Domain specific over-expression of a peptide encoded by an I-band domain of the human TTN gene; the role of titin exons 248 – 250 in C2C12 myogenesis

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Abstract

In 1996, a form of Rippling Muscle Disease (RMD) was characterized as a heterogenic autoimmune neuromuscular disease. The mechanics of the main symptom, rolling contractions along the length of a muscle induced by stretching or tapping, are not yet understood, however, studies have suggested that the gigantic skeletal muscle protein titin may play a potential role. We hypothesize that an overexpression of a specific immunogenic titin domain would have an effect on the natural processes of cell growth, proliferation, and differentiation of C2C12 *Mus* musculus myoblast cells. A fusion plasmid was constructed to produce a fusion protein incorporating the immunogenic titin domain attached to a green fluorescent protein. C2C12 cultures were then transfected with the fusion plasmid and induced into differentiation over a two week period. Morphology and fluorescent patterns were analyzed using light and fluorescent microscopy techniques. Protein samples taken at various time points throughout the differentiation process were analyzed using SDS-PAGE. Techniques of the analysis of in vivo production of the immunogenic titin domain were developed. This preliminary data suggests that over-expression of an immunogenic titin domain has an effect on normal growth and differentiation of C2C12 cells. We have also shown that this method of study can be used to identify roles of not only this but other domains of the titin protein.

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Table of Contents

Chapters

List of Figures

Appendix 3: Supplementary Figures

List of Tables

Chapter 1: Introduction

1.1 – Rippling Muscle Disease

Rippling muscle disease (RMD) is a muscular ailment that affects the contractile ability of skeletal muscle cells. It was first characterized by Torbergson in 1975 as an autosomal dominantly inherited disease [22]. He surveyed a Scandinavian family over three generations and found five of those members experienced symptoms of RMD. Symptoms included muscle stiffness, pain, and cramping, usually in the lower extremities, after rest or during forced physical activity. However, the main, and most unusual, symptom that presented was a ripple-like muscle contraction that was induced by stretching or tapping the muscle [17, 22].

An intriguing feature of these involuntary muscle contractions were that they were found to act without motor unit action potentials (MUAPs). Torbergson reported that during analysis using electromyography recordings, no detectable MUAPs were identified. This led him to believe that contraction was not a result of depolarization of the muscle fiber membrane, but due to a defect in the muscle contractile machinery [22]. Bretag suggested, from his research in 1988 that the continuation of the electrically silent contractions may be the result of a potential role for stretch activated calcium channels [5, 12]. However, Ricker, in 1989, determined that the spread of the contractions was roughly ten times slower than those of normal muscle-fiber action potentials. These contractions were found to spread not only along the length of the muscle, but they also progress to laterally adjacent muscle fibers. He concluded that local contraction of a small number of sarcomeres may induce the contraction of surrounding sarcomeres [20].

In 1994, Stephen et al initially mapped a gene for RMD to chromosome 1, region 1q41 to 1q42. However, they also reported that the genetic analysis of several other families did not map

to this region, concluding that it is the defect of multiple genes that play a role in the phenotype of RMD [21]. Over the next couple of years, the caveolin-3 gene was found to be a factor of RMD along with multiple other muscular diseases [4]. Dotti et al, in 2006, identified another mutation in the Cav3 gene that participates in RMD. The skeletal muscles of a family of Italian nationality were examined and found to have a decreased amount of caveolin-3 protein. Caveolin-3 protein resides in skeletal muscle cell membranes and is essential in scaffolding and signaling functions. Genetic analysis showed a previously undocumented mutation within the scaffolding domain of the caveolin-3 gene in each of the affected family members. This specific region plays essential roles in homo-oligomerization and multiple signaling molecule interactions [6]. Another study by Lorenzoni et al found a mutation in the nucleotide at position 140 in the Cav3 gene. This missense mutation falls within the same scaffolding domain, a mere 14 nucleotides from the missense mutation previously described by Dotti et al. At this position, a highly conserved negatively charged Glutamine residue is replaced by a neutral Alanine residue. Due to other mutations found within this region in patients diagnosed with RMD, they suggest that this missense mutation also drastically compromises proper structure formation and thus, the ability of the protein. This alanine mutation was found in multiple patents diagnosed with RMD and allowed them to conclude that this region of the caveolin-3 protein is in fact significant to its function and RMD [16].

