated transcription factors OCT4, SOX2, c-MYC, or KLF4, were obtained from a plasmid repository and used as IVT templates. The mRNA was produced *in vitro* to include a 5' cap as well as a 3' polyA tail in order to mimic *in vivo* mRNA packaging. Primary cultures of bovine fetal fibroblasts were transfected with ivtRNA by way of a cation lipid delivery vehicle, Lipofectamine, for endocytotic uptake. This process allows the mRNA to bypass the phospholipid bilayer and enter the cell. The incorporation of modified bases during the *in vitro* transcription process was adopted to reduce cell immune response. Addition of small molecules to enhance the reprogramming process was evaluated as well. The success of ivtRNA transfection in bovine fetal fibroblast cells was determined through the measurement of cellular viability, mean fluorescence by flow cytometry under different concentrations of mRNA, and gene analysis measured by quantitative PCR.

CHAPTER ONE: INTRODUCTION

Different stem cell types have been described including Adult Stem Cells (ASCs), Embryonic Stem Cells (ESCs), Induced Pluripotent Stem Cells (iPSCs) and RNA-Induced Pluripotent Stem Cells (RiPSCs). Adult stem cells can be found in various types of adult, or non-embryonic, tissues and give rise to particular cell lines. The natural purpose of these cells is to replace diminishing cells during growth or injury (Odorico et al., 2001). Embryonic stem cells arise from the inner cell mass (ICM) of a developing blastocyst and can give rise to all cell lineages of the fetus proper; all three major germ layers are produced. ESCs can no longer differentiate into extra-embryonic, or placental, tissue (Odorico et al., 2001). The third type of stem cells is known as induced pluripotent stem cells or iPSCs. These cells are derived from reprogrammed somatic or differentiated cells, and can be utilized in place of ESCs to eliminate the destruction of embryos while harvesting the ICM. These cells closely resemble the ES cells in many aspects including the ability to differentiate into different cell-lineages and proliferation. RiPSCs are simply a type of iPSCs where synthetic *in vitro* transcribed messenger RNA is utilized for the induction for reprogramming. Induced pluripotent stem cells have become a very important tool for the scientific community to study the cellular differentiation process, genetic manipulations, and regenerative medicine.

Induced pluripotent stem cells can be obtained by an extended list of reprogramming tools. The reprogramming of somatic cells can be achieved through the delivery of exogenous transcription factors that stimulate genetic activity similar to that of embryonic stem cells. These exogenous transcription factors include, but are not limited to OCT4, SOX2, NANOG, c-MYC and KLF4 (Takahashi and Yamanaka, 2006). The introduction of these previously silenced embryonic transcription factors can up-regulate their own endogenous expression. While, theoretically, the

increased expression should be easily attainable, there are numerous cellular hurdles to overcome. These hurdles tend to lead to differences in efficiency and at times successes of various modes of cellular reprogramming to pluripotency. These modes of reprogramming utilize various cellular processes to increase endogenous expression of known pluripotency factors. Viral transduction is commonly used in research as the mode of choice for the delivery of these factors. The virus encoding the desired sequence is incubated with the target cells to be reprogrammed. The cells are reprogrammed quite efficiently and have desirable results, though the random insertion of viral particles into the host genome remains undesirable. Viruses with alternate modes of replication have been used to minimize this random insertion with mixed results, including incomplete reprogramming (Takahashi and Yamanaka, 2006; Fusaki et al., 2009). There have been other attempts to avoid this random insertion, such as the addition of specific proteins to the target cells (Kim et al., 2009a; Zhou et al., 2009; Cho et al., 2010). While this circumvents the insertion issue, it can be a difficult process to achieve due to the difficult purification processes. Synthetic messenger RNA transfection has the ability to avoid both random insertion into the host genome and rigorous purification protocols.

Synthetic RNA transfection has been an invaluable tool in understanding the mammalian genome due to its ability to deliver exogenous protein without mutagenic effects caused by double stranded DNA. A common problem associated with the introduction of exogenous mRNA into mammalian cells is the stimulated interferon response. This innate immune response can be avoided with the addition of modified bases during the *in vitro* transcription process of synthetically derived mRNA (ivtRNA). The bases cytidine triphosphate (CTP) and uridine triphosphate (UTP) are replaced with 5-methylcytidine-5'-triphosphate (5-Methyl-CTP) and pseudouridine-5'-triphosphate (Pseudo-UTP) during *in vitro* transcription. Cellular

reprogramming is achieved by the delivery of this ivtRNA into the cytoplasm. Previous cellular reprogramming experiments lacking modified bases resulted in increased toxicity and decreased cellular viability, which lead to the incorporation of modified bases (Warren et al., 2010). The decreased immune response by the inclusion of modified bases may be advantageous in a variety of applications, from the introduction of TALENS or zinc finger nucleases for genomic editing to increased efficiency of the development of induced pluripotent stem cells (Hockemeyer et al., 2011).

CHAPTER TWO: LITERATURE REVIEW

Embryonic Stem Cells

ESCs are derived from the inner cell mass (ICM) of a developing blastocyst. These cells will eventually differentiate into hundreds of various cell types that will unify to form an entire fully functional organism. The genetic programming needed to differentiate into a completely functional cell type is contained in each of these embryonic stem cells. There are very specific epigenetic triggers that must occur to determine which path is to be followed. The primary distinction of embryonic stem cells from trophectoderm cells is the change in cellular pathways and expression. The inner cells of the blastocyst receive cell surface signals impeding cellular blocks preventing pluripotent transcription factor Oct4 (Gilbert, 2010). The pluripotency of these embryonic stem cells is dependent on Oct4 gene expression. When Oct4 expression is decreased or prevented, differentiation pathways are activated.

Distinctive Properties of Embryonic Stem Cells

Embryonic stem cells possess a truly unique quality that allows complete differentiation of a cell down numerous developmental pathways. This quality is diligently sought after to cure and/or treat disease and further the success of regenerative medicine. This quality is known as pluripotency. Pluripotency can be defined as the ability of a cell to give rise to all three germ layers of the embryo proper while not producing the cells confined to extra-embryonic, placental tissue. Cells that have the ability to become both the embryo proper and the extra-embryonic tissue are known as totipotent. The cells that make up the zygote are totipotent and will eventually segregate into two specific cell types: ICM and trophoblast cells.

Stem cells possess another interesting quality of self-renewal. Few cells possess this remarkable feature. Many cells have a predetermined number of cell divisions and will eventually terminally differentiate; they will no longer self-renew. An example cell type expressing terminal differentiation is neuronal cells. These cells will develop and differentiate down a particular path and arrest at a pre-determined point. If damaged, these cells cannot repair themselves to be made new again. Regenerative medicine had focused on therapeutic care involving such damage, like spinal injuries, for some time now. The desire to capture these qualities and facilitate safe and efficient reproduction has been sought after by many research institutions. The study of numerous diseases as well as normal zoological development is made possible by the production of cells containing these qualities.

Mouse ESCs were first isolated and characterized in 1981. There were two characteristics of interest: infinite proliferation, and the ability develop into various specialized cell types (Evans and Kaufman (1981). Human ESCs were first isolated in 1998 by James Thomson at the University of Wisconsin. He utilized the characteristics developed previously to verify that the cells he had isolated were in fact ESCs. The cells possessed the ability to both infinitely proliferate and develop into various cell types (Thomson et al., 1998).

The disadvantage to possessing these cells is that in order to obtain them, the embryo harvested for embryonic stem cell retrieval is ultimately destroyed. Once the inner cell mass is removed from the blastocyst, it can no longer proceed with normal development to form an organism. This area of medicine has, of course, developed attention and ethical concerns in both the public and private sectors. Media and political attention have caused a decrease in research utilizing human embryos. The drive to eliminate embryo destruction led to the development of induced pluripotent, or reprogrammed cells.

Adult Stem Cells

Adult stem cells (ASCs) are present in the body of an individual and are used to replace cells lost due to growth or damage (Stein, 2011). Some tissues in the body have an abundant ability to self-renew, indicating an increased source of adult stem cells for replenishment; others do not regenerate as actively (Wagers and Weissman, 2004). These adult stem cells can be found in many tissues such as bone, marrow, skin, muscle, and fat. These cells are referred to as “adult” but are not restricted adult organisms and can occur in a variety of non-embryonic tissue.

A specialized form of ASCs used in research is hematopoietic (HSCs) or Mesenchymal stem cells. A single cell derived from bone marrow has the ability to give rise to different blood cell types (Till and McCulloch, 1961). ASCs can be characterized as multi-, oligo-, or unipotent, and can give rise to a limited number of cells—and, in some cases, only one very specific cell type (Wagers and Weissman, 2004). These cells are usually restricted to a particular cell type or group from which they are located, and have been the reason for limited clinical applications of ASCs.

Induced Pluripotent Stem Cells

Somatic or differentiated cells have the ability to be reprogrammed into a less differentiated, pluripotent-state and are referred to as induced pluripotent stem cells or iPSCs. These cells become like embryonic stem cells—regaining the ability to proliferate—and can be stimulated to become other cell types. IPSCs are a conduit for reprogramming methods resulting in a desired cell type. There are numerous epigenetic modifications that take place when a cell line transforms from a terminally differentiated state, such as a somatic cell to an embryonic-cell-like state. The cells gain the ability to change from unipotent to pluripotent: a precursor to many cell types instead of just one. The mechanisms within cellular reprogramming are intricate

and somewhat illusive. The understanding and manipulation of these mechanisms provide an array of scientific benefits. Attempts have been made in several species to reprogram somatic cells to pluripotency and become embryonic stem cell-like (ES cell-like) colonies. This feat was first completed in 1981 by culturing the inner cell mass of mouse embryos (Evans and Kaufman, 1981). Later, Yamanaka and colleagues established the known four-factor cocktail of transcription factors—Oct3/4, Sox2, c-Myc, and Klf4—needed to induce pluripotency in mice and human fibroblasts (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Due to the apparent conservation of the sequences, the induction of pluripotency has been successful in other species such as rat, pig, rhesus monkey, rabbit, and canine (Liu et al., 2008; Esteban et al., 2009; Ezashi et al., 2009; Liao et al., 2009; Honda et al., 2010; Shimada et al., 2010). Bovine embryonic fibroblasts recently have been reprogrammed to pluripotency utilizing a retroviral vector in 2011 (Han et al., 2011). The viral transfection process is very efficient, but has caused concern in the domestic animal industry due to its possible integration into the genome. Attempts are being made to eliminate viral components in the induction of pluripotent stem cells.

RNA Induced Pluripotent Stem Cells

A novel approach to eliminate the use of viral vectors in pluripotency induction is the use of *in vitro* transcribed mRNA (ivtRNA) as the pluripotency stimulating molecule. This ivtRNA encodes one of the known pluripotent transcription factors and can be combined with others for increased efficiency of induction. The ivtRNA is then encapsulated within a cationic lipid delivery vehicle for enhanced delivery into the cell. The host cell can then produce the transcription factor while eliminating the fear of viral contaminants. RNA-induced pluripotent stem cells can safely deliver the much sought-after qualities of ESCs while simultaneously eliminating the destruction of embryos.

Benefits of Stem Cells in Research

Stem cells possess the ability to differentiate into separate cell lineages and can contribute greatly to the scientific community. The benefits of safe iPSCs are numerous. One of the benefits is regenerative medicine, which is designed to replace lost or damaged cells due to illness or injury (Hipp and Atala, 2008). The benefits affect human therapeutic applications, alleviated ethical concerns with the destruction of embryos for stem cell harvest, provision of a higher quality and less differentiated cell to be used for SCNT, and higher quality genetically modified livestock with no viral carryover. The ES-like colonies can be developed with both efficient production and safe transmission lacking integration into the host genome. The elimination of viral integration and increased colony production are useful research goals for commercial applications.

Gene Expression of Embryonic Stem Cells

OCT4

There are numerous pluripotent transcription factors associated with embryonic stem cells. These include OCT4, SOX2, c-MYC, and NANOG. OCT4 is a part of the POU (Pir-Oct-Unc) transcription factor family that regulates gene expression by binding to a specific domain containing an AGTCAAAT consensus sequence (Schöler et al., 1991; Pesce and Schöler, 2001; Jin et al., 2002). Many sources suggest the presence of OCT4 is required to obtain any of the other transcription factors, and is responsible for maintaining pluripotency (Niwa et al., 2000; Pesce and Schöler, 2001; Jin et al., 2002; Babaie et al., 2007). Overexpression of OCT4 can stimulate ESCs to differentiate into endoderm or mesoderm type cells (Niwa et al., 2000; Rodriguez et al., 2007) while reduced expression induces trophectoderm differentiation (Niwa et al., 2000; Hay et al., 2004; Matin et al., 2004; Zaehres et al., 2005; Babaie et al., 2007). The

diagram below illustrates the changes in OCT4 expression throughout development (Jin et al., 2002). Positive OCT4 expression

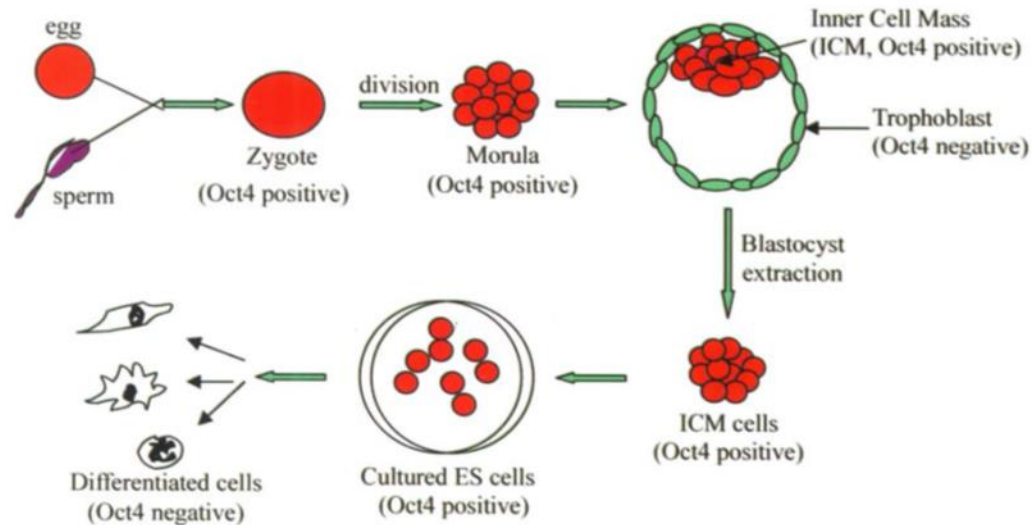


Figure 2.1 OCT4 Expression During Development (Jin et al., 2002)

can be seen in the zygote and morula, but later expression becomes isolated to the ICM of the blastocyst. The outer trophoblastic cells are expected to test negative for OCT4. Once ESCs differentiate into separate lineages, the OCT4 expression is suppressed.

Due to the importance of OCT4 in maintaining pluripotency, it is crucial to limit its expression when cells require differentiation instead of proliferation. “Oct4 activity must be tightly regulated to ensure the continuity of the germline and proper differentiation of various tissues and organs” (Jin et al., 2002). OCT4 works in conjunction with other transcription factors to assist in maintaining pluripotency via stimulation and repression of important genes within the genome. OCT4 is a part of the POU (Pir-Oct-Unc) transcription factor family that regulates gene expression by binding to a specific domain containing an AGTCAAAT consensus sequence (Schöler et al., 1991; Pesce and Schöler, 2001; Jin et al., 2002).

SOX2

Another transcription factor proven important in early development of ESCs is SOX2. This factor plays a role in maintaining pluripotency but can also participate in the differentiation of ESCs. SOX2 expression is not restricted to the inner cell mass and epiblast, but is also found to be expressed in neural tissues, extra-embryonic ectoderm, gut endoderm, esophagus, and trachea (Wood and Episkopou, 1999; Avilion et al., 2003; Williamson et al., 2006; Adachi et al., 2011). Prior to implantation, SOX2 plays a significant role in the formation of trophoctoderm and neural development (Avilion et al., 2003; Kelberman et al., 2006; Taranova et al., 2006). Although expression has been reported in numerous locations, SOX2 continues to play a fundamental role in maintaining the inner cell mass. Overexpression of SOX2 can lead to non-specific lineage differentiation, neuronal formation, and/or massive cell death (Mitsui et al., 2003; Zhao et al., 2004; Kopp et al., 2008; Adachi et al., 2011). If repressed, SOX2 can induce the formation of trophoctoderm (Masui et al., 2007). An impediment in the continuation of the ICM maintenance as well as defective development of trophoblast cells was observed in Sox2-deficient mice (Avilion et al., 2003). These observations suggest the widely diverse role of SOX2 in maintenance and differentiation during the earliest stages of development.

NANOG

There is another very important transcription factor that exists within the pluripotency network known as NANOG. This transcription factor is essential in blocking differentiation in embryonic stem cells. The development of ESCs occurs in cycles of expansion, or proliferation, and differentiation. There are specific signals that must be present for the cell to follow one path or another. The combined expression of OCT4 and SOX2 transcription factors is thought to activate NANOG by binding to the NANOG promoter thus activating transcription. NANOG

gains the ability to, in turn, further activate itself, OCT4, and SOX2 (Chickarmane et al., 2006; Wu et al., 2006; Storm et al., 2007; Takao et al., 2007). A continuous loop is formed containing numerous interweaving pathways that would both activate and repress expression in a very specific manner. A recent paper suggests possible auto-repression of NANOG to regulate transcription switching independent of OCT4 and SOX2 (Navarro et al., 2012). It is suggested that “in contrast to the accepted model, [there is] a negative correlation between the level of *Nanog* mRNA and protein...and the level of transcription of the endogenous *Nanog* locus...suggesting that NANOG negatively affects transcription [of the] *Nanog* gene” (Navarro et al., 2012). Once NANOG expression is repressed, its absence stimulates its own production; but when in abundance, NANOG again undergoes auto-repression through unknown binding sites (Navarro et al., 2012). OCT4 and SOX2 have been shown to activate NANOG transcription through joint binding, but the OCT4/SOX2 stimulation pathway may be independent of the NANOG-*Nanog*-induced pathway (Navarro et al., 2012).

c-MYC

In 2006, Takahashi and Yamanaka isolated four transcription factors needed to stimulate pluripotency in somatic cell reprogramming. These four factors were OCT4, SOX2, NANOG, and c-MYC. The first three are known to have a close relationship for intertwining activation and repression of one another, as previously stated. Transcription factor c-MYC plays a role in the efficiency in which these factors can induce pluripotency in somatic cells, but it is not actually required for the process to take place (Nakagawa et al., 2008; Wernig et al., 2008). However, c-MYC is a known oncogene that has the potential to become mutated and stimulate infinite proliferation. It is this fact that guides researchers to refine an efficient production process for the

induction of pluripotency that excludes the c-MYC transcription factor from the reprogramming cocktail for fear of inducing cancer.

KLF4

The Kruppel-like factor 4 is a key factor in cellular reprogramming to pluripotency (Evans et al., 2007). This factor is tightly bound to the OCT4 and SOX2 synergistic network of signaling pathways and influences the production of the much needed transcription factor, Nanog (Wei et al., 2009). KLF4 can play the role of both an activator and repressor of the transcriptional pathway to pluripotency by regulating proliferation and differentiation (Evans et al., 2007). This transcription factor includes three zinc fingers at the C terminus that play a crucial role in the activation and transcription of the Nanog promoter (Wei et al., 2009). KLF4 has been reported to be a tumor suppressor within gastrointestinal cancers (Dang et al., 2000). It is interesting to note, however, that there are negative forms of KLF4 that can suppress or decrease cellular reprogramming to pluripotency. Studies have reported that the introduction of these negative forms can significantly reduce cellular reprogramming (Wei et al., 2009). Numerous research strategies attempt to eliminate KLF4 from the reprogramming cocktail due to its identification as an oncogene associated with breast cancer (Foster et al., 2000). This factor has both tumor-suppressive and tumor-stimulatory characteristics and may lead to its final elimination within the reprogramming cocktail, but its intimate relationship with pluripotency factors OCT4 and SOX2 will secure its position in many reprogramming protocols.

Somatic Cell Nuclear Transfer

The process of somatic cell nuclear transfer fuses an enucleated oocyte with a fully differentiated somatic cell to produce an embryo, and even live offspring (Gurdon et al., 1958; Campbell et al., 1996; Wilmut et al., 1997). The donor cell must undergo a series of epigenetic

changes to become reprogrammed into an embryonic state when placed inside or fused with an oocyte. Once the cell has been reprogrammed, developmental processes can then occur to develop cells of many lineages. If a donor cell population to be used for SCNT has already been reprogrammed, it could jumpstart the developmental process, thus increasing efficiency.

Early experiments tested the theory of cells maintaining the genetic material required to support different cellular lineages. This was evaluated by transplanting nuclei from living cells into the eggs of frogs (Briggs and King, 1953; Gurdon et al., 1958; Gurdon, 1962a). The birth of Dolly in 1997 exhibited the idea that a differentiated cell maintained the genetic ability to support the complete development and maturation of an adult mammal through the process of somatic cell nuclear transfer (Wilmot et al., 1997). The environment within the oocyte supports the epigenetic reprogramming of the transferred nucleus and has the potential to become totipotent, developing into a fully developing embryo. The concern in this process is incomplete cellular reprogramming as the transferred nucleus undergoes epigenetic modifications. These early incomplete reprogramming processes often lead to problematic placental development, large offspring syndrome, and shorter life span (Yang et al., 2007; Gurdon and Wilmot, 2011). In earlier amphibian experiments, the success of the development process was thought to be influenced by the donor cell for transfer (Briggs and King, 1960). The earlier in the developmental pathway a donor cell is harvested, the more normality exhibited in the transfer development (Briggs and King, 1957; Gurdon, 1962b). The concern leads to the requirement of a less differentiated cell as the nuclear donor for somatic cell nuclear transfer. This less-differentiated cell can be obtained from embryonic cells, though they may not be available for isolation. A completely reprogrammed somatic cell exhibiting embryonic stem cell characteristics and gene expression is thought to be an alternate source of nuclei for transfer. It is

at a less-differentiated state and requires less reprogramming stimulation by the oocyte.

Efficiency of SCNT and decrease in problematic developmental concerns has the potential to be achieved with this previously reprogrammed nucleus.

Reprogramming by Viral Transfection

While true embryonic stem cells are isolated from the inner cell mass of the embryo, numerous processes have been defined to obtain cells whose characteristics are nearly identical. Cellular reprogramming by viral transduction is the most accepted method for reprogramming. The viral particles are easily accessible and can be altered for very specific uses. The viral particles encoding desired sequences are introduced to the cell culture where they are taken up by the cells. The sequences are integrated into the genome and are expressed by the cell to allow reprogramming. When compared to mRNA transfection this approach is characterized by a considerable decline in workload. The transduction is conducted once or twice at the beginning of the reprogramming trial compared to daily mRNA transfections during the weeks of cellular reprogramming. The daily transfections not only are more labor intensive, but also increase the likelihood of contamination with excessive handling. The viral method demonstrates increased efficiency when compared to other reprogramming methods, though efficiency can be measured in various ways. The total number of cells reprogrammed from the starting material remains around 1% but can be increased considerably compared to other methods. According to a recent paper, the mRNA reprogramming method is equally or more efficient than the accepted viral methods (Warren et al., 2010).