Since its discovery, RMD has, in a few rare cases, been associated with myasthenia gravis (MG). Carl Ansevin's work was the first to characterize RMD as a heterogenetic, autoimmune neuromuscular disease. Here antibodies that would normally target foreign invaders of the body now direct their attack against components of the muscle cell. In this case, the rippling muscle symptom is a result of an autoimmune response and both disease symptoms decrease with immunosuppressive therapies aimed at MG. Ansevin and associates studied a

patent who presented with rippling muscle symptoms. He had nine siblings, all of which never presented symptoms of RMD, nor did his parents or other relatives. Months after he first exhibited symptoms, the patent was diagnosed with Myasthenia Gravis, an autoimmune neuromuscular disease. He was given immunosuppressive treatments for the myasthenia gravis and found that the symptoms of both MG and RMD dissipated [1].

In 1999, Muller-Felber et al documented two more patients who presented with the autoimmune form of RMD. Within each of these case studies both patients had an activation of an immune response (i.e. bacterial infection or an intrinsic bronchial asthma) from five months to two years before the onset of RMD symptoms, which was then followed by diagnosis of MG. Neither patient had an observable increase in acetocholine receptor antibodies, a primary indication of MG, at the time of RMD diagnosis; however they did display an elevation of skeletal muscle antibody titers. Following the diagnosis of MG, each was treated with immunosuppressive therapy that conclusively terminated the rippling symptom [17].

Another study completed in 1999 by Walker et al. set out to analyze the sera of patients diagnosed with both RMD and MG to determine which elements of a skeletal muscle myocyte may be targeted by autoantibodies. They found autoantibodies from these patients had an affinity for larger skeletal muscle polypeptides. Due to the lack of MUAPs of the rippling phenomenon and the fact that the larger proteins within skeletal muscle cells are targeted, it was suggested that there is an induction of mechanosensitivity of calcium channels. Two calcium channels, the ryanodine receptor and the dihydropyridine receptor, fit the findings and were thus proposed to have some involvement in the mechano-sensitive nature of the rippling muscle symptom [26].

In 2006, Watkins et al. continued this investigation by examining autoimmune rippling muscle disease (ARMD) patient sera to establish which large polypeptides may play a role in the

disease. Their findings identified five proteins: titin Isoform N2A, ATP synthase 6, protein phosphatase 1 regulatory subunit 1 (PPP1R3), enolase and aldolase (**Table 1**). The final two proteins, having nothing to do with muscle contraction, were disregarded. Of the ten immunoreactive clones highlighting the five mentioned proteins, six were reactant with the titin isoform N2A, which is an exceptionally large sarcomeric protein [27]. This lead them to believe it was this protein, that has functionally important roles in structure of the sarcomere and muscle function, that may be the key to the rippling phenomena.

Possible autoantigen	Sequence Gen Bank Id. #	# of clones with identity	Immunoreactive clones		
PPP1R3	NM005398	1	pRMMG-1		
Titin N2-A	NP596869.1	6	pRMMG-5,-6,-8, -9,-11	pMG10-1	
ATP synthase	NP776050	1	pRMMG-4		
Enolase	NP001967.1	1		pMG10-2	
Aldolase	CAA30979.1	1			pSNMG-15

Clones are identified by the patient's sera used to identify the immunoreactive peptides produced by that cDNA clone. (RMMG, rippling muscle myasthenia gravis patient; MG, myasthenia gravis patient; SNMG, seronegative myasthenia gravis patient.)

Table 1. List of proteins subject to autoantibody attack within RMD / MG diagnosed patients and their respective immunoreactive clones. Watkins, Thomas C., et al. Journal of Cellular Biology 99 2006 (79-87)

Further bioinformatic analysis of the titin immunoreactive clones illustrated the location of each on the titin polypeptide. **Figure 3** shows two distinct immunogenic regions of titin that possess sensitivity to autoantibodies of ARMD. The first localization corresponded to the main immunogenic region (MIR). The titin MIR is located near the transition from A-band to I-band, and has been found to be the site of a majority of autoantibody attack in patients possessing myasthenia gravis with a thymoma [11]. The second region that was emphasized is located

within the A-band, near the M-line, where titin is closely associated with myosin. The interest in this region and the pRMMG6 sequence is due to its seemingly uniqueness to ARMD.