Problems Associated with Viral Transfection

The possible integration into the host genome is the major concern in viral-mediated reprogramming. The many human and/or domestic animal applications do not allow genetic carry-over of viral material. The numerous studies performed utilizing viral reprogramming generated mixed conclusions concerning the removal of viral particles with extended culture. Viral particles are difficult to completely eliminate from the cell lineage.

DNA-Free Transfection Methods; Cell-Penetrating Peptide Moieties and RNA-Based Viral Systems

The mode of transmission most commonly utilized is viral transmission due to its increased efficiency in the production of pluripotent stem cell lines. Other modes include, but are not limited to, serial protein transduction (Kim, D. et al., 2009; Zhou et al., 2009), non-integrating Sendai virus (Fusaki et al., 2009), chemical induction paired with a single transcription factor (Li, Y. et al., 2011), and the novel approach of synthetic modified mRNA delivery (Warren et al., 2010). These methods all utilize different mechanisms for successful cell integration. The method of transmission to be utilized commercially or on a grand scale should be efficient in reprogramming and formation of iPSC colonies, as well as safe for the host genome post-transmission. The viral method is highly efficient, but it is the least safe of all methods. The possible integration into the host genome is of the greatest concern, especially if the cells are to be used in human therapeutic treatments or in the formation of genetically modified livestock. The random insertion of the viral components into the host genome carries numerous possible genetic complications. Protein transduction is safe as it pertains to the host genome, but is difficult to harvest and purify in quantities required for reprogramming procedures (Zhou et al., 2009). The Sendai virus has an RNA-based life cycle and theoretically should not integrate into the host genome. However, the purification processes are very rigid to ensure no viral carryover.

This harsh process can decrease the overall efficiency of the Sendai virus as a carrier vehicle (Fusaki et al., 2009). However, synthetic mRNA can be directly administered to induce pluripotency without integration into the host genome and provide efficient iPSC production (Warren et al., 2010).

In Vitro Transcribed Messenger RNA Delivery for Reprogramming

Previous research has focused on inducing pluripotency of somatic cells to avoid the destruction of embryos due to its obvious ethical concerns. The addition of transcription factors normally present in the embryonic cells is fundamental to the reprogramming process. The methods of including these factors have varied from viral transmission, bacterial plasmid introduction, and addition of cellular extracts in culture. While all have been successful in developing pluripotent stem cells, growing concerns center on factor carry-over from these methods. The addition of synthetically derived messenger RNA encoding known pluripotent transcription factors can eliminate these genetic concerns. Desired sequences are synthesized from a template and packaged to ensure acceptance by the target cell. The transcripts contain a 5' guanosine cap analogue and 3' polyadenylation similar to the packaging acquired *in vivo* for translation. The *in vitro* transcribed transcript, ivtRNA, is delivered into the cell by either lipid-mediated delivery vehicle or electroporation. Once the sequence crosses the cell membrane, it can be taken up by the cellular machinery for translation. The increase in desired transcription factors has the ability to increase or decrease endogenous production. Repeated transfections using exogenous synthetic messenger RNA is required to achieve this effect. The encouraging characteristic of ivtRNA is that it does not integrate into the host genome and does not carry over into subsequent generations.

Enhanced Reprogramming Using Small Molecules

Reprogramming of somatic cells has proven to be inefficient, leading to the discovery of small molecules for enhanced reprogramming. The small molecules can either replace current transcription factors, or simply be added to the culture medium for increased reprogramming efficiency. The replacement of oncogenic transcription factors such as c-Myc and Klf4, as well as the elimination of viral vectors, can be useful in therapeutic applications. The eventual elimination of possible genetic carryover and integration is the goal of small molecule reprogramming methods. A recent study may have overcome this barrier by reprogramming mouse somatic cells to pluripotency by small molecules alone (Hou et al., 2013).

Small molecules have the potential to stimulate or inhibit critical developmental pathways for the induction or maintenance of pluripotency, and are typically organic compounds measuring less than 500 Daltons in size. These small molecules can be genetic factors, signaling molecules, and chemical inhibitors that can replace and/or enhance the predetermined core transcription factors required for reprogramming. Some non-oncogenic transcription factors can replace oncogenic transcription factors in reprogramming. Klf4 and c-Myc can be replaced with Nanog and Lin28 respectively (Yu et al., 2007). Certain cell types readily express core transcription factors endogenously and require less exogenous stimulation to achieve reprogramming. Neural progenitor cells (NPCs) contain endogenous Sox2 in relatively high levels and can be reprogrammed to pluripotency without addition of exogenous Sox2 transcription factors (Eminli et al.; Duinsbergen et al., 2008; Jeong Beom et al., 2008).

The addition of small molecules can enhance a reprogramming procedure by affecting various cellular processes. One process commonly targeted is the G9a histone methyltransferase pathway (HMT). Histone modifications such as lysine methylation can greatly affect chromatin

accessibility during mitosis and transcription. These modifications can have various developmental effects. Specifically, histone H3 Lysine9 (H3K9) methylation is responsible for chromatin repression for developmentally important genes and is catalyzed by G9a HMT. According to a previous study, “the euchromatic H3-K9 methylation regulated by G9a is involved in the transcriptional silencing of developmentally regulated genes” (Tachibana et al., 2002). The inhibition of G9a HMT can lead to the up-regulation of pluripotent transcription factors previously silenced. DZNep, a S-adenosyl homocysteine (SAH) hydrolyse inhibitor, has been reported to increase reprogramming efficiency when combined with other small molecules (Hou et al., 2013). The increase in SAH interferes with the lysine methylation process required for repression. One small molecule that has been shown to inhibit G9a histone methyltransferase (HMT) is BIX-01294, which does not compete with cofactor S-adenosylmethionine (SAM) (Shi et al., 2008). BIX-01294 has been reported to enhance cellular reprogramming to pluripotency and has the ability to replace Sox2 when reprogramming mouse neural progenitor cells (NPCs) (Shi et al., 2008). The chemical inhibitor, NuP0148 (NuPotential, Baton Rouge, LA), inhibits G9a HMT by binding to the S-adenosylmethionine (SAM) binding site within the enzyme. This inhibits the transfer of a methyl group to the histone lysine tail. The obstruction of the SAM binding site may be advantageous to simply increase the ratio of SAH to SAM present within the system. Developing small molecule inhibitors requires specificity and the SAM binding site seems to be an exceptionally good landscape. This inhibition then leads to increased reprogramming by reactivating the previously silenced Oct4 heterochromatin region. The reactivation of the gatekeeper transcription factor, Oct4, jumpstarts cellular reprogramming to pluripotency.

In addition, glycogen synthase kinase 3 (GSK3) and mitogen-activated protein kinase (ERK1/2) pathways can be inhibited to promote cellular reprogramming. The combined inhibition of these pathways can lead to reactivation and stabilization of the core pluripotent transcriptional circuitry required for differentiated cellular reprogramming. GSK3 inhibitor CHIR99021 combined with ERK1/2 inhibitor PD0325901 has been shown to enhance reprogramming to pluripotency (Silva et al., 2008).

Safety and Efficiency of Reprogramming to Pluripotency: Future Outlook

Pluripotency can be achieved by numerous reprogramming methods, all of which result in different yields and standards of efficiency. The following diagram has been modified to illustrate the position these methods place in terms of safety and efficiency (González et al., 2011). Efficiency is not a term uniformly agreed upon, but generally refers to a yield of colonies from a limited amount of resources and time. Safety refers to the genetic carryover associated with the reprogramming method. The viral methods have proven to be the most efficient in terms of colony yield, but have yet to completely eliminate genetic carryover. Methods of reprogramming such as the addition of proteins associated with pluripotency are quite safe. Concern with genetic integration and long-term expression is absent; however, the complicated processes associated result in low yield. A previous study utilized synthetic messenger RNA, or ivtRNA, as a reprogramming tool with efficiency results comparable to viral methods (Warren et al., 2010). The lack of genetic integration and/or random insertion places this reprogramming method at the peak of efficiency and safety.

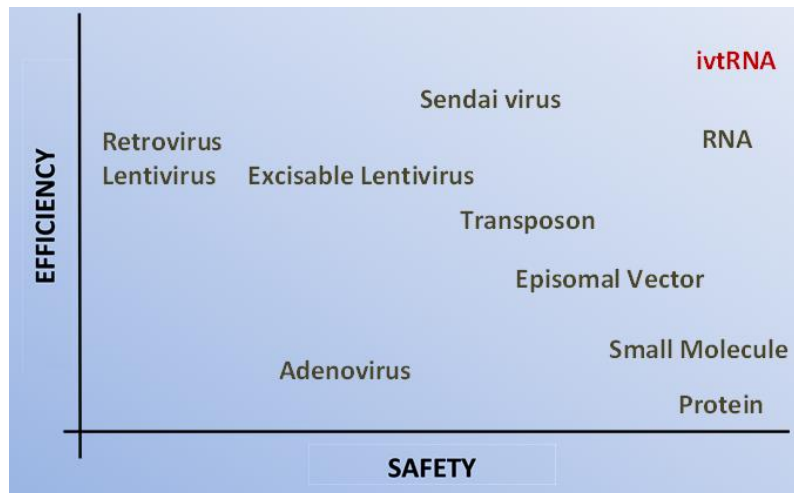


Figure 2.2 Efficiency and Safety Comparison of Cellular Reprogramming Methods (modified from (González et al., 2011))

CHAPTER THREE: RESPONSE OF BOVINE FETAL FIBROBLASTS TO PLURIPOTENCY INDUCTION WITH IN VITRO TRANSCRIBED MRNA

Introduction

Cellular reprogramming can be achieved by the delivery of mRNA into a biological system. The introduction of RNA can either up-regulate or “knock-down” gene expression in a very specific manner. The introduction of exogenous mRNA eliminates the transcription process required with the introduction of DNA into the cell. The new sequence can be delivered into the cytoplasm for immediate translation. There are two methods of mRNA delivery: electroporation and cationic lipid-mediated delivery vehicles (Van Tendeloo et al., 2001; Audouy et al., 2002). Electroporation is the process in which genetic material is introduced into the cell by pores in the cytoplasm caused by electric pulses. This process efficiently delivers the genetic material, but is not optimal for repeated uses. Though electroporation has proven to be successful, we utilized the second approach due to the need of repeated exposure to exogenous factors.

Repeated electroporation can damage the cells and hinder proliferation and expansion, thus inhibiting reprogramming. Cationic delivery vehicles are liposome-type molecules that will spontaneously interact with the cell surface to introduce genetic material to the cytoplasm. This method utilizes the fact that cells cultured *in vitro* have a net negative charge (Dwarki et al., 1993). The lipid delivery vehicles contain a positive charge and are naturally attracted to the cell surface. Incubation of cationic lipid-delivery vehicles containing genetic information with target cells can initiate cellular reprogramming with the uptake of mRNA.

The method of mRNA introduction can be easily managed, but maintaining sufficient volumes of RNA can be problematic. RNA rapidly degrades and is less stable than its DNA counterpart. Messenger RNA can be synthesized and quantified *in vitro* to obtain an adequate

amount for the transfection. This *in vitro* transcribed messenger RNA is also packaged similar to the form found *in vivo* in the cytoplasm. The addition of a 5' cap and poly-A tail is utilized in the *in vitro* transcription process to mimic its natural form to be translated.

Reprogramming and the induction of pluripotency using *in vitro* transcribed mRNA transfection have proven successful in human and murine cell lines (Warren et al., 2010). The objective of this study was to deliver ivtRNA to bovine fetal fibroblasts in a manner consistent with induced pluripotency. Green fluorescent protein (GFP) ivtRNA was delivered and fluorescence was measured by flow cytometry to determine a desirable transfection concentration and time course. Cellular viability was examined to address cytotoxicity.

The stimulation of endogenous bovine OCT gene expression is critical in the cellular reprogramming process. OCT4 has been referred to as the “Gatekeeper in the beginnings of mammalian development” (Pesce and Schöler, 2001). OCT4, a part of the POU domain, remains expressed throughout development, but down-regulation becomes simultaneous with cellular differentiation (Jin et al., 2002). The up-regulation of endogenous OCT4 is necessary to jumpstart the reprogramming network, including SOX2 and Nanog. OCT4 transcription factor is normally expressed early within embryo development—almost exclusively within the blastomeres. As the organism develops, OCT4 expression becomes confined to germ cell expression (Pesce and Schöler, 2001). The differentiation process requires activation of specific genes and silencing of others. As the developmental process continues, OCT4 expression becomes limited in the organism as a whole. The silencing of genes required for pluripotency and rapid cell division is mandatory as a cell becomes more specialized. The specialized—or differentiated—cell becomes distinct, producing transcripts specific to its cell type. The down-regulation of embryonic genes becomes necessary for the cell to become specialized.

In vitro transcribed mRNA transfection has proven an invaluable tool in understanding the mammalian genome due to its ability to deliver exogenous protein without mutagenic effects that may be caused by double-stranded DNA. However, a common problem associated with the introduction of exogenous mRNA into mammalian cells is the stimulated interferon response. Toll-like receptors on the surface of mammalian cells are able to recognize foreign genetic patterns and initiate an immune response (Uematsu and Akira, 2007). This response involves a change in gene expression to ultimately result in antigen-specific immunity (Takeda and Akira, 2005). Toll-like receptors have the ability to recognize a vast number of organisms in a very specific manner, including viruses, and proceed in step for their elimination (Uematsu and Akira, 2007). The signaling pathway associated with this invoked immune response is quite elaborate and has the ability to become lethal to cells in culture. Experiments within this manuscript utilizing ivtRNA lacking modified bases resulted in toxicity to the point of early termination of the time course. Previous cellular reprogramming experiments lacking modified bases resulted in increased toxicity and a decrease in cellular viability, which lead to the incorporation of modified bases (Warren et al., 2010). Essentially, the innate immune response can be avoided with the inclusion of modified bases during the *in vitro* transcription process of *in vitro* derived mRNA. The bases cytidine triphosphate (CTP) and uridine triphosphate (UTP) are replaced with 5-methylcytidine-5'-triphosphate (5-Methyl-CTP) and pseudouridine-5'-triphosphate (Pseudo-UTP) during the *in vitro* transcription process. Cellular reprogramming is achieved via the delivery of this modified ivtRNA into the cytoplasm of a cell by a cationic delivery vehicle, Lipofectamine. The innate immune response is not invoked and the target cells continue to proliferate.

The process of cellular reprogramming may be enhanced with the inclusion of small molecules in the experimental regime. These small molecules have the ability to promote or enhance reprogramming by direct involvement in the reprogramming mechanisms themselves. The small molecules, or inhibitors, utilized include MEK inhibitor PD0325901, GSK3 inhibitor CHIR99021, and G9a histone deacetylase inhibitor NuP0148. The combination of PD0325901 and CHIR99021 has been shown to promote pluripotency when combined with LIF (Silva et al., 2008). PD0325901 has been reported to promote growth in iPSCs and possibly inhibit growth in non-iPSCs (Shi et al., 2008). Histone deacetylase inhibitors have been shown to enhance cellular reprogramming in bovine somatic cells (Staszkiewicz et al., 2013). The combination of these inhibitors has the potential to enhance the reprogramming process and possibly lead to the replacement of pluripotent transcription factors all together.

Primary endpoint of this evaluation is to report the induced expression of endogenous pluripotency genes. This induction is the first step in cellular reprogramming to pluripotency. Stimulation of endogenous pluripotency transcription factors suggests that the epigenetic modifications required for altered gene expression have been initiated, and the target cells are on the path to becoming ESC-like cells.

Materials and Methods

Templates were obtained from Addgene as plasmids containing sequences encoding GFP or human OCT4, SOX2, KLF4, c-MYC. Plasmid inserts were excised by restriction enzyme prior to in vitro transcription utilizing mMESSAGE mMACHINE Kit (High Yield Capped RNA Transcription Kit) according to manufacturer protocol. The reaction was packaged with the Poly (A) Tailing Kit (Applied Biosystems AM1350) followed by purification using MEGAclear spin columns (Applied Biosystems AM1908). KMOS (Klf4, c-Myc, Oct4, Sox2) stoichiometry of

1:1:3:1 cocktail was utilized. RNA transfections were carried out using Lipofectamine® RNAiMAX Reagent. Modified 2X NTP/CAP (5-methylcytidine-5'-triphosphate (5-Methyl-CTP) and pseudouridine-5'-triphosphate (Pseudo-UTP)) was substituted for manufacturer supplied 2X NTP/CAP during *in vitro* transcription of modified mRNA. All cells transfected were bovine fetal fibroblasts (BFF), passage 2-5, cultured at 37°C and 5% CO₂ in specified media.

Cell Culture

All target cells were obtained from previously isolated primary cultures (site angelica). Primary cultures of fibroblasts were established from eight 50-day-old bovine fetuses recovered from a local abattoir according to previous protocol (Giraldo et al., 2009). With the exception of the primary culture, the fibroblasts were passaged at 80% confluence. Cultures were passaged by releasing cells with trypsin (0.25%), counted using a hemacytometer and re-seeded at an initial concentration of 100,000 cells/flask. These bovine fetal fibroblasts (BFFs) were expanded prior to treatment in each experiment.

Cell Cryopreservation

Fibroblast cells were frozen and thawed as needed. For cell freezing, the fibroblasts were resuspended in DMEM supplemented with 10% BCS and 10% dimethyl sulfoxide (DMSO) and cooled at 1.0°C/min until reaching -80°C before storage in liquid nitrogen. Approximately 1,000,000 cells were frozen in 1 ml of freezing medium per cryovial. Cells were thawed by holding the cryovial for 10 sec at room temperature followed by submersion in 38°C water. Thawed cells were washed once in culture medium before being replated.

RNA Transfection

Lipofectamine® RNAiMax Reagent mediated transfection was carried out within suggested concentration ranges. Manufacturer protocol suggests 0.5-1.5 µl of Lipofectamine

reagent per well of 24-well plate leading to the utilization of 1.0 μ l Lipofectamine reagent per well. The protocol suggests 6 pmol of RNA per well of 24-well plate, but various concentrations were evaluated (1 pmol, 2 pmol, and 4 pmol) according to previous study suggestions (Warren et al., 2010). RNA 100 ng/ μ L was diluted per manufacturer instructions and components were pooled and incubated 15 minutes at room temperature prior to being dispensed to culture media. All cells transfected were bovine fetal fibroblasts, passage 2-5, cultured at 37°C and 5% CO₂ in specified media.

Flow Cytometry

Single cell suspension in PBS without calcium and magnesium in a range of concentrations (50,000-800,000 cells/mL) were evaluated with SPECIFIC MACHINE. The relative fluorescence was used as (actual number used from software) for comparison. Mean intensity?

Cellular Viability

Cellular viability was measured by counting a final number of cells per well following seeding of constant number of cells for all wells. Cells were counted using a hemocytometer and phase contrast microscope. The numbers represented the total number of cells within each well at the time of measurement.

Gene Expression

Cells were extracted from each well followed by mRNA isolation using Dynabeads® mRNA DIRECT™ Kit. Each sample mRNA was eluted in 15 μ l RNase-free water and placed directly into Bio-Rad iScript™ cDNA Synthesis Kit reaction. The iScript™ kit utilized reverse transcriptase to transform mRNA into a more stable form, cDNA. The cDNA transcribed from each sample was evaluated for relevant gene expression with q-PCR.

Q-PCR

Basic PCR reactions consisted of 25 ul Jumpstart Red Mix, 2 ul forward primer, 2 ul reverse primer, 10 ul cDNA, and 11 ul dH₂O for a total of 50 ul. PCR was performed with a Hotstart of 94°C (2 min), denature 94°C (30 sec), annealing 60°C (30 sec), extension 72°C (1 min) for 30 cycles followed by a final extension at 72°C (5 min). The final PCR products were evaluated by gel electrophoresis using 1% agarose gel containing ethidium bromide. The gel was observed under UV light using BIO-RAD Universal Hood II and density gradient was evaluated with Quantity One Analysis Software.

Q-PCR procedure utilized EvaGreen® based method. Bio-Rad SsoFast™ EvaGreen® Supermix was used with primers from Table A.1. Q-PCR was performed with an enzyme activation at 95°C (1 min) followed by 40 repeats of denature at 95°C (5 sec) and annealing at 60°C (30 sec). A final denature and extension step was performed at 95°C (1 min) and 55°C (1 min) respectively. A melt-curve analysis was carried out by repeating 80 times and increasing 5°C every 10 seconds beginning at 55°C.

Statistical Analysis

Data were analyzed for all preliminary GFP experiments using SAS statistical software, GLM ANOVA, Levene's Test for homogeneity of variance. Tests for normality, Shapiro-Wilks, performed and no transformations were made. Cell viability of modified transfection experiments were evaluated using an ANOVA under normal assumptions. Relative gene expression was evaluated with REST statistical software. The program uses the geometric mean of multiple reference genes to normalize the results against genes of interest (GOI) since ratios

are used for data evaluation; this is called the Normalization Factor. Individual expressions are calculated relative to each reference gene and averaged using the geometric mean.

$$Expression = GOI\ concentration / GEOMEAN (refConc1, refConc2)$$

Estimates of concentrations vary exponentially due to the equation form of $c = A \times e^{C_T}$.

Experimental Design

Experiment 1

The 24-well plate was prepared with five treatment groups arranged in columns containing four wells each; Control, No RNA, Low, Medium, High. The “Control” group contains 5×10^4 bovine fetal fibroblasts grown in normal growth medium; “No RNA” contains bovine fetal fibroblasts treated with Lipofectamine lacking ivtRNA. The remaining three treatment groups contained bovine fetal fibroblasts transfected with three separate concentrations of ivtRNA-Lipofectamine complex and were cultured in normal growth medium (Dulbecco’s Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin (P/S)). All wells were seeded at a density of 5×10^4 of bovine fetal fibroblasts BEZ2 passage 2 (P2). Treatment groups were transfected with specific concentrations of ivtRNA encoding GFP utilizing lipofectamine as the transfection reagent. GFP relative expression was measured by flow cytometry 24-hours post-transfection. The measurement of cellular viability was investigated by determining the number of surviving cells following the transfection of a constant number of cells in each well.

Experiment 2

A 24-well plate was seeded with BFF P4 bovine fetal fibroblasts at the same density with normal growth medium. The treatment groups were in columns containing four wells each. The first column was the control group containing target cells grown under normal conditions. All

other columns were transfected with 2pmol of GFP ivtRNA per well. The second column was evaluated at 12 hours post-transfection and remaining columns were evaluated in 12-hour increments. The treatment groups were as follows: Control, 12-Hour, 24-Hour, 36-Hour, 48-Hour, and 60-Hour. The control group was evaluated simultaneously with the 60-Hour treatment group. Cellular viability was evaluated by counting cells after seeding a constant number of cells in each well.

Experiment 3

A 24-well plate was seeded with BFF P6 at 5×10^4 cells per well. The GFP ivtRNA was used to transfect the target cells over an extended period. The plate was divided into columns containing four wells each as treatment groups Control, 3-day, 6-day, 9-day, 12-day and 15-day. All treatment groups other than the control were transfected with 2pmol per well of GFP ivtRNA every other day. Cells were evaluated for mean fluorescence using flow cytometry and cellular viability was assessed by cell counting.

Experiment 4

In vitro transcribed OCT4 mRNA was delivered to bovine fetal fibroblasts BFF P5, followed by examination for endogenous expression. Previous concentration transfection conditions were used to transfect the cells every other day for 12 days with known pluripotency factor OCT4. Endogenous bOCT4 gene expression was measured by total RNA extraction and reverse transcription to cDNA, followed by PCR utilizing bovine OCT4 primers and bovine PolyA primers as a control.

Experiment 5

Bovine fetal fibroblasts BFF P1 were seeded at a density of 2.5×10^4 cells per well. Treatment groups were transfected with modified ivtRNA encoding green fluorescent protein

(GFP) to evaluate the effects on cellular viability and fluorescence. The cellular viability was measured by counting a final number of cells after seeding a constant number of cells in all treatment groups. The control group consisted of bovine fetal fibroblasts cultured in normal growth medium (DMEM, 10% FBS, 1% P/S). A no RNA (NR) group was held under the same conditions with the addition of the transfection reagent, Lipofectamine, to account for toxicity due to the transfection reagent alone. The cells were transfected every other day for 12 days and were evaluated on days 3, 6, 9 and 12 for viability and fluorescence by flow cytometry.

Experiment 6

This experiment consisted of bovine fetal fibroblasts BFF transfected with modified ivtRNA cocktails encoding multiple pluripotency factors; 3Factor (KOS) or 4Factor (KMOS) combinations. The controls were identical to the previous experiments, but treatment groups were transfected with modified *in vitro*-transcribed mRNA encoding either three factors (3F: OCT4, SOX2, KLF4) or four factors (4F: OCT4, SOX2, KLF4, c-MYC). Transfections were performed every other day for 24 days with media changed every day. Cells were evaluated for toxicity by cell counting at the end of the experiment.

Experiment 7

Reprogramming experiments were carried out to evaluate possible change in gene expression of bovine fetal fibroblasts. The target cells, BFF, were seeded at a density of 1×10^4 cells per well containing irradiated mouse embryonic fibroblast (iMEF) feeder layers. Cells were transfected every day with KMOS cocktail containing modified ivtRNA with media changed daily. Cells were transfected daily for 21 days and cultured in either +/-3i media (+3i contains three inhibitors PD0325901, CHIR99021, NuP0148 and -3i lacks inhibitors). Pictures were taken

of all wells on Days 15 and 21. The cells were harvested, counted, and total RNA was recovered on Day 21. Gene expression was evaluated with quantitative PCR.

Results

Experiment 1: GFP ivtRNA Concentration Evaluation

Bovine fetal fibroblast cells transfected with 2 pmol (medium mRNA concentration) displayed the highest relative fluorescence with $p < 0.001$ (graph 3.1). The cellular viability proved to be variable in this particular evaluation, which can be expected with this type of treatment (graph 3.2).

An optimum ivtRNA concentration was established by selecting the concentration that yielded the highest relative fluorescence. The optimum concentration of GFP ivtRNA (2 pmol per well) was used to transfect cells, and GFP expression was measured every 12 hours post transfection to determine an expression time course from a single transfection. The relative fluorescence peaked between 24 and 36 hours post transfection (fig. 3.3). Cell viability decreased when compared to the control, with transfected treatments having significantly fewer cells than controls at all time points (fig. 3.4). Cell viability was measured by counting the final number of cells in each well after seeding a constant number of cells. A notable observation was the decrease in cellular viability once the Lipofectamine containing the GFP mRNA was added to the cell culture, when compared to the control group. Each treatment group receiving ivtRNA transfection experienced a significant amount of cytotoxicity when compared to the control group ($p < 0.001$).

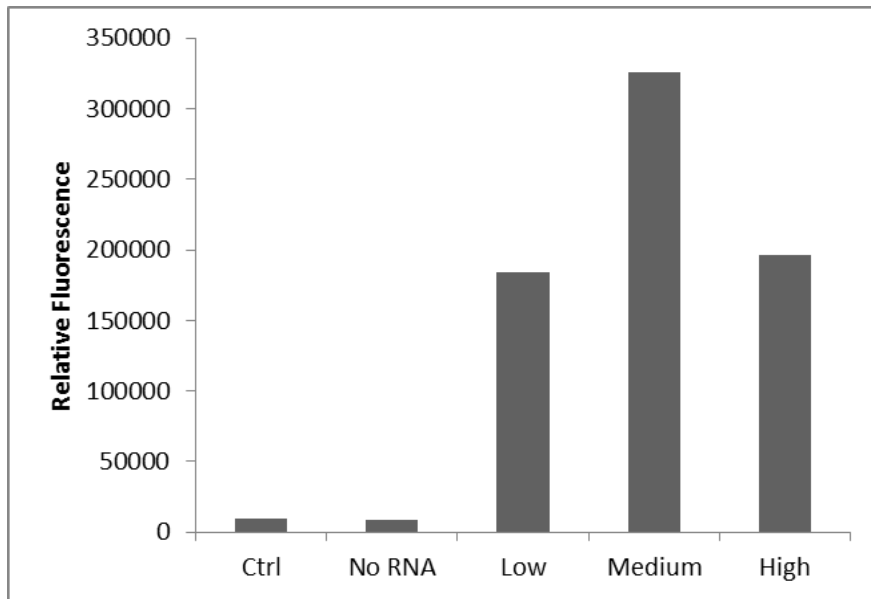


Figure 3.1 GFP ivtRNA Concentration Fluorescence

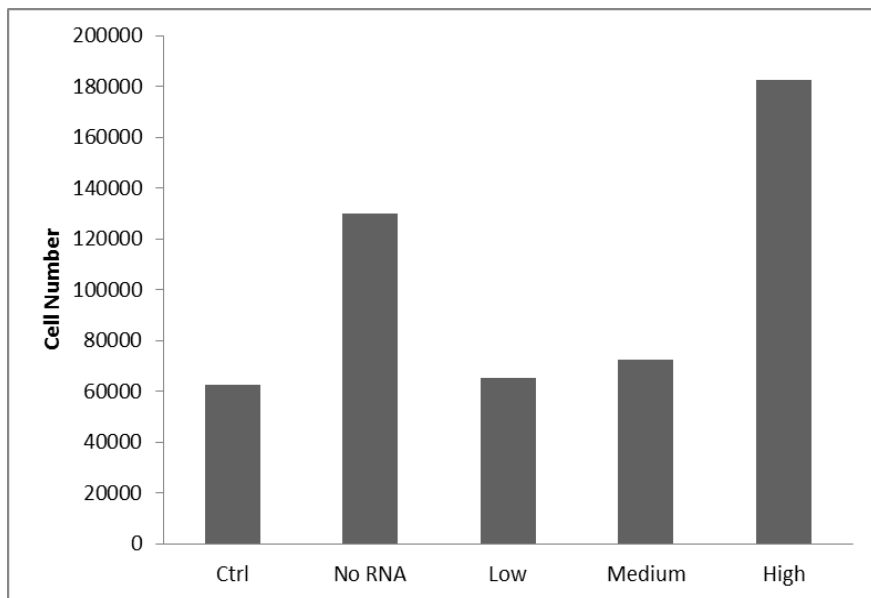


Figure 3.2 GFP ivtRNA Effect on Cellular Viability

Experiment 2: GFP ivtRNA Time Course Evaluation

Once an optimal concentration of mRNA for transfection was established, the time course for delivery was addressed. GFP expression was measured every 12 hours post-transfection by the mean fluorescence reading given by flow cytometry. Gene expression peaked between 24 and 36 hours and slowly declined until the final measurement of 60 hours (Fig. 3.3). The final measurement was not significantly different from the first 12-hour measurement. No significant difference was observed between the measurements taken at 24 and 36 hours, but these measurements were significantly different from the rest.

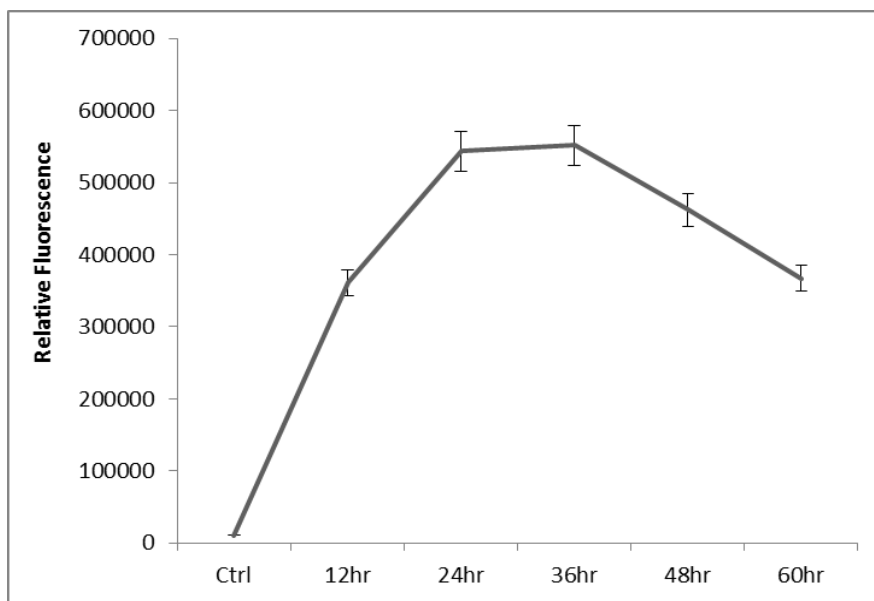


Figure 3.3 GFP ivtRNA Expression Time Course

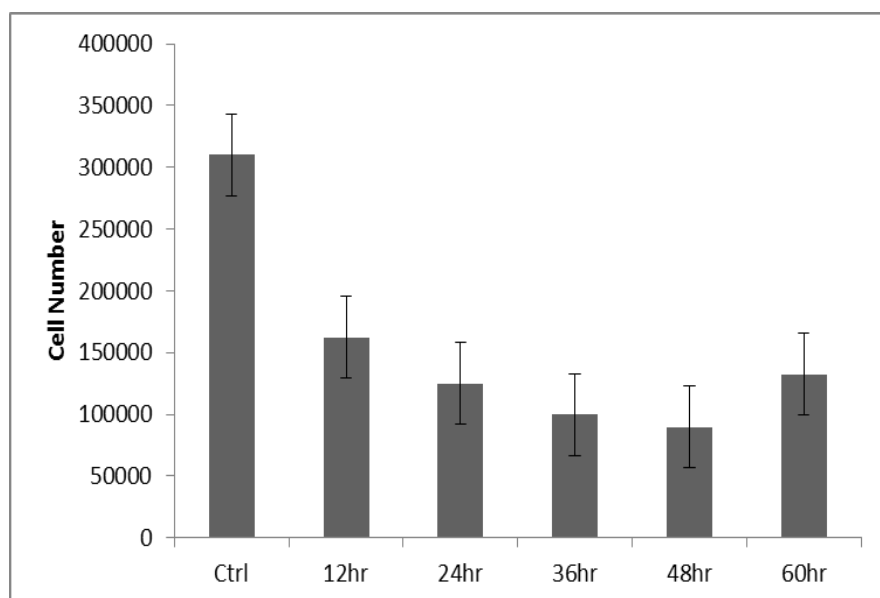


Figure 3.4 GFP ivtRNA Expression Time Course Cellular Viability

Experiment 3: GFP ivtRNA Extended Time Course Evaluation

Cells were transfected every other day with GFP ivtRNA, and expression was determined by flow cytometry on days 3, 6, and 9. The GFP was examined every 3 days with flow cytometry, but cells were no longer viable by day 12. Repeated transfections measured every three days illustrated greater fluorescence within treatment groups when compared to controls, but did not increase fluorescence with repeated transfections (fig. 3.5). Cell count of all treatment groups illustrates a decrease in cellular viability of ivtRNA treated groups when compared to both controls (fig. 3.6). No difference was observed in viability of all cells treated with ivtRNA encoding GFP when compared to controls ($p=0.9$). A significant difference was observed in fluorescence on all time points when compared to controls (day3 $p=0.004$, day6 $p=0.004$, day9 $p=0.007$, day12 $p=0.04$).

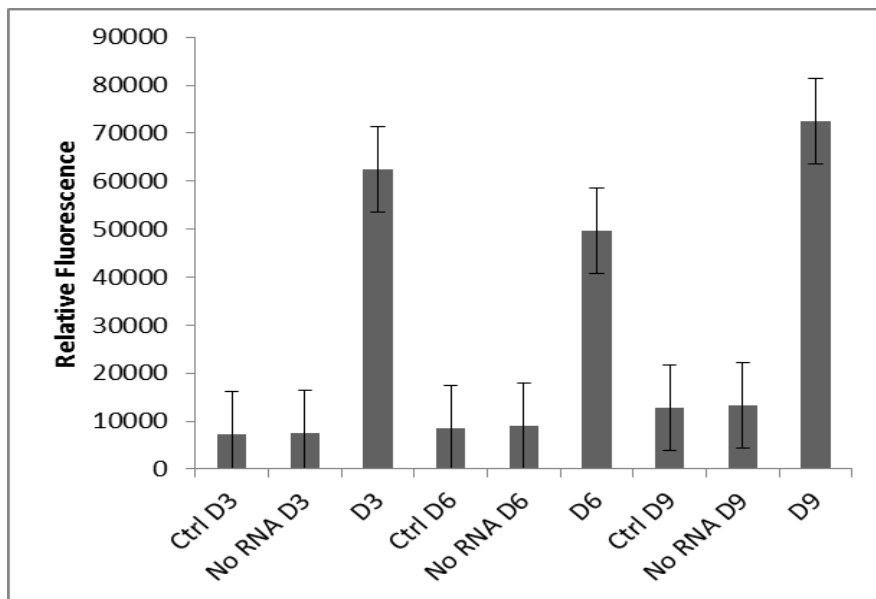


Figure 3.5 GFP ivtRNA Expression Extended Time Course

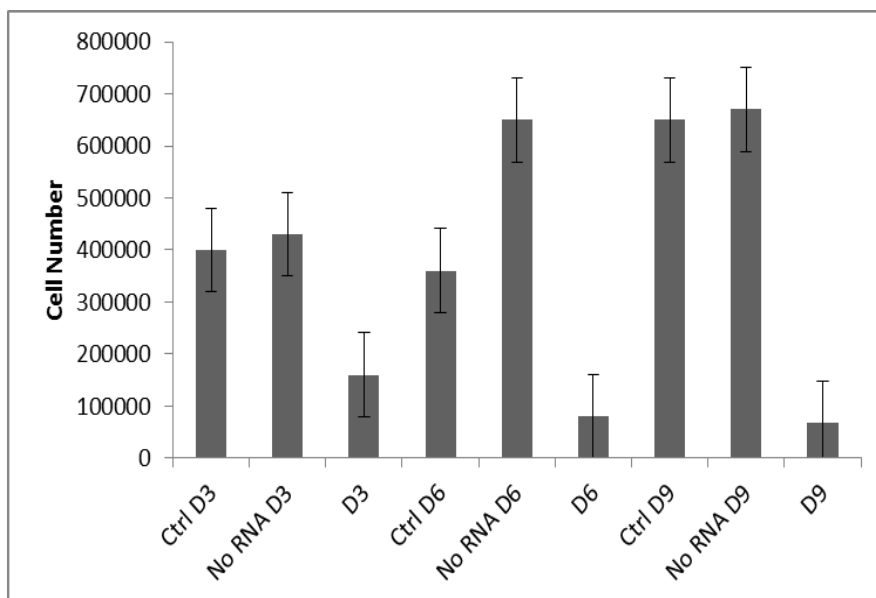


Figure 3.6 GFP ivtRNA Extended Time Course Cellular Viability

Experiment 4: OCT4 ivtRNA Transfection and Endogenous Activation

A PCR gradient was used to determine an optimal annealing temperature for the bovine OCT4 primers where no binding to the human OCT4 sequence could occur. As the annealing temperature increased, the affinity for the human oct4 plasmid decreased (fig. 4.1). The optimal temperature where the primers bind to the bovine sequence ONLY was then used in subsequent PCR reactions to determine the presence of endogenous OCT4. As the annealing temperature increases, the affinity for the human oct4 plasmid decreases. The maximum temperature for the primers to anneal to the bovine oct4 plasmid—and not to the human oct4 plasmid—is between 58.9 and 63.3 degrees Celsius.

Bovine fetal fibroblasts were transfected with ivtRNA encoding human Oct4 every other day. RNA was isolated on day 12 and analyzed by semi-quantitative RT-PCR for expression of the endogenous OCT4. Expression was quantitated by comparing intensities of electrophoresis bands using poly-adenylate polymerase (PAP) gene for standardization. Expression levels were expressed as the ratio of intensities of electrophoresis bands. The low concentration of hOCT4 mRNA transfection in the bovine fibroblast cells up-regulated endogenous bOCT4 mRNA expression measured qualitatively by comparing the intensity per mm² of the bands present on the gel (fig.4.2). The ratio of bOCT4 to bPolyA was inverted when compared to the two control groups (fig.4.2) indicating up-regulation. The ratio (OCT4/PAP) was 0.7 for untreated controls, 0.68 for cells transfected without RNA, and 1.2 for cells transfected with 140 ng per well ivtRNA encoding hOCT4. Expression was not detected in higher concentration treatments due to lack of cell survival. PCR conditions previously optimized did not amplify the human OCT4 mRNA, thus detecting the presence of endogenous OCT4. The increased ratio of 1.2 suggests the stimulation of endogenous OCT4 expression. This can be seen by the inverse of the band

intensity seen in U5 and U6 when compared to the band intensity ratio of U1&U2 and U3&U4.

There is an inverse in the concentration ratio of the treated group.

Table 3.1 Optimal PCR Conditions for Binding Bovine OCT4 Sequence

Annealing Temp. °C	Bovine OCT4 Plasmid	Human OCT4 Plasmid	Control No Plasmid
65.0	+	-	-
63.3	++	-	-
58.9	+++	-	-
55.8	+++	+	-

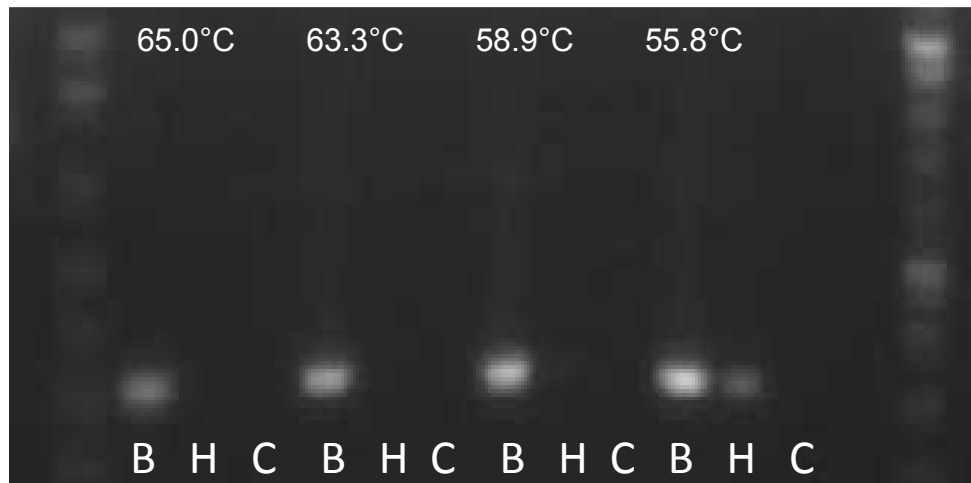


Figure 3.7 PCR Gradient with Decreasing Annealing Temperature

Figure Legend 3.7

B: Bovine OCT4 plasmid

H: Human OCT4 plasmid

C: Control, no plasmid

(Bovine OCT4 primers used with all reactions)

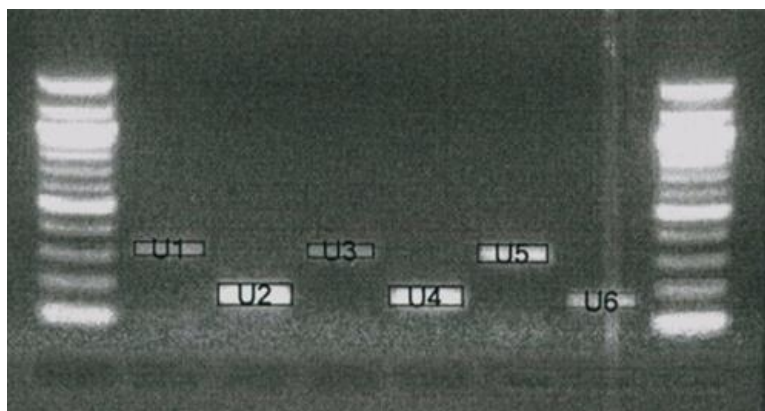


Figure 3.8 Semi-Quantitative RT-PCR displaying endogenous Oct4 up-regulation containing PAP standardization

Figure Legend 4.2

U1—Control Bovine OCT4
 U2—Control Poly-Adenylate Polymerase
 U3—No RNA Bovine OCT4
 U4—No RNA Poly-Adenylate Polymerase
 U5—Low Bovine OCT4
 U6—Low Poly-Adenylate Polymerase

Table 3.2 Expression Comparison to Housekeeping Gene

Sample	Primers	Mean Value INT	INT Ratio
Control	bOCT4	166.64	0.7
Control	PAP	237.92	
No RNA	bOCT4	157.76	0.68
No RNA	PAP	233.13	
Low	bOCT4	216.27	1.2
Low	PAP	182.18	

Experiment 5: Modified GFP ivtRNA Transfection Evaluation

The introduction of synthetic mRNA containing modified bases maintains cellular viability when compared to controls. No difference in viability of all cells treated with synthetic mRNA encoding GFP was observed when compared to controls ($p=0.9$). There was a significant

difference in fluorescence on all time points when compared to controls (day3 $p=0.004$, day6 $p=0.004$, day9 $p=0.007$, day12 $p=0.04$).