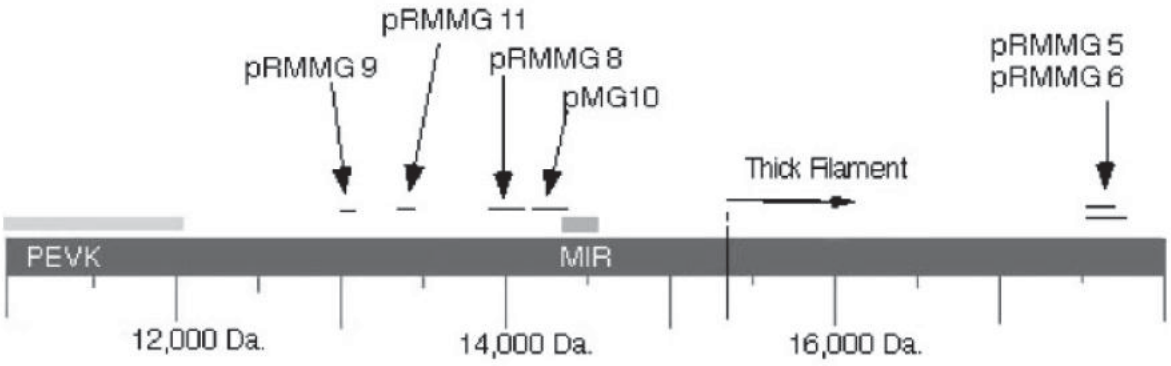


Figure 1. Two distinct immunogenic domains within the A-band and I-band regions of titin. Six clones within the study completed by Watkins show identification with the titin protein. Four recognize a region mapped to the I band while 2 map to a position within the A-band region Watkins, Thomas C., et al. Journal of Cellular Biology 99 2006 (79-87)

Characterization of the pRMMG6 clone was reported in a study completed by Zelinka et al. The immunogenic titin domain was incorporated into a PGEX-3X plasmid that resultantly produced a fusion protein linking the pRMMG6 protein product to glutathione S-transferase. The titin portion of the constructed fusion plasmid was confirmed to retain its immunoreactivity. The cDNA sequence (GenBank ID: EU 428784) identifies with the N2-A isoform (GenBank ID: NM 133378). The majority of the pRMMG6 clone sequence was found to span the exons 248 (90 % coverage), 249 (100 % coverage) and a portion of 250 (23.4 % coverage) and translate to an amino acid sequence that makes up two fibronectin 3 (FN3) domains and part of an adjacent immunoglobulin (Ig) domain. BLAST analysis also confirmed a 93.9 % homology to the mouse N2-A isoform. Additional bioinformatic analysis amended the previous location of the

immunogenic titin domain to, in fact, reside within the I-band region close to the I/A-band junction [29].

1.2 – Titin

Titin is the third most abundant protein found in vertebrate striated muscle and the largest known single polypeptide to date. It is made up of has a molecular weight of 3000 kDa and approximately 20,000 amino acid0 residues. It spans one half of the sarcomere with its amino-terminal domain anchored within the Z-disc, and its carboxy-terminal domain contained by the M-line [9]. **Figure 2** [7, 15] shows the orientation of the titin protein in the sarcomere. The Z-disc region of titin contains binding sites for α-actin, and T-cap/telethonin. I band titin incorporates repeated immunoglobulin (Ig) -like domains surrounding a PEVK region. The PEVK region contains a large number of Proline (P), Glutamine (E), Valine (V), and Lysine (K) amino acid residues. The variation in length of the PEVK region contributes to the multiple isoforms of titin. The cardiac isoforms N2B and N2BA tend to have a shorter PEVK region than that of the skeletal muscle isoform N2A. Throughout the A band, titin forms numerous interactions with myosin, giving this section a structurally stiff quality. It is made up of two separate repeat patterns of Ig-like domains and fibronectin type 3 (FN3) domains. Near its carboxy-terminal domain in the M-line, titin contains a kinase domain [15].