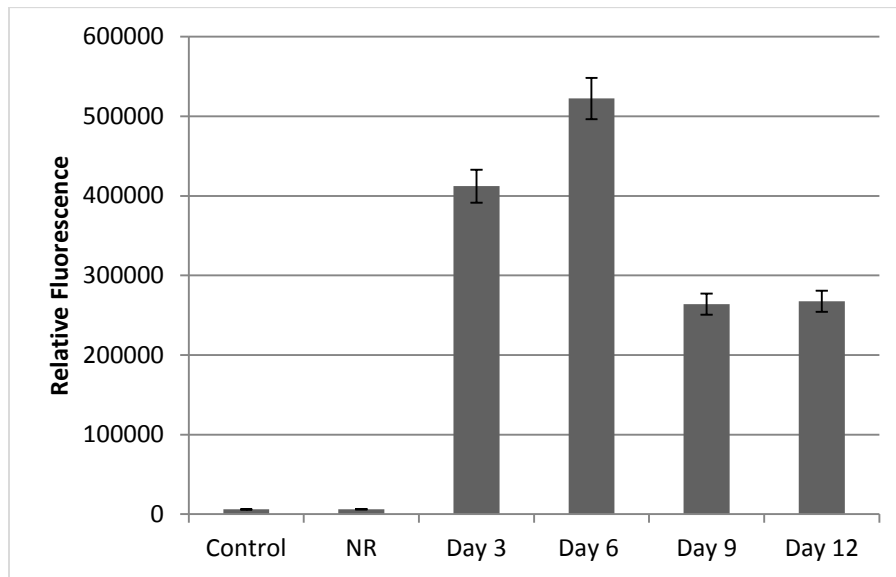


Figure 3.9 Relative Fluorescence of Modified GFP ivtRNA Transfection

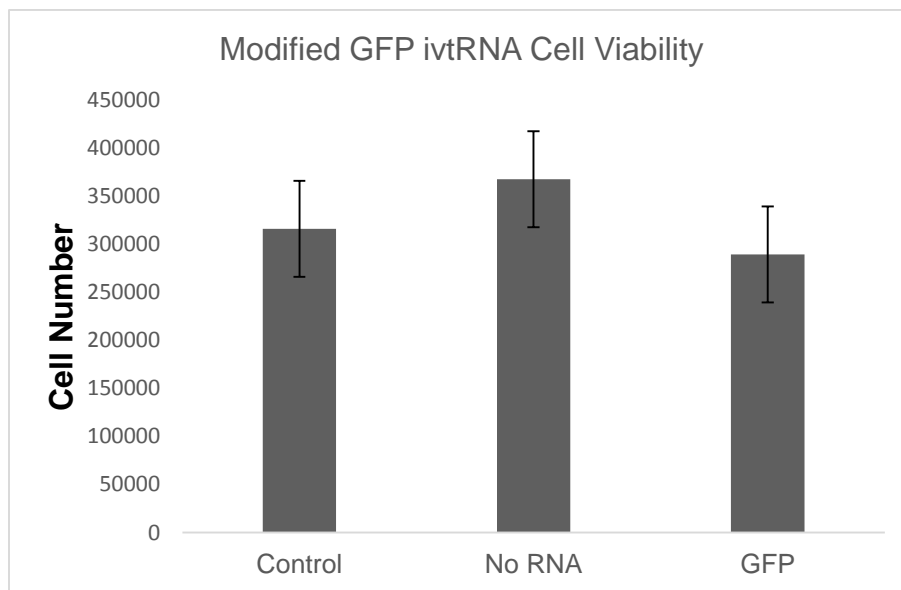


Figure 3.10 Measurement of Cellular Viability with Modified GFP ivtRNA Transfection

Experiment 6: Three Factor and Four Factor Modified ivtRNA Time Course Evaluation

The experiment consisted of bovine fetal fibroblasts transfected with modified synthetic mRNA encoding pluripotency factors. The controls were identical to the previous experiment, but treatment groups were transfected with modified synthetic mRNA encoding either three factors (3F-KOS: OCT4, SOX2, KLF4) or four factors (4F-KMOS: OCT4, SOX2, KLF4, c-MYC). The treated cells were transfected every other day and evaluated on day 24 for cellular viability. No difference was observed in cellular viability in all treatment groups when compared to controls ($p=0.2$) (fig. 5.1). The introduction of synthetic mRNA containing modified bases maintains cellular viability when compared to controls.

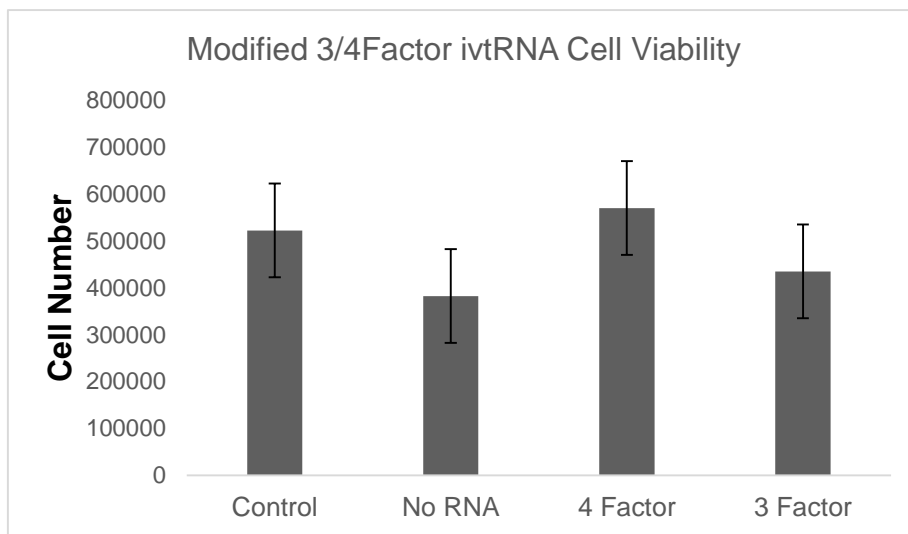


Figure 3.11 Measurement of Cellular Viability Post Modified 4F/3F ivtRNA Transfection

Experiment 7: Modified Four Factor Transfection With and Without Inhibitors

Expression of Oct4 was up-regulated in both treatment groups when compared to controls. The Ct values were evaluated by REST© Software (Pfaffl et al., 2002). The hypothesis test performed in this program determines whether there is a significant difference between controls and treatment groups while taking into account reaction efficiency and normalization of

multiple reference genes. The expression values obtained are a result of the gene of interest concentration divided by the reference gene concentration. The transfected group lacking inhibitors (-3i) had a significant increase in Oct4 expression with a value of 334.055 ($p=0.007$) when compared to controls. Nanog was not different than controls with expression value of 3.88 ($p=0.136$). The transfected group including inhibitors (+3i) had a significant increase in Oct4 expression with a value of 55.827 ($p=0.004$) when compared to controls. Nanog was significantly down-regulated with an expression value of 0.077 ($p=0.020$) when compared to controls. All values were normalized against both PAP and GAPDH, each having a reaction efficiency near 100%.

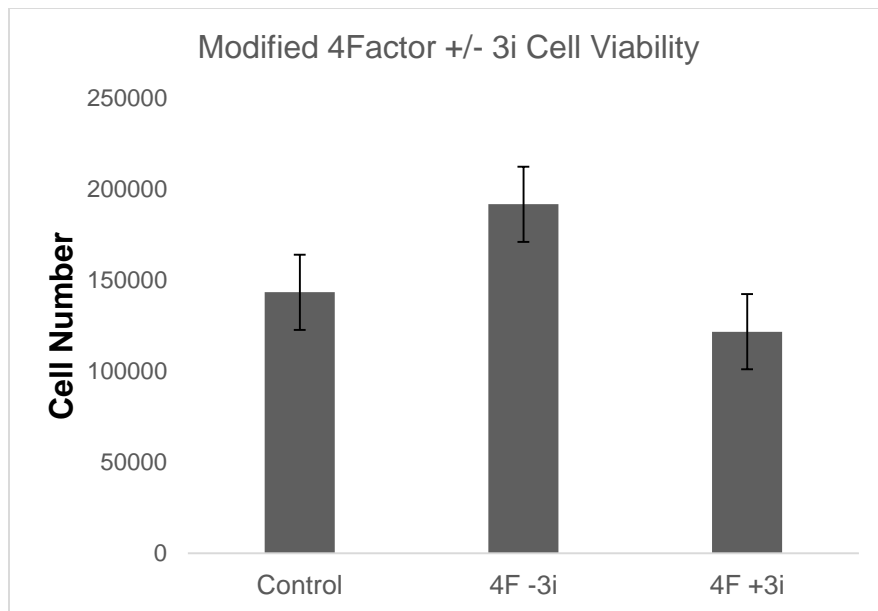


Figure 3.12 Cell Viability after 21 day transfection with 4F KMOS +/-3i culture media

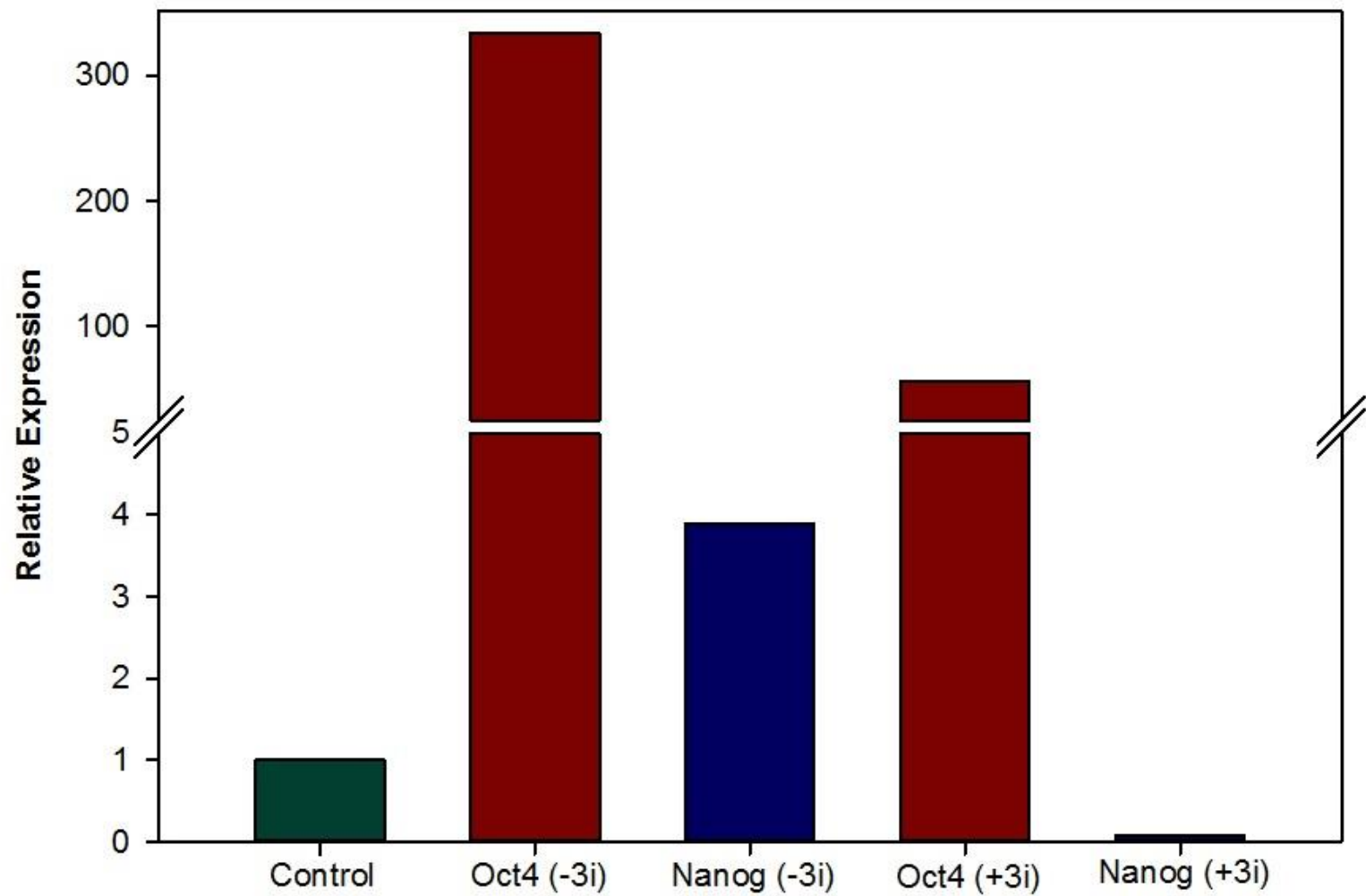


Figure 3.13 Relative Expression of Oct4 and Nanog normalized against housekeeping genes PAP and GAP

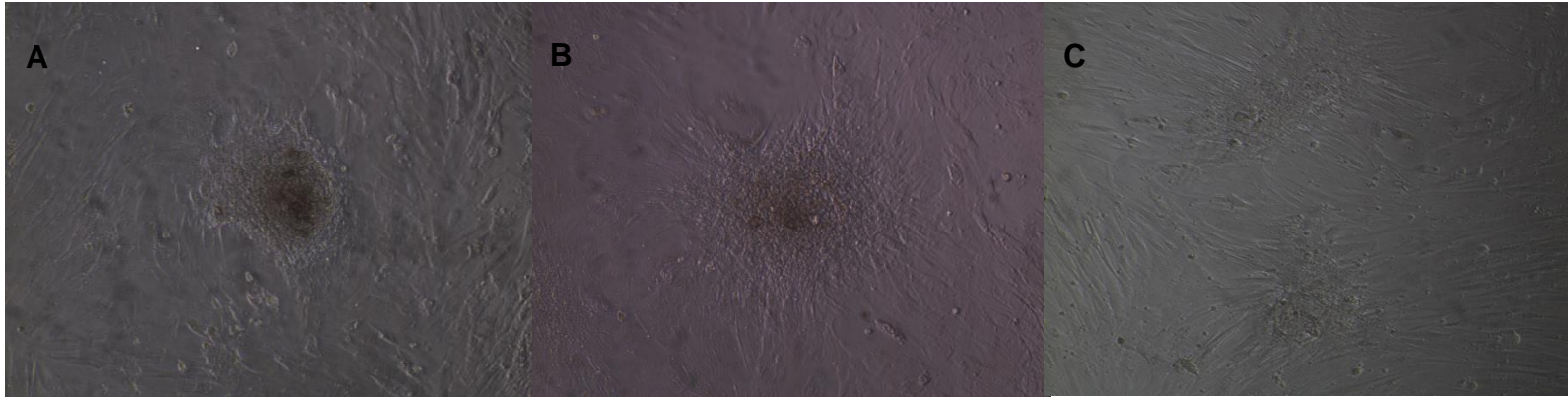


Figure 3.14: BFF cells 15 days culture and daily transfections: (A) 4F -3i Day 15, (B) 4F +3i Day 15, (C) Control Day 15, photos obtained from EVO phase contrast microscope

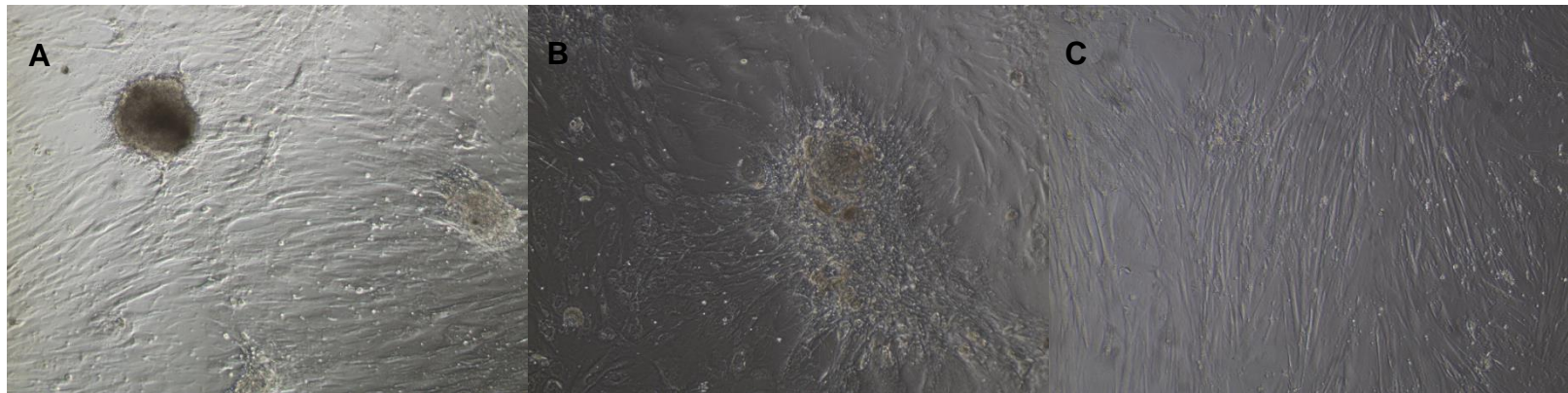


Figure 3.15: BFF cells 21 days culture and daily transfections: (A) -3i Day 21, (B) +3i Day 21, (C) Control Day 21, photos obtained from EVO phase contrast microscope

Discussion

The addition of exogenous *in vitro*-transcribed messenger RNA can be successful in influencing relative gene expression within bovine fetal fibroblasts. These mammalian cells were capable of incorporating foreign transcripts encoding green fluorescent protein into their normal cellular processes, which resulted in expression. Relative gene expression was influenced on a cellular level, indicating the cells ability to be reprogrammed with the addition of exogenous transcripts.

The increased levels of toxicity and the decrease in cellular viability were possibly due to the invoked interferon response that is normally initiated when confronted with viral infection.

During cellular reprogramming, exogenous transcription factors are introduced to the cells of interest to stimulate the production of endogenous transcripts. The presence of endogenous expression is often difficult to determine, and specific measures are carried out to distinguish endogenous from exogenous transcripts. If no endogenous stimulation occurs, the cessation of exogenous transcripts will cause the cell to return to normal gene expression. If, in fact, endogenous expression is stimulated and exogenous transcripts are halted, the cell should continue to translate the endogenous sequence. The stimulation of endogenous expression could be the first steps in complete reprogramming regimes.

Transfection of bovine fibroblast cells with the low concentration of hOCT4 mRNA, up-regulated endogenous bOCT4 mRNA expression. It was measured qualitatively by comparing the intensity per mm² of the bands present on the agarose gel. Repeated transfections with ivtRNA resulted in toxicity unrelated to the transfection agent. The increased toxicity was thought to be in response to the addition of exogenous material invoking an innate immune response. Cellular viability decreased with introduction of ivtRNA. The control group, NR,

introduced to the transfection reagent alone did not experience the levels of toxicity of those treatment groups receiving *in vitro* transcribed messenger RNA. This addition of exogenous material mirrors that of viral replication processes, and the toxicity is a natural cellular response. Experiments utilizing ivtRNA including modified bases reduced cell toxicity of ivtRNA. These modified bases aid in the decrease of the immune response from the target cells and may improve the reprogramming process. The cells will maintain their viability and thus survive the treatment regime. The expected viability concern was eliminated by incorporating these modified bases. The modified bases decrease the interferon response normally stimulated with the introduction of exogenous messenger RNA. Further experiments were implemented with modified ivtRNA encoding pluripotency factors OCT4, SOX2, KLF4 and c-MYC during the transcription process to enhance reprogramming.

CHAPTER FOUR

SUMMARY AND CONCLUSIONS

Numerous epigenetic barriers must be overcome during the process of cellular reprogramming. These barriers have the ability to impede the stimulated up-regulation of embryonic transcription factors within somatic cells. The complete induction of pluripotency is the ability to overcome a plethora of epigenetic modifications. These barriers are more difficult to be carried out than a simple cell lineage switch. There are cells that can be transformed from one cell type to another, such as B cells into macrophages, with the stimulation from a single stimulus with 100% efficiency within a 48-hour time period (Xie et al., 2004). This level of efficiency does not occur within the induction of pluripotency. Mouse fibroblasts were reprogrammed 18 days after transduction (Li et al., 2011), human ESC derived fibroblasts displayed tight morphology and distinct colony borders by the end of the second week of transfection (Warren et al., 2010), and germinal vesicle extract treated cells (GV-ETC) exhibited colony formation in 7 days (Miyamoto et al., 2009). There are numerous methods and combinations of stimuli that have improved efficiency, but none have come close to the previously mentioned 48-hour conversion. A complete understanding of the primary mechanisms to overcome allows improvement in the reprogramming efficiency.

Transfection with ivtRNA into bovine fetal fibroblasts was not well defined which required preliminary experiments to be performed. These fundamental experiments outlined the parameters for transfection. Transcripts encoding GFP were obtained as a template from Addgene plasmid 26822 containing the sequence for eGFP, or Enhanced Green Fluorescent Protein. A concentration of ivtRNA for transfection needed to be established. Lipofectamine® RNAiMAX Reagent protocol suggests 6 pmol RNA per well 24-well plate, as well as a range of 0.6-30 pmol per well. A previous experiment supplied 1200 ng (1.71 pmol) per well in a 6-well

plate (Warren et al., 2010). A range of concentrations was developed from the suggested concentrations to establish uniformity of low (1 pmol), medium (2 pmol), and high (4 pmol) concentrations per well. When scaled down from 6-well format to 24-well format, the final amount of ivtRNA supplied to each well was low (140 ng), medium (280 ng), and high (560 ng). The preliminary GFP ivtRNA transfection supported the medium 2 pmol per well concentration as the optimal concentration which compares to the 1.71 pmol concentration previously mentioned. However, the endogenous expression of Oct4 in the “unmodified” experiment was up-regulated within the low concentration group of 1 pmol per well. It is thought this was due to the increased toxicity of increased concentrations of unmodified ivtRNA which was seen in previous experiments (Warren et al., 2010).

Once the optimal concentration of 2 pmol ivtRNA per well was established, a time course for transfections was evaluated. The GFP ivtRNA time course evaluation experiment displayed peak GFP relative expression between 24 and 36 hours, leading to the decision to transfect every other day instead of everyday (Warren et al., 2010). The time course was utilized throughout most experiments, though possibly under an incorrect assumption. The Addgene plasmid 26822 encodes for eGFP, or “enhanced” GFP, which is a recombinant form utilized for its extended fluorescence and increased stability (Cormack et al., 1996). The peak time of GFP relative expression measured may be due to an extended ivtRNA half-life when compared to Oct4 ivtRNA half-life. The delay in transfection time when using pluripotency transcription factors may not have been advantageous. The benefit of daily transfections was not evaluated until the final experiment, and could possibly have been the source of limited results of reprogramming. However, the increase or maintained cellular viability post-transfection with modified ivtRNA was greater than previously reported (Warren et al., 2010). There was no difference between

transfected cells and control cells or cells treated with the transfection reagent alone. Cellular viability maintenance may be attributed to the less rigorous transfection regime.

One of the first embryonic transcription factors that must be stimulated is pluripotency factor OCT4 (Kim et al., 2009b). The decrease in OCT4 expression leads to differentiation and cell specification; therefore, its stimulation is required for the intricate pluripotency network to become active (Pesce and Schöler, 2001). The delivery of Oct4 alone induced pluripotency in adult neural stem cells (Kim et al., 2009b). The abundance of expression in early embryonic development has long been silenced in a somatic cell line. Methylation of the Oct4 promoter is the key to managing gene expression. The promoter is highly methylated in somatic cells due to pluripotency silencing, and completely unmethylated in embryonic cells (Gidekel and Bergman, 2002; Simonsson and Gurdon, 2004). The barriers that are overcome to stimulate this gatekeeper of pluripotency are necessary for the induction of other embryonic stem cell transcription factors. Endogenous Oct4 expression was indeed stimulated, indicating the initiation of the reprogramming process within these bovine fetal fibroblasts. The epigenetic modifications required for the induction of pluripotency were in fact engaged. Cell morphology was likewise affected during the process. The cells began to change and form tightly bound groups of cells—many with distinct borders.

The reprogramming regime including inhibitors (+3i) during culture did not differ from transfected culture lacking inhibitors (-3i) in terms of up-regulation of embryonic transcription factor Oct4. The near 100-fold increase in endogenous Oct4 expression is consistent with mouse fibroblasts reprogrammed to iPSCs compared to mouse ESCs (Zhou and Zeng, 2013). Nanog, however, was down-regulated in the +3i treatment group. In a previous study, the inclusion of MEK inhibitor, PD0325901, and GSK3 inhibitor, CHIR99021—in combination during the

reprogramming of cells that have been recently infected, have had a negative effect on the required gene expression (Feng et al., 2009). The timing of culture introduction to these inhibitors may play a role in whether or not the reprogramming is enhanced or inhibited. The epigenetic remodeling the cells are undergoing must be at an optimal time point for enhancement to take place. Inhibitor concentration is also a contributing factor for efficient and successful reprogramming. The concentrations of CHIR99021 and PD0325901 utilized were consistent with previous studies (Yu et al., 2011) and NuP0148 concentration was estimated due to its recent development and current concentration evaluation experiments currently being carried out by NuPotential, LLC. The alteration of this final concentration or combination of concentrations may still need to be evaluated in future experiments.

The stimulation of endogenous OCT4 expression is encouraging to the reprogramming process. Somatic cells with the ability to become reprogrammed to be ESC-like cells have the ability to aid in therapeutic applications and other research endeavors. If these cells are in fact reprogrammed to a less differentiated state, they might also provide a more efficient nuclear donor for somatic cell nuclear transfer. This less-differentiated cell would require less epigenetic modifications by the recipient oocyte. The overall success of SCNT would then increase yielding positive results for embryos to transfer. An increase in SCNT efficiency would be advantageous in livestock production schemes.

LITERATURE CITED

- Adachi, K., H. Suemori, N. Nakatsuji, and E. Kawase. 2011. The Role of SOX2 in Maintaining Pluripotency and Differentiation of Human Embryonic Stem Cells.
- Audouy, S. L., L. M. H. de Leij, D. Hoekstra, and G. Molema. 2002. In Vivo Characteristics of Cationic Liposomes as Delivery Vectors for Gene Therapy. *Pharm Res* 19: 1599-1605.
- Avilion, A. A., S. K. Nicolis, L. H. Pevny, L. Perez, N. Vivian, and R. Lovell-Badge. 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes & Development* 17: 126-140.
- Babaie, Y., R. Herwig, B. Greber, T. C. Brink, W. Wruck, D. Groth, H. Lehrach, T. Burdon, and J. Adjaye. 2007. Analysis of Oct4-Dependent Transcriptional Networks Regulating Self-Renewal and Pluripotency in Human Embryonic Stem Cells. *STEM CELLS* 25: 500-510.
- Briggs, R., and T. J. King. 1953. Factors affecting the transplantability of nuclei of frog embryonic cells. *Journal of Experimental Zoology* 122: 485-505.
- Briggs, R., and T. J. King. 1957. Changes in the nuclei of differentiating endoderm cells as revealed by nuclear transplantation. *Journal of Morphology* 100: 269-311.
- Briggs, R., and T. J. King. 1960. Nuclear transplantation studies on the early gastrula (*Rana pipiens*): I. Nuclei of presumptive endoderm. *Developmental biology* 2: 252-270.
- Campbell, K. H. S., J. McWhir, W. A. Ritchie, and I. Wilmut. 1996. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 380: 64-66.
- Chickarmane, V., C. Troein, U. A. Nuber, H. M. Sauro, and C. Peterson. 2006. Transcriptional Dynamics of the Embryonic Stem Cell Switch. *PLoS Comput Biol* 2: e123.
- Cho, H.-J., C.-S. Lee, Y.-W. Kwon, J. S. Paek, S.-H. Lee, J. Hur, E. J. Lee, T.-Y. Roh, I.-S. Chu, S.-H. Leem, Y. Kim, H.-J. Kang, Y.-B. Park, and H.-S. Kim. 2010. Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. *Blood* 116: 386-395.
- Cormack, B. P., R. H. Valdivia, and S. Falkow. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173: 33-38.
- Dang, D. T., K. E. Bachman, C. S. Mahatan, L. H. Dang, F. M. Giardiello, and V. W. Yang. 2000. Decreased expression of the gut-enriched Krüppel-like factor gene in intestinal adenomas of multiple intestinal neoplasia mice and in colonic adenomas of familial adenomatous polyposis patients. *FEBS Letters* 476: 203-207.
- Duinsbergen, D., M. Eriksson, P. A. C. t Hoen, J. Frisé, and H. Mikkers. 2008. Induced pluripotency with endogenous and inducible genes. *Experimental Cell Research* 314: 3255-3263.

- Dwarki, V. J., R. W. Malone, and I. M. Verma. 1993. [43] Cationic liposome-mediated RNA transfection. In: W. Ray (ed.) *Methods in Enzymology* No. Volume 217. p 644-654. Academic Press.
- Eminli, S., J. Utikal, K. Arnold, R. Jaenisch, and K. Hochedlinger. Reprogramming of Neural Progenitor Cells into Induced Pluripotent Stem Cells in the Absence of Exogenous Sox2 Expression.
- Esteban, M. A., J. Xu, J. Yang, M. Peng, D. Qin, W. Li, Z. Jiang, J. Chen, K. Deng, M. Zhong, J. Cai, L. Lai, and D. Pei. 2009. Generation of Induced Pluripotent Stem Cell Lines from Tibetan Miniature Pig. *Journal of Biological Chemistry* 284: 17634-17640.
- Evans, M. J., and M. H. Kaufman. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292: 154-156.
- Evans, P. M., W. Zhang, X. Chen, J. Yang, K. K. Bhakat, and C. Liu. 2007. Krüppel-like Factor 4 Is Acetylated by p300 and Regulates Gene Transcription via Modulation of Histone Acetylation. *Journal of Biological Chemistry* 282: 33994-34002.
- Ezashi, T., B. P. Telugu, A. P. Alexenko, S. Sachdev, S. Sinha, and R. M. Roberts. 2009. Derivation of induced pluripotent stem cells from pig somatic cells. *Proc Natl Acad Sci U S A* 106: 10993-10998.
- Feng, B., J.-H. Ng, J.-C. D. Heng, and H.-H. Ng. 2009. Molecules that Promote or Enhance Reprogramming of Somatic Cells to Induced Pluripotent Stem Cells. *Cell stem cell* 4: 301-312.
- Foster, K. W., A. R. Frost, P. McKie-Bell, C.-Y. Lin, J. A. Engler, W. E. Grizzle, and J. M. Ruppert. 2000. Increase of GSK3β Messenger RNA and Protein Expression during Progression of Breast Cancer. *Cancer Research* 60: 6488-6495.
- Fusaki, N., H. Ban, A. Nishiyama, K. Saeki, and M. Hasegawa. 2009. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci* 85: 348-362.
- Gidekel, S., and Y. Bergman. 2002. A Unique Developmental Pattern of Oct-3/4 DNA Methylation Is Controlled by a cis-demodification Element. *Journal of Biological Chemistry* 277: 34521-34530.
- Gilbert, S. F. 2010. *Developmental Biology*. Ninth Edition ed. Sinauer Associates, Inc., Sunderland, Massachusetts, USA.
- Giraldo, A. M., J. W. Lynn, M. N. Purpera, T. D. Vaught, D. L. Ayares, R. A. Godke, and K. R. Bondioli. 2009. Inhibition of DNA methyltransferase 1 expression in bovine fibroblast cells used for nuclear transfer. *Reproduction, Fertility and Development* 21: 785-795.

- González, F., S. Boué, and J. C. I. Belmonte. 2011. Methods for making induced pluripotent stem cells: reprogramming à la carte. *Nature Reviews Genetics* 12: 231-242.
- Gurdon, J. B. 1962a. Adult frogs derived from the nuclei of single somatic cells. *Developmental biology* 4: 256-273.
- Gurdon, J. B. 1962b. The transplantation of nuclei between two species of *Xenopus*. *Developmental biology* 5: 68-83.
- Gurdon, J. B., T. R. Elsdale, and M. Fischberg. 1958. Sexually Mature Individuals of *Xenopus laevis* from the Transplantation of Single Somatic Nuclei. *Nature* 182: 64-65.
- Gurdon, J. B., and I. Wilmut. 2011. Nuclear Transfer to Eggs and Oocytes. *Cold Spring Harbor Perspectives in Biology* 3.
- Han, X., J. Han, F. Ding, S. Cao, S. S. Lim, Y. Dai, R. Zhang, Y. Zhang, B. Lim, and N. Li. 2011. Generation of induced pluripotent stem cells from bovine embryonic fibroblast cells. *Cell Res* 21: 1509-1512.
- Hay, D. C., L. Sutherland, J. Clark, and T. Burdon. 2004. Oct-4 Knockdown Induces Similar Patterns of Endoderm and Trophoblast Differentiation Markers in Human and Mouse Embryonic Stem Cells. *STEM CELLS* 22: 225-235.
- Hipp, J., and A. Atala. 2008. Sources of Stem Cells for Regenerative Medicine. *Stem Cell Reviews and Reports* 4: 3-11.
- Hockemeyer, D., H. Wang, S. Kiani, C. S. Lai, Q. Gao, J. P. Cassady, G. J. Cost, L. Zhang, Y. Santiago, J. C. Miller, B. Zeitler, J. M. Cherone, X. Meng, S. J. Hinkley, E. J. Rebar, P. D. Gregory, F. D. Urnov, and R. Jaenisch. 2011. Genetic engineering of human pluripotent cells using TALE nucleases. *Nat Biotechnol* 29: 731-734.
- Honda, A., M. Hirose, M. Hatori, S. Matoba, H. Miyoshi, K. Inoue, and A. Ogura. 2010. Generation of Induced Pluripotent Stem Cells in Rabbits: POTENTIAL EXPERIMENTAL MODELS FOR HUMAN REGENERATIVE MEDICINE. *Journal of Biological Chemistry* 285: 31362-31369.
- Hou, P., Y. Li, X. Zhang, C. Liu, J. Guan, H. Li, T. Zhao, J. Ye, W. Yang, K. Liu, J. Ge, J. Xu, Q. Zhang, Y. Zhao, and H. Deng. 2013. Pluripotent Stem Cells Induced from Mouse Somatic Cells by Small-Molecule Compounds. *Science* 341: 651-654.
- Jeong Beom, K., H. Zaehres, W. Guangming, L. Gentile, K. Ko, V. Sebastiano, M. J. Araújo-Bravo, D. Ruau, H. Dong Wook, M. Zenke, and H. R. Schöler. 2008. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature* 454: 646-650.
- Jin, G. P., Z. Y. Chang, H. R. Scholer, and D. Pei. 2002. Stem cell pluripotency and transcription factor Oct4. *Cell Res* 12: 321-329.

- Kelberman, D., K. Rizzoti, A. Avilion, M. Bitner-Glindzicz, S. Cianfarani, J. Collins, W. K. Chong, J. M. W. Kirk, J. C. Achermann, R. Ross, D. Carmignac, R. Lovell-Badge, I. C. A. F. Robinson, and M. T. Dattani. 2006. Mutations within Sox2/SOX2 are associated with abnormalities in the hypothalamo-pituitary-gonadal axis in mice and humans. *The Journal of Clinical Investigation* 116: 2442-2455.
- Kim, D., C. Kim, J. Moon, Y. Chung, M. Chang, B. Han, S. Ko, E. Yang, K. Cha, R. Lanza, and K. Kim. 2009a. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell stem cell* 4: 472 - 476.
- Kim, J. B., V. Sebastiano, G. Wu, M. J. Araúzo-Bravo, P. Sasse, L. Gentile, K. Ko, D. Ruau, M. Ehrich, D. van den Boom, J. Meyer, K. Hübner, C. Bernemann, C. Ortmeier, M. Zenke, B. K. Fleischmann, H. Zaehres, and H. R. Schöler. 2009b. Oct4-Induced Pluripotency in Adult Neural Stem Cells. *Cell* 136: 411-419.
- Kopp, J. L., B. D. Ormsbee, M. Desler, and A. Rizzino. 2008. Small Increases in the Level of Sox2 Trigger the Differentiation of Mouse Embryonic Stem Cells. *STEM CELLS* 26: 903-911.
- Li, Y., Q. Zhang, X. Yin, W. Yang, Y. Du, P. Hou, J. Ge, C. Liu, W. Zhang, X. Zhang, Y. Wu, H. Li, K. Liu, C. Wu, Z. Song, Y. Zhao, Y. Shi, and H. Deng. 2011. Generation of iPSCs from mouse fibroblasts with a single gene, Oct4, and small molecules. *Cell Res* 21: 196-204.
- Liao, J., C. Cui, S. Chen, J. Ren, J. Chen, Y. Gao, H. Li, N. Jia, L. Cheng, and H. Xiao. 2009. Generation of induced pluripotent stem cell lines from adult rat cells. *Cell stem cell* 4: 11.
- Liu, H., F. Zhu, J. Yong, P. Zhang, P. Hou, H. Li, W. Jiang, J. Cai, M. Liu, K. Cui, X. Qu, T. Xiang, D. Lu, X. Chi, G. Gao, W. Ji, M. Ding, and H. Deng. 2008. Generation of Induced Pluripotent Stem Cells from Adult Rhesus Monkey Fibroblasts. *Cell stem cell* 3: 587-590.
- Masui, S., Y. Nakatake, Y. Toyooka, D. Shimosato, R. Yagi, K. Takahashi, H. Okochi, A. Okuda, R. Matoba, A. A. Sharov, M. S. H. Ko, and H. Niwa. 2007. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat Cell Biol* 9: 625-635.
- Matin, M. M., J. R. Walsh, P. J. Gokhale, J. S. Draper, A. R. Bahrami, I. Morton, H. D. Moore, and P. W. Andrews. 2004. Specific Knockdown of Oct4 and β 2-microglobulin Expression by RNA Interference in Human Embryonic Stem Cells and Embryonic Carcinoma Cells. *STEM CELLS* 22: 659-668.
- Mitsui, K., Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, M. Maruyama, M. Maeda, and S. Yamanaka. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113: 631-642.

- Miyamoto, K., T. Tsukiyama, Y. Yang, N. Li, N. Minami, M. Yamada, and H. Imai. 2009. Cell-Free Extracts from Mammalian Oocytes Partially Induce Nuclear Reprogramming in Somatic Cells. *Biology of Reproduction* 80: 935-943.
- Nakagawa, M., M. Koyanagi, K. Tanabe, K. Takahashi, T. Ichisaka, T. Aoi, K. Okita, Y. Mochiduki, N. Takizawa, and S. Yamanaka. 2008. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotech* 26: 101-106.
- Navarro, P., N. Festuccia, D. Colby, A. Gagliardi, N. P. Mullin, W. Zhang, V. Karwacki-Neisius, R. Osorno, D. Kelly, M. Robertson, and I. Chambers. 2012. OCT4/SOX2-independent Nanog autorepression modulates heterogeneous Nanog gene expression in mouse ES cells. *EMBO J* advance online publication.
- Niwa, H., J.-i. Miyazaki, and A. G. Smith. 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature genetics* 24: 372-376.
- Pesce, M., and H. R. Schöler. 2001. Oct-4: Gatekeeper in the Beginnings of Mammalian Development. *STEM CELLS* 19: 271-278.
- Pfaffl, M. W., G. W. Horgan, and L. Dempfle. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic acids research* 30: e36.
- Rodriguez, R. T., J. M. Velkey, C. Lutzko, R. Seerke, D. B. Kohn, K. S. O'Shea, and M. T. Firpo. 2007. Manipulation of OCT4 Levels in Human Embryonic Stem Cells Results in Induction of Differential Cell Types. *Experimental Biology and Medicine* 232: 1368-1380.
- Schöler, H. R., T. Ciesiolka, and P. Gruss. 1991. A nexus between Oct-4 and E1A: implications for gene regulation in embryonic stem cells. *Cell* 66: 291-304.
- Shi, Y., C. Desponts, J. T. Do, H. S. Hahm, H. R. Schöler, and S. Ding. 2008. Induction of Pluripotent Stem Cells from Mouse Embryonic Fibroblasts by Oct4 and Klf4 with Small-Molecule Compounds. *Cell stem cell* 3: 568-574.
- Shimada, H., A. Nakada, Y. Hashimoto, K. Shigeno, Y. Shionoya, and T. Nakamura. 2010. Generation of canine induced pluripotent stem cells by retroviral transduction and chemical inhibitors. *Molecular Reproduction and Development* 77: 2-2.
- Silva, J., O. Barrandon, J. Nichols, J. Kawaguchi, T. W. Theunissen, and A. Smith. 2008. Promotion of Reprogramming to Ground State Pluripotency by Signal Inhibition. *PLoS Biol* 6: e253.
- Simonsson, S., and J. Gurdon. 2004. DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. *Nat Cell Biol* 6: 984-990.
- Staszkiwicz, J., R. A. Power, L. L. Harkins, C. W. Barnes, K. L. Strickler, J. S. Rim, K. R. Bondioli, and K. J. Eilersten. 2013. Silencing Histone Deacetylase-Specific Isoforms

Enhances Expression of Pluripotency Genes in Bovine Fibroblasts. Cellular Reprogramming.

- Stein, B., Luong, Shi, Smith, Vasquez. 2011. Human Stem Cell Technology and Biology "A Research Guide and Laboratory Manual". John Wiley & Sons, Inc., Hoboken, New Jersey.
- Storm, M. P., H. K. Bone, C. G. Beck, P.-Y. Bourillot, V. Schreiber, T. Damiano, A. Nelson, P. Savatier, and M. J. Welham. 2007. Regulation of Nanog Expression by Phosphoinositide 3-Kinase-dependent Signaling in Murine Embryonic Stem Cells. *J. Biol. Chem.* 282: 6265-6273.
- Tachibana, M., K. Sugimoto, M. Nozaki, J. Ueda, T. Ohta, M. Ohki, M. Fukuda, N. Takeda, H. Niida, H. Kato, and Y. Shinkai. 2002. G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev* 16: 1779-1791.
- Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131: 861-872.
- Takahashi, K., and S. Yamanaka. 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* 126: 663-676.
- Takao, Y., T. Yokota, and H. Koide. 2007. β -Catenin up-regulates Nanog expression through interaction with Oct-3/4 in embryonic stem cells. *Biochemical and Biophysical Research Communications* 353: 699-705.
- Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *International Immunology* 17: 1-14.
- Taranova, O. V., S. T. Magness, B. M. Fagan, Y. Wu, N. Surzenko, S. R. Hutton, and L. H. Pevny. 2006. SOX2 is a dose-dependent regulator of retinal neural progenitor competence. *Genes & Development* 20: 1187-1202.
- Thomson, J. A., J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall, and J. M. Jones. 1998. Embryonic Stem Cell Lines Derived from Human Blastocysts. *Science* 282: 1145-1147.
- Till, J. E., and E. A. McCulloch. 1961. A Direct Measurement of the Radiation Sensitivity of Normal Mouse Bone Marrow Cells. *Radiation Research* 14: 213-222.
- Uematsu, S., and S. Akira. 2007. Toll-like Receptors and Type I Interferons. *Journal of Biological Chemistry* 282: 15319-15323.
- Van Tendeloo, V. F. I., P. Ponsaerts, F. Lardon, G. Nijs, M. Lenjou, C. Van Broeckhoven, D. R. Van Bockstaele, and Z. N. Berneman. 2001. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive

- pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. *Blood* 98: 49-56.
- Wagers, A. J., and I. L. Weissman. 2004. Plasticity of Adult Stem Cells. *Cell* 116: 639-648.
- Warren, L., P. D. Manos, T. Ahfeldt, Y.-H. Loh, H. Li, F. Lau, W. Ebina, P. K. Mandal, Z. D. Smith, A. Meissner, G. Q. Daley, A. S. Brack, J. J. Collins, C. Cowan, T. M. Schlaeger, and D. J. Rossi. 2010. Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified mRNA. *Cell stem cell* 7: 618-630.
- Wei, Z., Y. Yang, P. Zhang, R. Andrianakos, K. Hasegawa, J. Lyu, X. Chen, G. Bai, C. Liu, M. Pera, and W. Lu. 2009. Klf4 Interacts Directly with Oct4 and Sox2 to Promote Reprogramming. *STEM CELLS* 27: 2969-2978.
- Wernig, M., A. Meissner, J. P. Cassady, and R. Jaenisch. 2008. c-Myc Is Dispensable for Direct Reprogramming of Mouse Fibroblasts. *Cell stem cell* 2: 10-12.
- Williamson, K. A., A. M. Hever, J. Rainger, R. C. Rogers, A. Magee, Z. Fiedler, W. T. Keng, F. H. Sharkey, N. McGill, C. J. Hill, A. Schneider, M. Messina, P. D. Turnpenny, J. A. Fantes, V. van Heyningen, and D. R. FitzPatrick. 2006. Mutations in SOX2 cause anophthalmia-esophageal-genital (AEG) syndrome. *Human Molecular Genetics* 15: 1413-1422.
- Wilmut, I., A. E. Schnieke, J. McWhir, A. J. Kind, and K. H. S. Campbell. 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature* 385: 810-813.
- Wood, H. B., and V. Episkopou. 1999. Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. *Mechanisms of Development* 86: 197-201.
- Wu, Q., X. Chen, J. Zhang, Y.-H. Loh, T.-Y. Low, W. Zhang, W. Zhang, S.-K. Sze, B. Lim, and H.-H. Ng. 2006. Sall4 Interacts with Nanog and Co-occupies Nanog Genomic Sites in Embryonic Stem Cells. *Journal of Biological Chemistry* 281: 24090-24094.
- Xie, H., M. Ye, R. Feng, and T. Graf. 2004. Stepwise Reprogramming of B Cells into Macrophages. *Cell* 117: 663-676.
- Yang, X., S. L. Smith, X. C. Tian, H. A. Lewin, J. P. Renard, and T. Wakayama. 2007. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nature genetics* 39: 295-302.
- Yu, J., K. F. Chau, M. A. Vodyanik, J. Jiang, and Y. Jiang. 2011. Efficient feeder-free episomal reprogramming with small molecules. *PLoS One* 6: e17557.
- Yu, J., M. A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. L. Frane, S. Tian, J. Nie, G. A. Jonsdottir, V. Ruotti, R. Stewart, I. I. Slukvin, and J. A. Thomson. 2007. Induced

- Pluripotent Stem Cell Lines Derived from Human Somatic Cells. *Science* 318: 1917-1920.
- Zaehres, H., M. W. Lensch, L. Daheron, S. A. Stewart, J. Itskovitz-Eldor, and G. Q. Daley. 2005. High-Efficiency RNA Interference in Human Embryonic Stem Cells. *STEM CELLS* 23: 299-305.
- Zhao, S., J. Nichols, A. G. Smith, and M. Li. 2004. SoxB transcription factors specify neuroectodermal lineage choice in ES cells. *Molecular and Cellular Neuroscience* 27: 332-342.
- Zhou, H., S. Wu, J. Y. Joo, S. Zhu, D. W. Han, T. Lin, S. Trauger, G. Bien, S. Yao, Y. Zhu, G. Siuzdak, H. R. Scholer, L. Duan, and S. Ding. 2009. Generation of induced pluripotent stem cells using recombinant proteins. *Cell stem cell* 4: 381-384.
- Zhou, Y. Y., and F. Zeng. 2013. Integration-free Methods for Generating Induced Pluripotent Stem Cells. *Genomics, proteomics & bioinformatics*.

APPENDIX A PROTOCOLS

mMESSAGE mMACHINE® PROTOCOL

1. Thaw frozen reagents. Place RNA Polymerase Enzyme Mix on ice. Vortex the 10X Reaction Buffer and 2X NTP/CAP to ensure contents are resuspended and place the 2X NTP/CAP on ice. Centrifuge in microfuge briefly prior to removing caps.
2. Assemble transcription reaction at room temperature. Add the water and ribonucleotides to the tube prior to adding the 10X Reaction Buffer, followed by the addition of linear DNA template and Enzyme Mix.
 - a. 10 µl 2X NTP/CAP, 2 µl 10X Reaction Buffer, 6 µl (0.1-1ug) Linear DNA template, 2 µl Enzyme Mix
3. Mix thoroughly by gently flicking the tube or pipetting up and down. Centrifuge briefly to bring the reaction to the bottom of the tube.
4. Incubate at 37°C for 2 hours for maximum yield (1 hour incubation has an 80% yield)
5. OPTIONAL: Add 1 µl TURBO DNase and mix thoroughly. Incubate 15 min at 37°C to remove template DNA.
6. Reaction goes immediately into Poly(A) Tailing Kit

AMBION® POLY(A) TAILING KIT PROTOCOL

1. Begin with a completed, room temperature, DNase-treated mMessage mMachine reaction (20 µl in 1.5ml tube). DO NOT add EDTA to the reaction to inactivate the DNase.
2. Remaining at room temperature, add the following reagents in the order they are listed to the 20 µl mMessage mMachine reaction:
 - a. 20 µl mMessage mMachine reaction, 36 µl Nuclease-free water, 20 µl 5X E-PAP Buffer, 10 µl 25 mM MnCl₂, 10 µl 10 mM ATP

3. Remove 0.5 μ l of the reaction prior to adding the enzyme to run as a control on a gel against the tailed reaction.
4. Add 4 μ l of E-PAP and mix gently. The final reaction should be 100 μ l.
5. Incubate for 1 hour at 37°C.
6. Reaction can go directly into the MEGAclean™ Kit procedure or can be placed on ice or stored at -20°C until for later use.
7. During the incubation period, prepare a denaturing agarose gel for electrophoresis. The agarose-formaldehyde gel is used to compare the tailed reaction to the untailed original.
 - a. Heat the samples to 75°C for 10 minutes.
 - b. Load the samples onto the gel and run in 1X MOPS buffer at 5 volts/cm until the bromophenol blue dye is near the bottom of the gel.
 - c. Examine in a UV light. The tailed reaction should be approximately 150 bases longer than the untailed original.

FOMALDEHYDE AGAROSE GEL ELECTROPHORESIS

1.2 g agarose

10 ml 10X FA gel buffer (components listed below)

Add RNase-free water to 100ml

1. Heat to melt agarose. Cool to 65°C in a water bath.
2. Add 1.8 ml of 37% (12.3 M) formaldehyde and 1 μ l of a 10 mg/ml ethidium bromide stock solution. Mix thoroughly and pour into gel mold.
3. Equilibrate the gel for at least 30 minutes in 1X FA gel running buffer prior to running the gel.
4. Run the gel at 5-7 V/cm in 1X FA gel running buffer.

MEGACLEAR™ KIT PROCEDURE

1. Begin with 100 µl Poly(A) Tailing Reaction (or bring RNA sample up to 100 µl by adding Elution Solution and mix gently).
2. Add 350 µl of Binding Solution Concentrate and mix gently by pipetting.
3. Add 250 µl of 100% ethanol and mix gently by pipetting.
4. Insert a filter cartridge into a Collection and Elution tube supplied by the manufacturer.
5. Apply the RNA sample to the filter cartridge.
6. Centrifuge at 10,000-15,000 x g for 1 minute to pass mixture through the filter.
7. Discard flow-through and reuse the Collection and Elution Tube during the following wash steps.
8. Be sure ethanol has been added to the wash solution before use. Apply 500 µl Wash Solution and centrifuge at 10,000-15,000 x g for 1 minute to pass the Wash Solution through the filter.
9. Repeat with a second 500 µl Wash Solution.
10. Discard flow-through and centrifuge for 30 seconds at 10,000-15,000 x g to remove any Wash Solution.
11. Elute RNA with 50 µl of Elution Solution
 - a. OPTION 1: Place filter cartridge into a new Collection/Elution Tube. Apply 50 µl Elution Solution to the center of the filter and close the cap. Incubate in heat block at 65-70°C for 5-10 minutes. Recover eluted RNA by centrifuging for 1 minute at 10,000-15,000 x g. *To maximize recovery, repeat with a second 50 µl of elution solution in the same tube.
 - b. OPTION 2: Pre-heat 110 µl of Elution Solution to 95°C. Apply 50 µl of pre-

heated Elution Solution to the center of the filter and close the cap. Centrifuge for 1 minute at 10,000-15,000 x g. *To maximize recovery, repeat with a second pre-heated 50 µl of Elution Solution into the same tube.

LIPOFECTAMINE® RNAiMAX REAGENT TRANSFECTION PROTOCOL

1. Plate target cells 24-hours prior to transfection in 500 µl growth medium each well of a 24-well plate. Cell should be 50-70% confluent for transfection.
2. Dilute ivtRNA in 50 µl of Opti-MEM® I Medium without serum per well in a 15 ml conical tube and mix gently. Label as Tube 1.
3. Gently mix Lipofectamine® RNAiMAX Reagent prior to use. Dilute 1 µl of Lipofectamine® RNAiMAX Reagent in 50 µl of Opti-MEM® I Medium per well in a separate 15 ml conical tube. Label as Tube 2.
4. Combine the contents of Tube 2 into the contents of Tube 1 and mix gently. Incubate for 15 minutes at room temperature.
5. Add adequate amount of RNA-Lipofectamine® complexes to each well and mix gently by rocking the plate back and forth several times. The total volume should be roughly 600 µl.
6. Incubate in CO₂ incubator at 37°C.
7. Change medium 4-6 hours after transfection.
8. Repeat transfection as desired.

R&D SYSTEMS® iMEF PLATING PROTOCOL

Catalog Number: PSC001, irradiated primary mouse embryonic fibroblast cells at passage 3, approximately 6×10^6 cells per vial, stored in LN

*Feeder layers should be plated at least 24 hours prior to seeding target cells.

1. Coat surface of dish with 0.1% sterile gelatin for 15 minutes.
2. Warm iMEF medium (high glucose DMEM, 10% fetal bovine serum, 2 mM L-Glutamine) to 37°C.
3. Thaw the cells quickly by warming vial in 37°C water bath. Immediately transfer contents of vial to 15 mL conical tube containing 5 mL of pre-warmed iMEF medium. Rinse vial with 1 mL of pre-warmed medium to ensure removal of all cells from vial.
4. Pellet cells at 200 x g for 5 minutes.
5. Remove excess gelatin from wells immediately prior to plating iMEF cells.
6. Resuspend iMEF cells in pre-warmed iMEF medium and transfer appropriate density of cells depending on the surface area of dish (approximately 1×10^6 cells/60 mm plate).
7. Incubate overnight in a 5% CO₂ incubator at 37°C.

DYNABEADS® RNA ISOLATION PROTOCOL

1. Harvest cells from culture flask and pellet as per standard procedure
2. Add 300 µl of Lysis/Binding buffer (100 mM Tris-HCL [pH 7.5], 500 mM LiCl, 10 mM EDTA [pH 8], 1% LiDS, 5 mM dithiothreitol [DTT]) to fresh cell pellet.
3. DNA-shearing step is advised if sample is greater than 500,000 cells. Strip cells using a 21-gauge needle and a 1 ml syringe.
4. Prepare 50 µl Dynabeads Oligo (dT)₂₅ per manufacturer instructions (Invitrogen Dynal As, Oslo, Norway, Cat.no. 610.11/610.12).
5. Add sample lysate to pre-washed Dynabeads (pre-washed in lysis/binding buffer).
6. Resuspend beads completely into sample lysate and incubate with continuous mixing (rolling mixer) for 5 minutes at room temperature. This allows the polyA tail to hybridize to the beads.

7. Place the vial on the magnet for 2 min and remove supernatant. If viscous, increase time to 10 minutes.
8. Wash beads/mRNA complex twice in 600 μ l Washing Buffer A (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS) at room temperature. Place on magnet to separate beads from solution between each wash step.
9. Wash beads/mRNA complex in 300 μ l Washing Buffer B (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 1 mM EDTA) at room temperature. Use the magnet to separate the beads from the solution.
10. Elute the RNA from the beads by adding 15 μ l of nuclease-free water, incubate at 70°C for 2 min. Immediately place tube on magnet, and transfer supernatant to a new RNase-free tube.
11. Use sample directly into cDNA protocol.

iSCRIPT CDNA SYNTHESIS PROTOCOL

1. Add 4 μ l of iScript reaction mix and 1 μ l of reverse transcriptase to a 15- μ l RNA sample from previous DYNABEADS isolation in a PCR tube (Bio-Rad Laboratories, Inc., Hercules, CA, USA).
2. Place PCR tubes into thermocycler and run cDNA protocol.
 - a. 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C, (final hold at 4°C)

RNEASY® PLUS MINI KIT (QIAGEN)

1. Harvest cells by trypsinization and pellet. Completely remove supernatant.
2. Disrupt cells with the addition of Buffer RLT Plus. Loosen pellet by flicking prior to adding 350 μ L Buffer RLT. Mix by vortexing or pipetting up and down.

3. Homogenize lysate by passing it through a 20-gauge needle 5 times in an RNase-free 1ml syringe.
4. Transfer homogenized lysate to a gDNA Eliminator spin column placed within 2 ml collection tube. Centrifuge 30 seconds at 8000 x g and discard column. Collection tube should contain flow-through to be used in following steps.
5. Add 350 µl of 70% ethanol to the flow-through in the collection tube. Mix by pipetting up and down.
6. Transfer 700 µl of sample to RNeasy® spin column placed within a 2 ml collection tube and close lid. Centrifuge for 15 seconds at 8000 x g. Discard flow-through.
7. Add 700 µl Buffer RW1 to the RNeasy® spin column placed into previous collection tube. Close lid and centrifuge for 15 seconds at 8000 x g. Discard flow-through.
8. Add 500 µl Buffer RPE to RNeasy® spin column and close lid. Centrifuge for 15 seconds at 8000 x g. Discard flow-through.
9. Add 500 µl Buffer RPE to RNeasy® spin column and close lid. Centrifuge 2 minutes at 8000 x g to ensure removal of ethanol. Remove column carefully not coming in contact with flow-through.
10. Place RNeasy® spin column into a new 2 ml collection tube and add 30 µl RNase-free water directly to spin column membrane. Close lid and centrifuge for 1 minute at 8000 x g for RNA elution. Repeat with an additional 30 µl to maximize recovery.

RT-PCR PROTOCOL

1. Each reaction is carried out in a total of 50 µl. Mix 25 µl of JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma Aldrich), 2 µl of (10 mM) forward primer, 2 µl

of (10 mM) reverse primer, 11 µl of water, and 10 µl of sample cDNA. Prepare master mixes prior to avoid repeated pipetting.

2. Place tubes into thermocycler with the following program:

a. Thermocycler Program

Hotstart	94°C	2 minutes
Denature	94°C	30 seconds
Anneal	60°C	30 seconds
Extend	72°C	1 minute
30 CYCLES		
Final Ext	72°C	5 minutes
Hold	4°C	indefinitely

b. Annealing temperature is specific to primers used in reaction.

3. Remove tubes from thermocycler and add 25 µl of each PCR product to a 1% agarose gel for electrophoresis.

qRT-PCR PROTOCOL

1. Set up plate template.

2. Prepare master mixes for each gene. Each reaction is carried out in a 20 µl reaction (10 µl of SsoFast™ Evagreen Supermix (Bio-Rad Laboratories, Inc. Hercules, CA, USA), 4 µl of nuclease-free water, 1 µl of (10 mM) forward primer, 1 µl of (10 mM) reverse primer, and 4 µl of sample cDNA, calibrator cDNA, or water as negative control.

3. Add 20 µl of each reaction into a single well of a 96-well plate with the designated plate set-up. Cover with sealing tape supplied by plate manufacturer. Be sure to completely seal each well using the rubber scraper or tool provided.

4. Place 96-well plate into thermocycler and run the following program:

a. Thermocycler Program

Cycle	Repeats	Step	Time	T°	Melt Curve
1	1	1	1 min	95	
2	40	1	5 sec	95	

		2	30 sec	60	
3	1	1	1 min	95	
4	1	1	1 min	55	
5	80	1	10 sec	55	yes, +5° each repeat

5. Evaluate post-run data for analysis and melting curve. If a calibrator dilution was performed, evaluate Standard Curve Efficiency, PCR Amp/Cycle graph, and Melt Curve Analysis.

Plasmid Preparation

Stab cultures containing plasmids encoding desired sequences were obtained from Addgene. The cultures were streaked on agar plates containing ampicillin 100ug/ml concentration. Clones were chosen from each plate by inserting a pipette tip into a single colony and inserting it into 3ml of Terrific Broth containing ampicillin 100ug/ml in a 15 ml culture tube. The selected clones were prepared as mini-preps with overnight culture at 37° C shaking at 225 RPM in C24 Incubator Shaker (New Brunswick Scientific, Classic Series).

Mini Prep Isolation

All tubes were cloudy post-incubation, indicating growth of the selected clones. Mini-preps were performed on all tubes using SIGMA GenElute Plasma Mini Prep Kit. Cells were pelleted from 1.5 ml of previous overnight culture for 1 minute at 12,000 x g in table top centrifuge (Thermo, Heraeus Pico 17 Centrifuge). Supernatant was discarded and cells were resuspended by pipetting up and down in 200 µl of Resuspension Solution provided in the kit. The cells were then lysed with the addition of 200 µl of Lysis Solution and inverting the tubes gently to mix. The tubes were then allowed to equilibrate for ≤ 5 minutes. The cleared lysate was then prepared for isolation with the addition of 350 µl of Neutralization Solution and inverted 4-6 times to mix. The tubes were then centrifuged at maximum speed of 13,000 x g for 10 minutes. The binding column was prepared by adding 500 µl of Column Preparation Solution and

centrifuged at 12,000 x g for 1 minute. The cleared lysate was then transferred to the prepared binding columns to bind the plasmid DNA for isolation. The column containing the cleared lysate was centrifuged for 1 minute at 12,000 x g and the flow-through was discarded. Contaminants were removed by adding 750 µl of Wash Solution (ethanol added prior to first use of kit) to the column followed by centrifugation at 12,000 x g for one minute. The flow through was discarded and was centrifuged for an additional minute to remove to dry the column. Each column was transferred to a new collection tube and the plasmid DNA was eluted in 75ul of molecular grade water and was stored at -20°C.

Clone Selection

Diagnostic cuts in the DNA were performed with restriction enzyme XbaI on each clone. A master mix was prepared when applicable (buffer provided by manufacturer, molecular grade water, XbaI restriction enzyme) for all mini-prep isolations. The master mix was then divided and 85ul was placed in 12 separate 1.5 ml centrifuge tubes followed with the insertion of 15ul DNA for each clone. The digest was performed in a hot water bath of 37°C for 1 hour and 15 minutes. The tubes were removed from the water bath and 15ul of loading buffer was added to each tube and mixed gently by pipetting up and down. A 1% agarose gel was prepared and 25 µl of each digest were loaded into the wells for electrophoresis evaluation. The bands were evaluated with the additional 1 kilobase (1Kb) and 100 base pair (100bp) ladders added to the outside lanes. The correct diagnosis was reached with the GFP plasmid dropping out a 904 bp sequence and OCT4 plasmid dropping out a 1200 bp sequence.

Clones were selected and were removed from the refrigerator and 2 ml of Terrific Broth containing ampicillin was added. The cultures were allowed to shake at 225 RPM at 37°C for 1

hour. The remaining 50 ml of broth was then added and the culture was incubated overnight at the same conditions in a 500 ml Erlenmeyer flask.

Midi Prep Plasmid Purification

Qiagen Midi Preps were then carried out by pelleting 50 ml of overnight culture at 6000 x g for 15 minutes at 4°C. Supernatant was removed and the pellet was resuspended in 4 ml of Buffer P1, followed by the addition of 4 ml of Buffer P2. The mix was thoroughly made homogenous by inverting vigorously 5 times followed by 5-minute incubation at room temperature. The QIAfilter Cartridge was prepared during this incubation period. Buffer P3 was added at 4 ml and mix thoroughly by inverting vigorously 5 times. The lysate was poured into the barrel of the QIAfilter Cartridge and was incubated for 10 minutes at room temperature. QIAGEN-tip 100 was equilibrated by adding 4 ml of Buffer QBT. This buffer was released by gravity flow. The lysate in the barrel of the QIAfilter Cartridge was expelled into the prepared QIAGEN-tip 100 by inserting the plunger into the barrel and filtering the lysate into the prepared tip. The cleared lysate was allowed to flow through the tip by gravity flow, followed by 2 x 10 ml wash with Buffer QC. The DNA was eluted with 5 ml of Buffer QF and precipitated with the addition of 3.5 ml of room-temperature isopropanol. The solution was mixed and centrifuged at 15,000 x g for 30 minutes at 4°C. The supernatant was decanted and the DNA pellet was washed with 2 ml of room temperature 70% ethanol and centrifugation at 15,000 x g for 10 minutes. The supernatant was decanted and the pellet was allowed to air-dry for 5-10 minutes. The DNA was re-dissolved in 500 µl TE for each preparation and placed in -20°C for storage. DNA was quantified by absorbance by SmartSpec™ Plus Spectrophotometer (BioRad) and TE buffer.

Diagnostic restriction digests were performed on Midi Preps for validation using XbaI restriction enzyme. The digests were performed overnight at 37°C in a warm water bath. The digests were evaluated using gel electrophoresis and a 1% agarose gel.

Synthesizing Messenger RNA

Plasmids containing template sequence were linearized using restriction enzyme XbaI. The restriction digest was terminated with the addition of 1/10th the volume of 3 M Na acetate and 2 volumes of ethanol. The solution was mixed well and stored in -20°C for 15 minutes. The DNA was pelleted by centrifugation at 13,000 x g for 15 minutes. The supernatant was removed and the tube centrifuged again, and any residual fluid was removed with a fine-tipped pipet. The pellet was resuspended in 6 µl TE and was carried over into the mMESSAGE mMACHINE Kit (High Yield Capped RNA Transcription Kit). The reaction was performed in a total of 20 µl, including 10 µl of 2X NTP/CAP, 2 µl 10X Reaction Buffer, 6 µl linear template DNA, and 2 µl of Enzyme Mix. The solution was mixed by gently flicking the tube and was incubated for 2 hours at 37°C in a warm water bath.

The previously completed 20ul reaction was carried over to the Poly (A) Tailing Kit (Applied Biosystems AM1350) for further RNA packaging. The tailing agents were added in order (36 µl Nuclease-free water, 20 µl 5X *E*-PAP Buffer, 10 µl 25 mM MnCl₂, and 10 ul mM ATP, 4 µl *E*-PAP) to the room temperature 20ul mMessage mMachine reaction yielding a total of 100 µl. This tailing reaction was incubated at 37°C for 1 hour in a warm water bath.

The tailing reaction was purified using MEGAclean Kit (Applied Biosystems AM1908). The RNA sample was first subjected to 350 µl of Binding Solution Concentrate and was mixed gently by pipetting up and down. 250 µl of 100% ethanol was added and mixed gently. A filter cartridge was inserted into a Collection Tube supplied by the kit. The RNA mixture was loaded

into the filter cartridge and is centrifuged for 1 minute at 13,000 x g in tabletop centrifuge. The flow-through was discarded and the same Collection Tube was used for the washing steps. The Wash Solution (previously added ethanol as directed) was applied 2 x 500 µl with centrifugation and discarding flow-through. The tubes were centrifuged once again to ensure removal of all wash solution. The RNA was then eluted into a new Collection Tube by adding 50 µl of pre-warmed (95°C) TE to the cartridge. The tube was centrifuged at 13,000 x g for 1 minute. This step was repeated within the same collection tube to maximize RNA recovered.

The ivtRNA was then quantified using NanoVue Spectrophotometer using TE as the blank for the machine. The solution was then diluted with an adequate volume of TE to obtain a working concentration of 100 ng/ml.

Expansion of Cell Cultures

Established bovine fetal fibroblast cell line BEZ2 (passage 2-5) was removed from liquid nitrogen and quickly placed in warm water bath at 37°C until thawed. Cells were removed from the cryovial and placed into 15ml conical tube containing 5ml of Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 1% penicillin/streptomycin (P/S). The cryovial was then rinsed with 1ml of the same medium to ensure removal of all cells. The conical tube was then centrifuged at 350 x g for 5 minutes to establish a cell pellet. The supernatant was removed and the cells were resuspended in 10 ml of medium and placed into a T75 for expansion. The T75 culture flask was incubated in 5% CO₂ at 37°C for two days. Once the cells were approaching confluency they were passaged with Trypsin (0.25 EDTA) and plated into 24-well plates at recommended seeding density of 50,000 cells per well. The plates were then incubated in 5% CO₂ at 37°C for 24 hours.

Lipofectamine Transfections

Previously plated bovine fetal fibroblasts reached 70% confluency and were ready to be transfected. Specific ivtRNA was diluted in Opti-MEM I Medium at in 15ml conical tubes. Lipofectamine RNAiMAX Reagent was diluted 1ul per 50ul Opti-MEM I Medium in a 15 ml conical tube. The diluted RNA complex was then combined with the Lipofectamine RNAiMAX Reagent and incubated for 15-20 minutes at room temperature. The RNAi-duplex-Lipofectamine RNAiMAX Reagent complexes were added to each well containing cells for transfection. The final volume was roughly 600ul per well. The plate was moved back and forth several times to ensure even distribution of ivtRNA-Lipofectamine mixture over all cells attached to well. The cells were incubated in 5% CO₂ at 37°C for 24 hours and all media was changed daily.

Flow Cytometry

Culture medium was aspirated from all wells, followed by a wash with PBS without calcium and magnesium. PBS was aspirated and cells were detached using Trypsin (0.25 EDTA). Normal growth medium containing serum was added to inactivate the Trypsin. The contents of each well were transferred into labeled 1.7ml centrifuge tubes. The cells were centrifuged at 400 x g for 8 minutes. The supernatant was completely aspirated and the cells were resuspended in 1ml PBS (without calcium and magnesium). A small volume (10 µl) was removed from each sample and placed in 90 µl of PBS for cell counting. The flow cytometer was prepared for sampling by the routine setup protocol prior to running any samples. Once the flow cytometer was ready, each sample was evaluated for 5 minutes with slow fluidics settings.

RNA Isolation

The cells were trypsinized from each well and treatment groups were combined into one 15ml tube each for unmodified ivtRNA experiments. The cells were isolated and evaluated by

well for all other experiments. The cells were pelleted and resuspended in 1 ml PBS without calcium and magnesium. This volume was transferred to 1.5 ml tubes and pelleted again at 400 x g for 8 minutes using a table top centrifuge. The mRNA was harvested using DYNA beads following the “mini” protocol. The mRNA was eluted in 15 ml of dH₂O, which went immediately into iScript to be converted into cDNA per manufacturer instructions, yielding a final volume of 20 µl. The final 20 µl reaction was divided in half for subsequent PCR reactions.

Polymerase Chain Reaction

PCR was used to determine the endogenous expression of bovine OCT4 compared to exogenous expression of human OCT4. Each PCR reaction consisted of 25 µl Jumpstart Red Mix, 2 µl forward primer, 2 µl reverse primer, 10 µl cDNA, and 11 µl dH₂O for a total of 50 µl. The final PCR products were evaluated by gel electrophoresis using 1.2% agarose gel including 100bp ladder on the outside lanes.

Establishing 4-Factor and 3-Factor Cocktails

A KMOS (KLF4, cMYC, OCT4, SOX2) and KOS (KLF4, OCT4, SOX2) cocktails were prepared using the working concentrations of each of the ivtRNA solutions. OCT4 was held at 3X the concentration of the other factors.

Calibrator Development

A positive control is required during the analysis of gene expression through quantitative PCR. This positive control normally comes in the form of a calibrator. Total RNA was isolated from BEZ2 P7 using RNeasy® Plus Mini Kit from Qiagen per manufacturer instructions. The RNA was immediately converted into cDNA using iScript. The calibrator is made up of cDNA from the target cells “spiked” with purified PCR product (QIAquick PCR Purification Kit) encoding specific genes. These genes include both the genes of interest, OCT4, SOX2, and

NANOG, as well as housekeeping genes PAP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The calibrator contains genes of interest at a final concentration of 2pg/μl and the housekeeping genes at a final concentration of 0.2pg/μl.

qPCR

Quantitative PCR was used to evaluate gene expression in the target cells. Total RNA was extracted from cells in all wells using DYNA beads according to the manufacturer's instructions. The final elution into 15ul dH₂O was placed directly into a complementary DNA reaction following the manufacturer's instructions, with a final volume of 20ul per sample. Prior to setting up PCR reactions, each sample was diluted with the addition of 50ul dH₂O. Each PCR reaction contained 4ul of sample cDNA. Measurements were carried out in triplicates and a no-template reaction as a negative control. Amplification, standard curves and gene expression efficiency were normalized against housekeeping genes PAP and GAPDH as internal controls.

Table A.1 PCR Primers

Primer	Sequence	Amplicon Length	Accession #
Oct4 sense	GGTTCTCTTTGGAAAGGTGTTC	223	NM_174580.2
Oct4 antisense	ACACTCGGACCACGTCTTTC		
Sox2 sense	AGGACTGAGAGAAAGAAGAAGAG	164	NM_001105463.2
Sox2 antisense	AAGAAAGAGGCAAACTGGAATC		
Nanog II sense	AATTCCCAGCAGCAAATCAC	215	NM_001025344.1
Nanog II antisense	CCCTTCCCTCAAATTGACAC		
Poly A sense	AAGCAACTCCATCAACTACTG	169	X63436
Poly A antisense	ACGGACTGGTCTTCATAGC		
GAPDH sense	CCTTCATTGACCTTCACTACATGGTCTA	127	U85042
GAPDH antisense	TGGAAGATGGTGATGGCCTTTCCATTG		

Table A.2: GFP ivtRNA Quantification

	Concentration (ug/ml)	A260/A280
GFP (1)	656.8	2.477

Table A.3 ivtRNA Concentrations

	RNAi duplex concentration *24-well plate	Volume per well (μl)
Low	1 pmol (140ng)	1.4
Medium	2 pmol (280ng)	2.8
High	4 pmol (560ng)	5.6

Table A.4 Plasmid DNA Quantification

	Concentration (ug/ml)	A260/A280
OCT4	12.3314	1.7169
GFP	13.2331	1.7738

Table A.5: hOCT4 ivtRNA Spectrophotometer Quantification

	Concentration (ug/ml)	A260/A280
hOCT4 (1)	596.0	2.656
hOCT4 (2)	592.4	2.649

Table A.6 ivtRNA Concentrations

	RNAi duplex concentration *24-well plate	µl per well
Low	1 pmol (140ng)	1.4
Medium	2 pmol (280ng)	2.8
High	4 pmol (560ng)	5.6

Table A.7 Plasmid DNA Quantification

	Concentration (ug/ml)	A260/A280
KLF4	12.3314	1.7169
c-MYC	13.2331	1.7738
SOX2	65.7967	1.9400

Table A.8: SOX2, KLF4, cMYC ivtRNA Quantification

Gene	Concentration (ug/ml)	A260/A280
SOX2	259.2	3.057
KLF4	402.4	2.661
cMYC	228.4	2.266
OCT4	260.3	2.936

Table A.9 Purified PCR Products

Gene	Concentration (ng/ml)	A260/A280
OCT4	4.7	1.500
SOX2	4.9	1.485
NANOG	15.5	1.667
PAP	14.8	1.561
GAPDH	17.0	1.735

APPENDIX B

MEDIA FORMULATIONS AND STOCK SOLUTIONS

Table B.1 List of Plasmids

Plasmid	Clones Selected From Plates	Clones Used for Mini-Prep	Clone Selected
26822pcDNA3.3_GFP	1-6	1-6	1
26816pcDNA3.3_OCT4	1-6	1-6	1
26817pcDNA3.3_SOX2	1-6	1-3, 6	2
26818pcDNA3.3_cMYC	1-6	1-6	6
26815pcDNA3.3_KLF4	1-6	1-6	1

NORMAL GROWTH MEDIUM

DMEM (Dulbecco's Modified Eagle Medium)

10% FBS (Fetal Bovine Serum; or Bovine Calf Serum (BCS) for non-experimental cell culture)

1% P/S (Penicillin/ Streptomycin)

iMEF CULTURE MEDIUM

DMEM

15% FBS

1% L-Glutamine

1% P/S

MODIFIED bESC MEDIUM

DMEM

15% FBS

1% L-Glutamine

1% P/S

1000 U/ml LIF

4 ng/ml bFGF

MODIFIED bESC MEDIUM +3i

DMEM

15% FBS

1% L-Glutamine

1% P/S

1000 U/ml LIF

4 ng/ml bFGF

0.5 μ M PD0325901

3 μ M CHIR99021

1.8 μ M NuP0148

10X FA Gel Buffer Composition

200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid)

50 mM sodium acetate

10 mM EDTA

1X FA Gel Running Buffer Composition

100 ml 10X FA gel buffer

20 ml 37% (12.3 M) formaldehyde

880 ml RNase-free water

Example:

Tube 1: 50ul OptiMEM x 17 wells = 850ul OptiMEM

2.8ul ivtRNA x 17 wells = 47.6ul ivtRNA

Tube 2: 50ul OptiMEM x 17 wells = 850ul OptiMEM

1ul Lipofectamine x 17 wells = 17ul Lipofectamine

The contents of Tube 2 is added to Tube 1 and incubated for 15-20 minutes. A volume of 103.8ul of mixture is added to each well after incubation is complete.

Note Transfections were done as forward transfections described by the Invitrogen Lipofectamine RNAiMAX Reagent protocol. The reverse transcription method was evaluated yielding similar results (data not shown). Due to the need of repeated transfections, the forward protocol was selected for all experiments.*

OCT4 Calibrator Evaluation

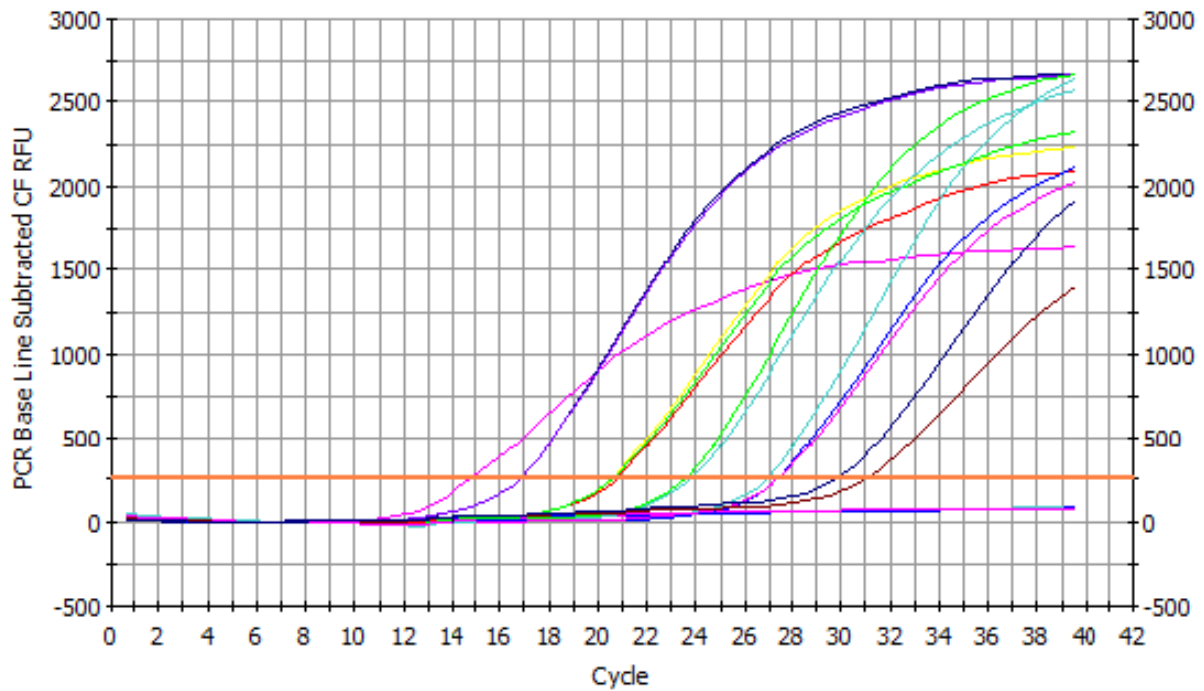


Figure B.1 OCT4 PCR Amp/Cycle Graph of SYBR-490

Correlation Coefficient: 0.997 Slope: -3.277 Intercept: 14.021 $Y = -3.277X + 14.021$
 PCR Efficiency: 101.9 %

□ Unknowns
 ♦ Standards

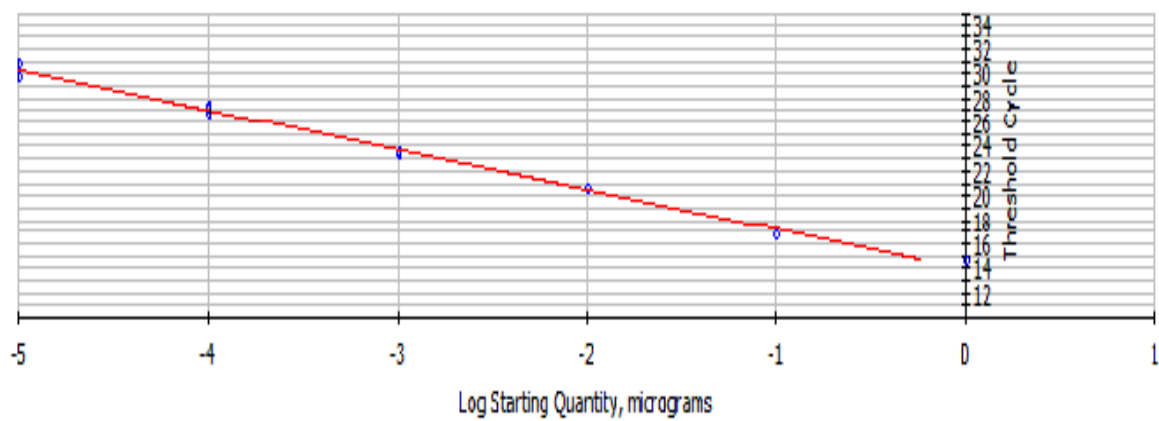


Fig. B.2 OCT4 Standard Curve

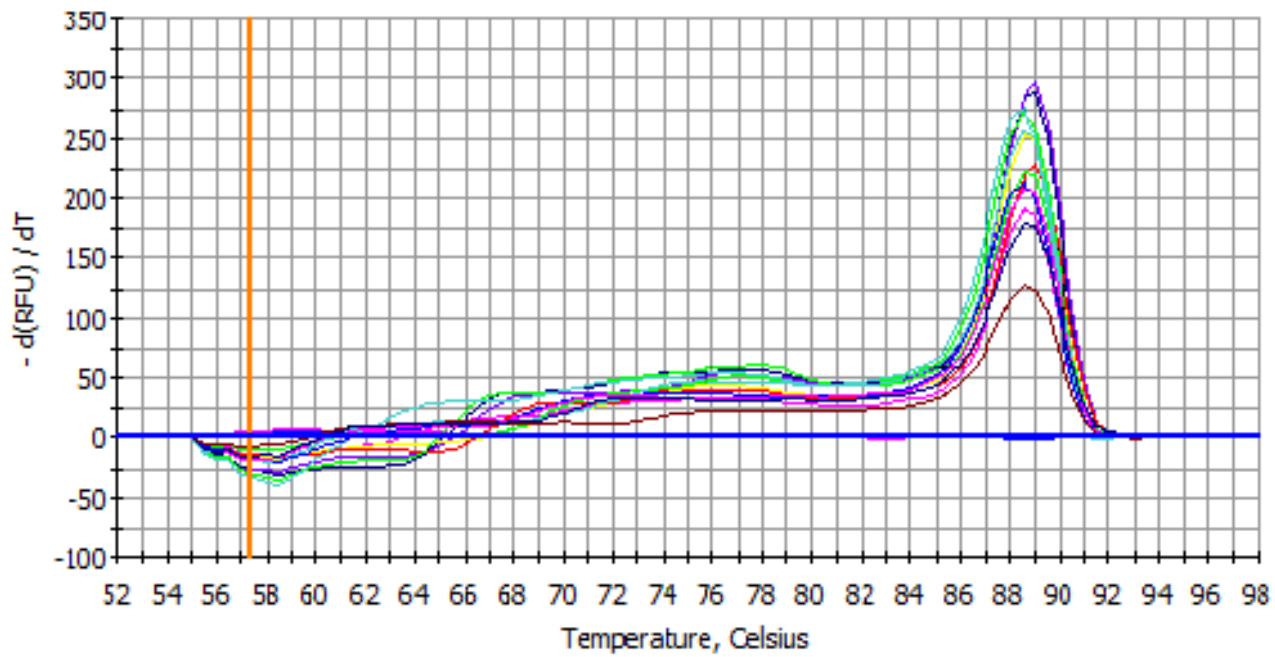


Figure B.3 OCT4 Melting Curve

SOX2 Calibrator Evaluation

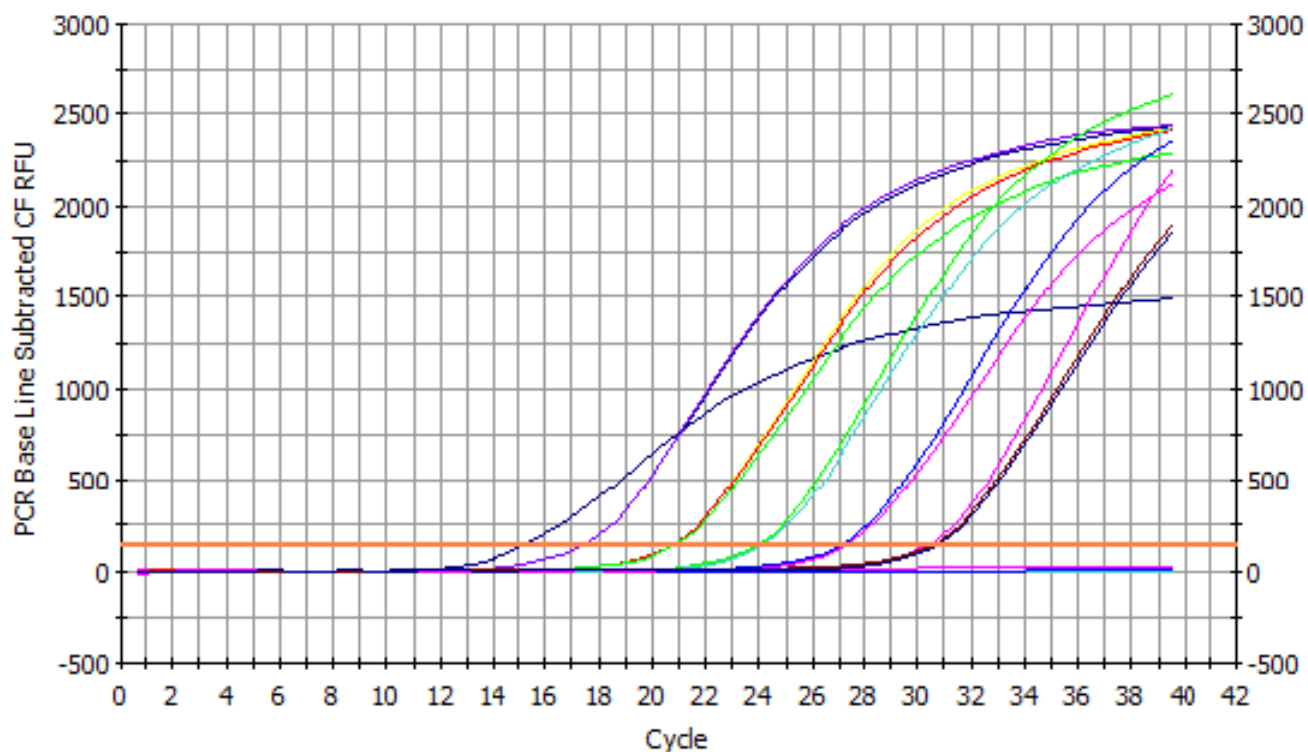


Figure B.4 SOX2 PCR Amp/Cycle Graph for SYBR-490

Correlation Coefficient: 0.999 Slope: -3.177 Intercept: 14.513 $Y = -3.177X + 14.513$
 PCR Efficiency: 106.4 %

□ Unknowns
 ● Standards

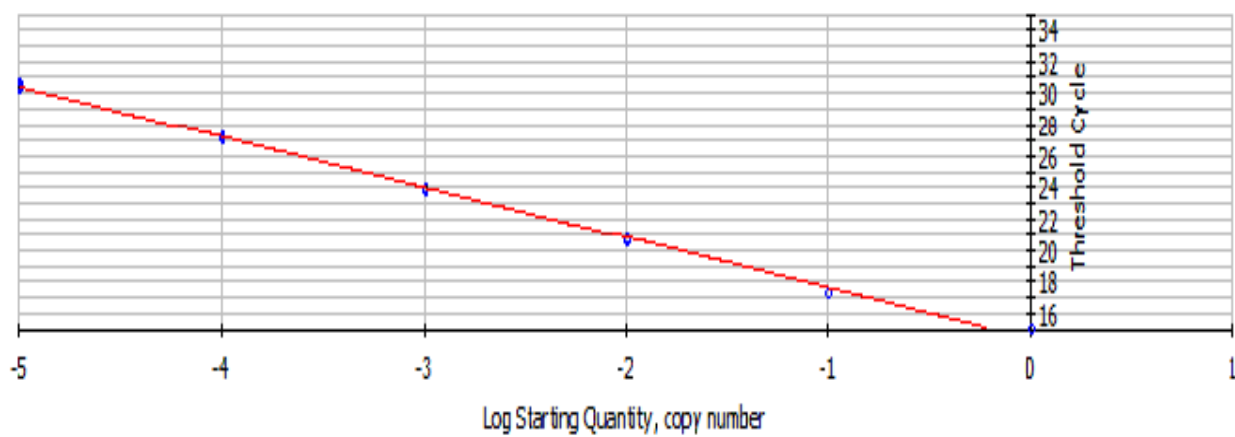


Figure B.5 SOX2 Standard Curve

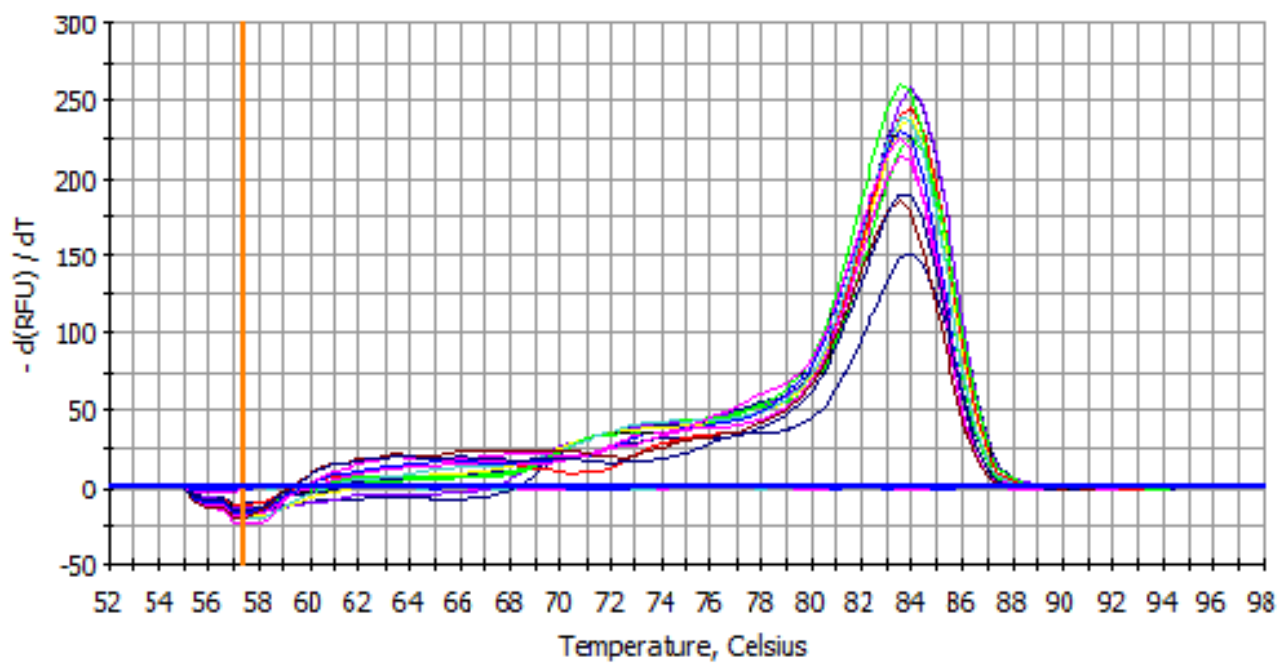


Figure B.6 SOX2 Melting Curve

NANOG Calibrator Evaluation

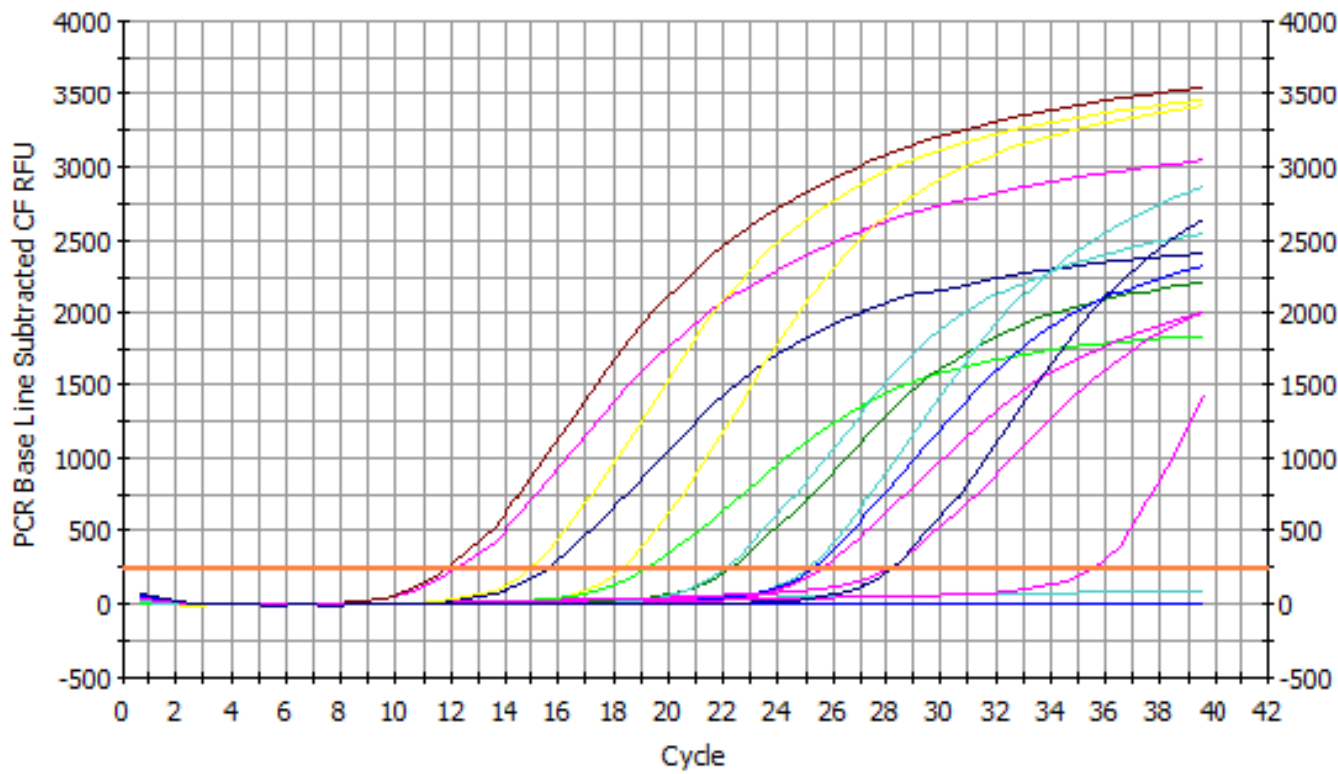


Figure B.7 Nanog PCR Amp/Cycle Graph for SYBR-490

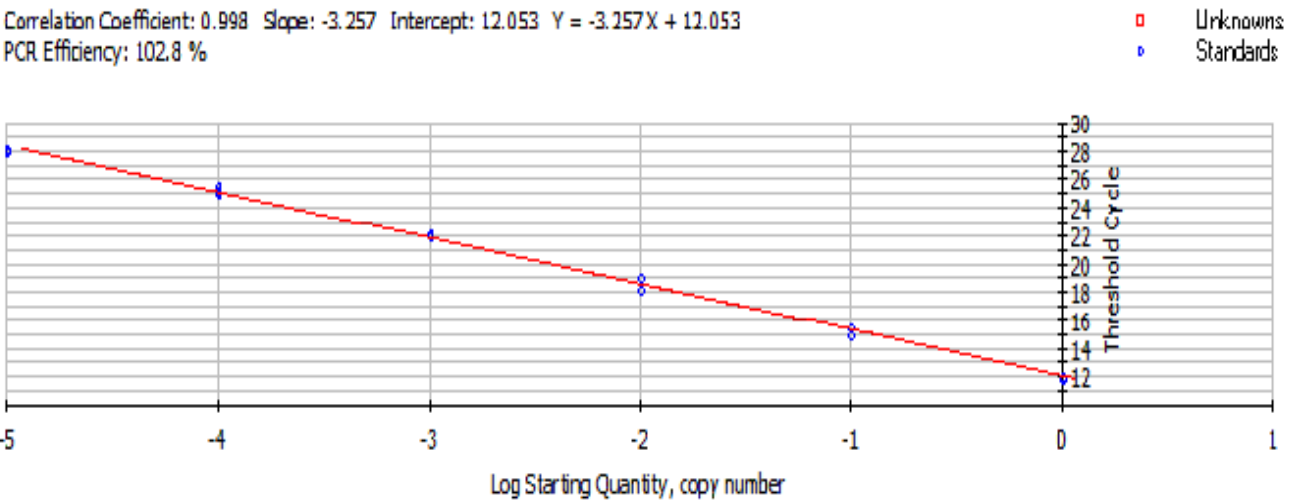


Figure B.8 Nanog Standard Curve

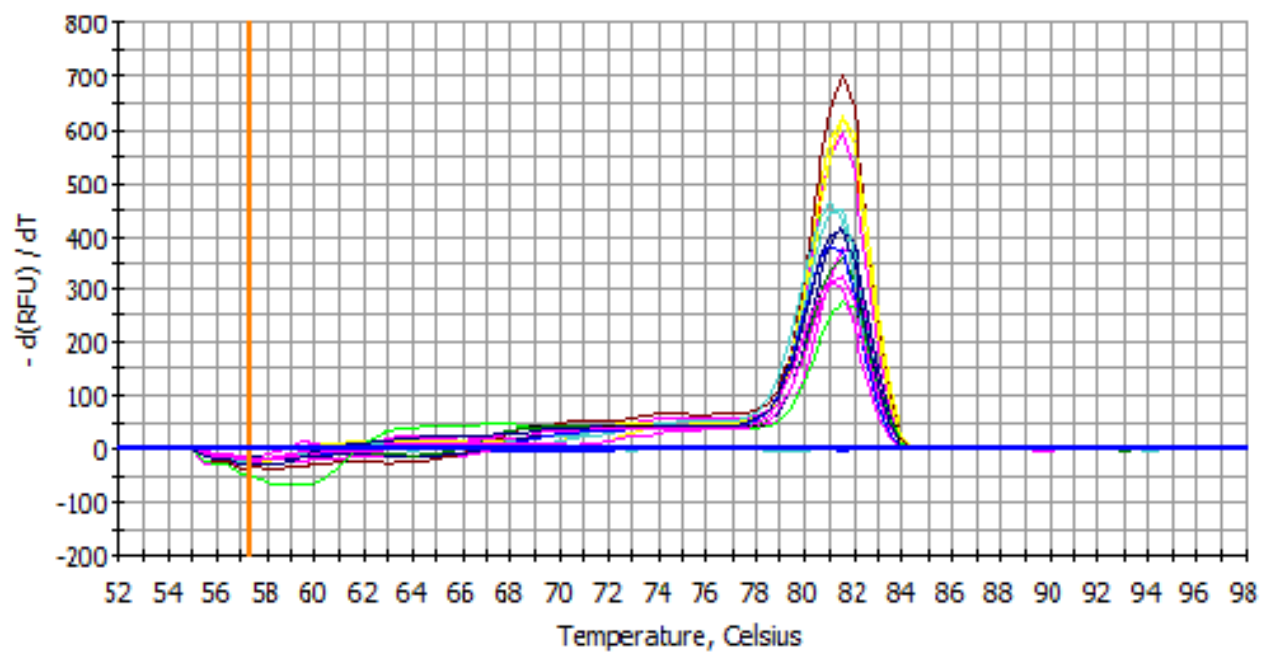


Figure B.9 Nanog Melting Curve

PAP CALIBRATOR EVALUATION

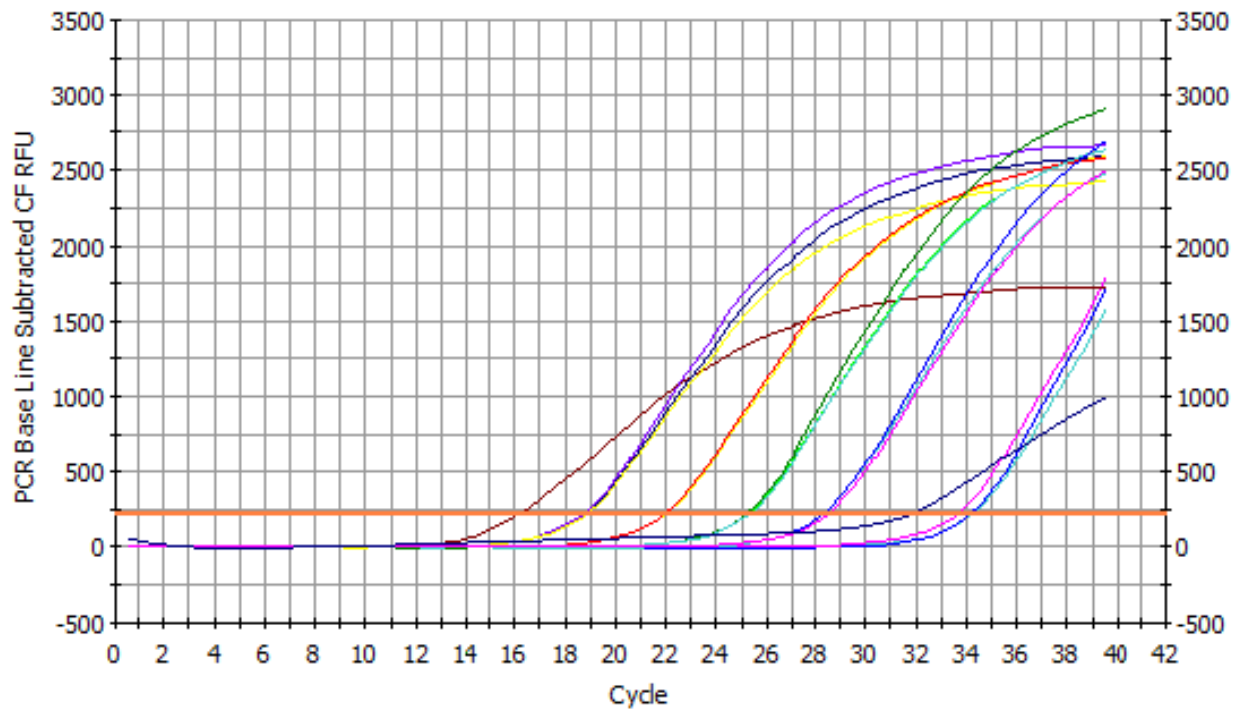


Figure B.10 PAP PCR Amp/Cycle Graph for SYBR-490

Correlation Coefficient: 1.000 Slope: -3.159 Intercept: 15.693 $Y = -3.159X + 15.693$
 PCR Efficiency: 107.3 %

Unknowns
 Standards

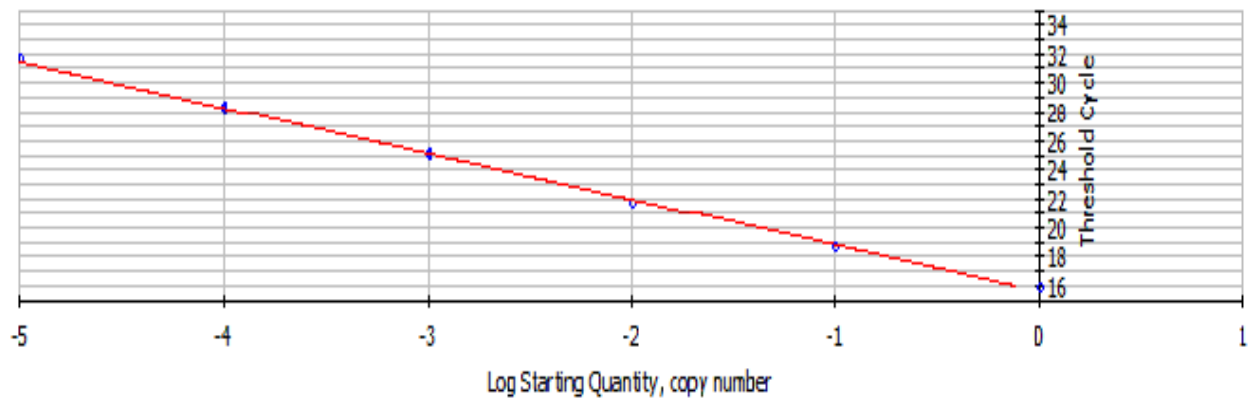


Figure B.11 PAP Standard Curve

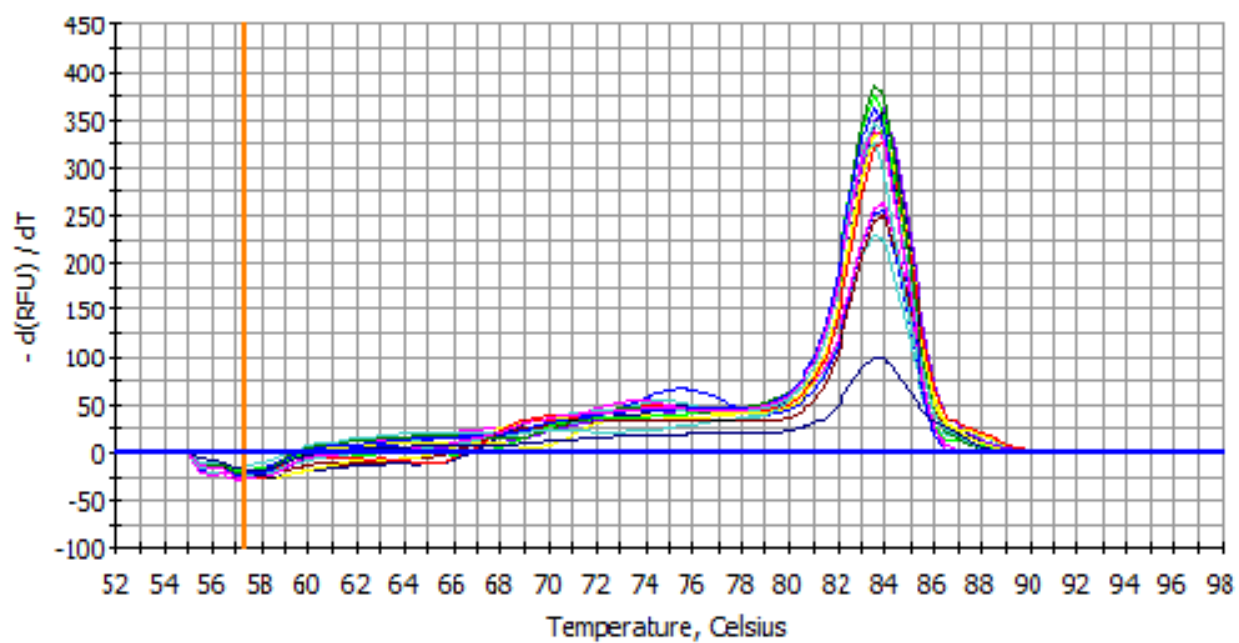


Figure B.12 PAP Melt Curve

GAPDH CALIBRATOR EVALUATION

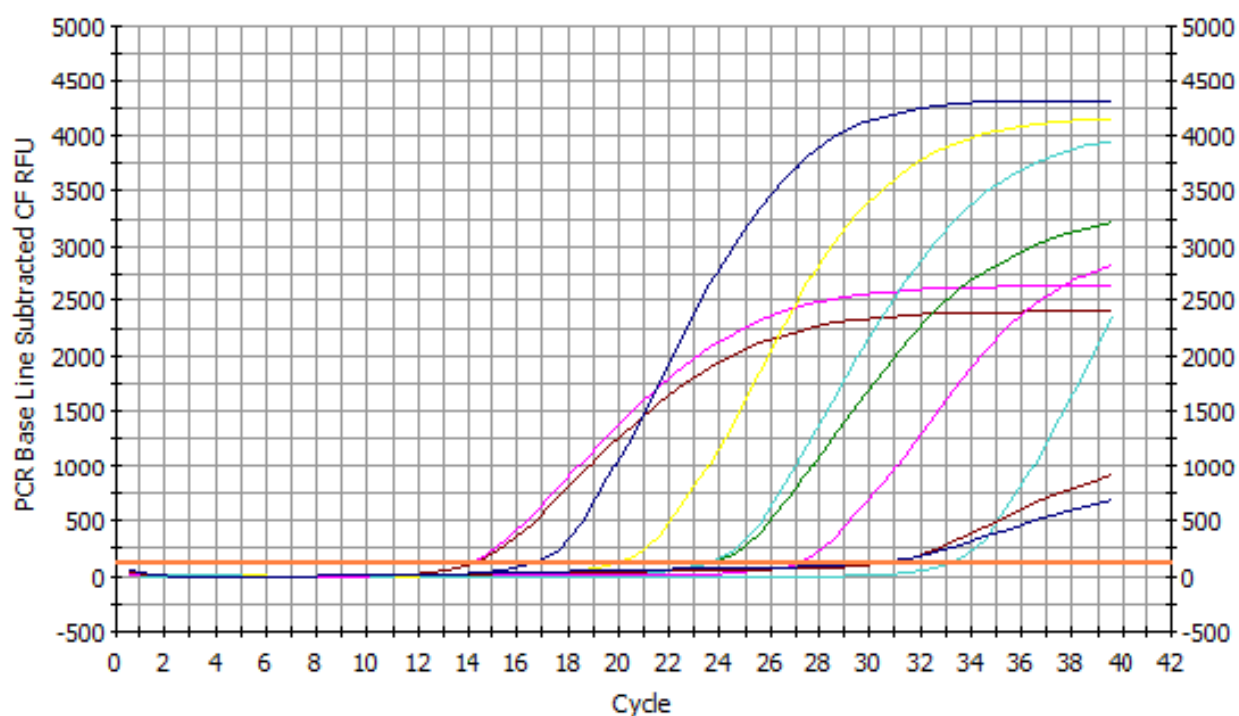


Figure B.13 GAPDH PCR Amp/Cycle Graph for SYBR-490

Correlation Coefficient: 0.998 Slope: -3.200 Intercept: 13.768 $Y = -3.200X + 13.768$
 PCR Efficiency: 105.3 %

Unknowns
 Standards

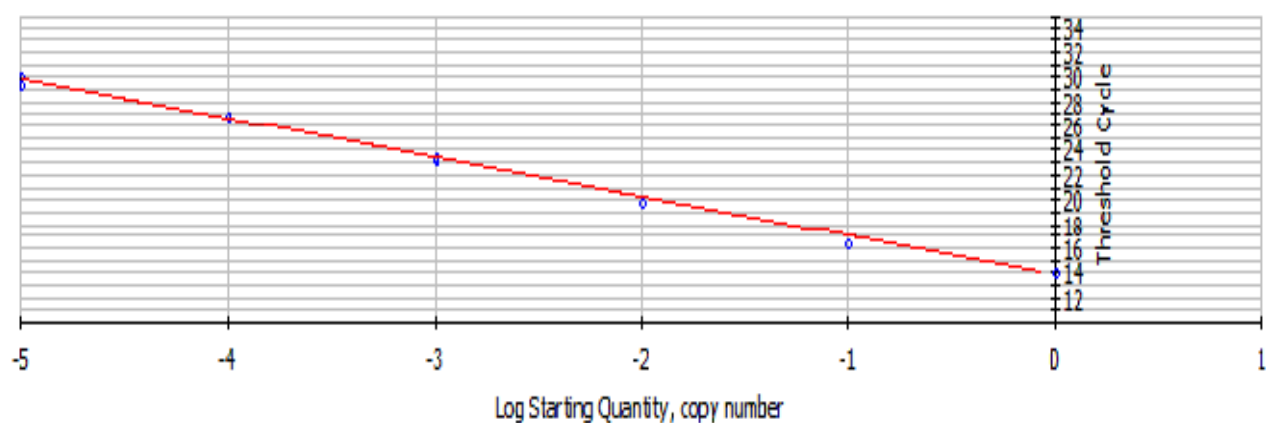


Figure B.14 GAPDH Standard Curve

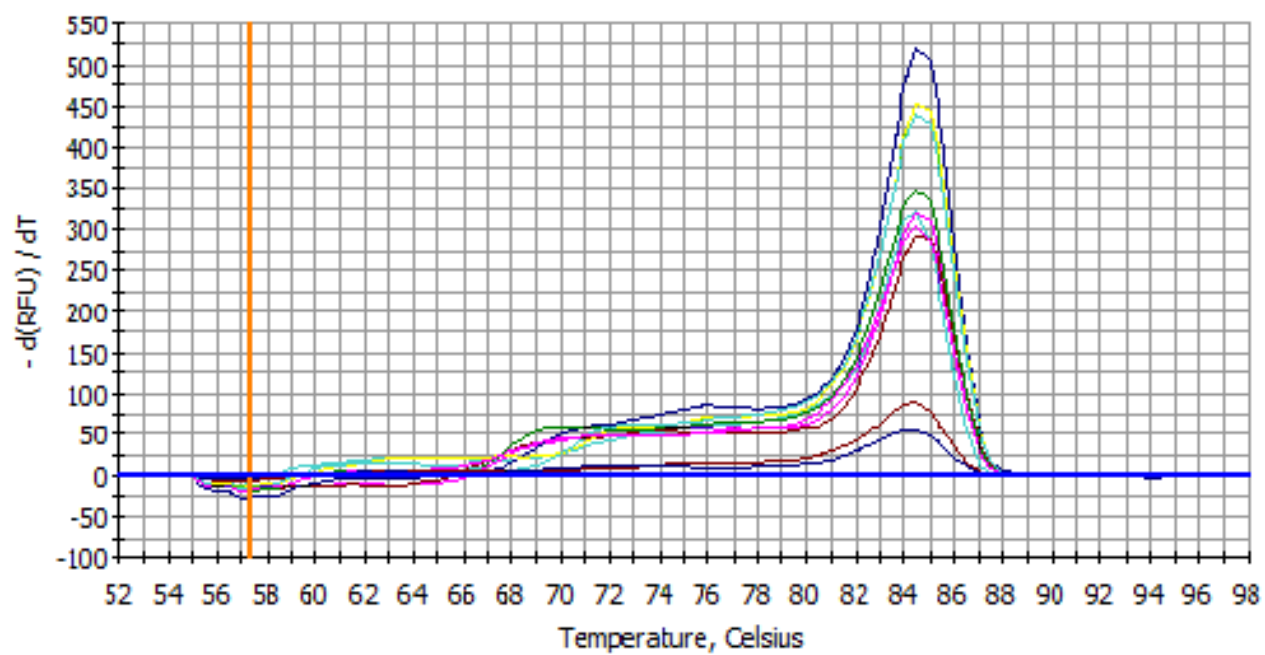


Figure B.15 GAPDH Melt Curve

VITA

Tricia Leigh Adams was born on March of 1982 to Karen James and Bobby Font Jr. in Baton Rouge, Louisiana. In 2000, she graduated with honors from Saint Amant High School in Saint Amant, Louisiana.

Following high school, she was married to a naval sailor, Eric Adams, and relocated several times around the United States to different naval bases. During the years spent moving and serving the country they were blessed with two beautiful children, Madelyn and Holden. In 2007, she and her family decided to settle down in Prairieville, Louisiana, and she pursued a Bachelor of Science degree in Biology from Southeastern Louisiana University. During her undergraduate studies, Tricia became interested in reproductive physiology and biotechnology. In her senior year, she participated in an undergraduate research as an internship followed by a fellowship at Louisiana State University under the supervision of Dr. Kenneth R. Bondioli. She also participated in an undergraduate research program at Southeastern Louisiana University under Dr. Penny Shockett. Tricia graduated Cum Laude in December of 2010 from Southeastern Louisiana University in Hammond, Louisiana.

Tricia entered the graduate program in reproductive physiology at Louisiana State University under the direction of Dr. Kenneth R. Bondioli in the fall of 2011 and is now a candidate for the degree of Master of Science in reproductive physiology in the Department of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.