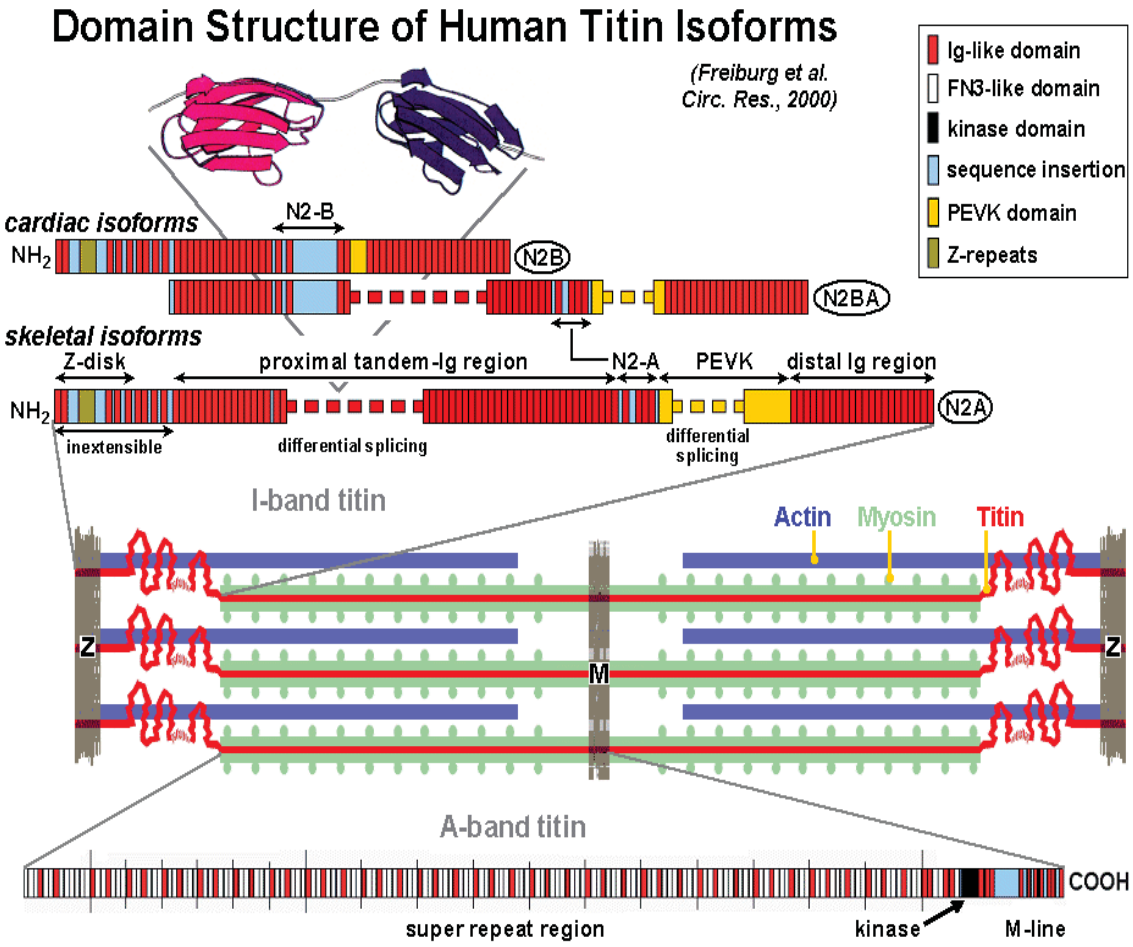


Figure 2. [7, 15] Titin within the sarcomere. Length-wise, two titin molecules span the sarcomere in a mirror like fashion (shown in red). Each protein has a carboxy-terminus located in the center m-line, and an amino-terminus located in its respective z-disc that borders the sarcomere.

The titin protein has been found to have a number of vital roles in the cell, from initial production during cell differentiation throughout the life of the adult skeletal muscle cell [14, 23, 25]. During development of muscle cells, it has been shown to play a vital role in the organization of other sarcomeric proteins [10]. Isaacs et al. revealed that titin production is amplified greater than four-fold within the first week of cell differentiation. After molecules are completely synthesized it immediately forms attachments to developing myotubes, giving it

stability in the developing cell [14]. In mature muscle cells it plays an essential role in the elasticity of the muscle cell [23].

1.3 – Titin during Differentiation

There is a common sequence of expression and organization of titin throughout the differentiation process. First, titin is synthesized as a single poly peptide into dot-like aggregates before the start of differentiation and it immediately begins to form associations with the cytoskeleton [25]. Biosynthetic studies have shown the approximated time required for synthesis of one titin protein is 1 to 1.5 hours [14]. As the differentiation process continues with cell elongation and fusion, sarcomeres begin to organize, and titin aggregates become arranged along and form attachments to stress fiber-like structures [24, 25]. This alliance creates an additional platform for other sarcomeric proteins, such as thick filaments, to form attachments and further construct the sarcomere. It has also been shown that the order of titin epitopes that reaches their final positions within the sarcomere begins by anchoring in its amino-terminal end in the Z-disk, progresses along the length of the protein, and ends with the carboxyl-terminal end affixing to the M-line. It is this unfolding process that must be fully completed before titin can provide proper measurement of the sarcomere and establish myosin filament length and position [24].

1.4 – Specific Aim of Research

Previous studies have shown that titin is responsible for many functions within developing myoblasts and adult skeletal muscle cells. Despite the volume of knowledge

of this protein many functions are still unknown, particularly those that may be related to the autoimmune form of RMD (ARMD). Therefore, the aim of this study is to over-express a specific domain of the human titin protein within differentiating C2C12 myoblast cells and observe localization of this domain, along with changes in morphology and development. A second objective is to develop methods that will aid in the continued research of this domain along with others found within the titin protein.

1.5 – Research Goals

The aim of this study is to identify potential roles of the immunogenic titin domain and the effects of over expression. The research goals are as followed:

1. To construct a fusion plasmid that will produce the immunogenic titin domain attached to a green fluorescent protein in frame.

2. To transfect C2C12 myoblasts with the fusion plasmid and track the immunogenic titin domain localization.

3. Observe changes in cell development and differentiation due to over expression of the titin domain.

4. Observe changes in protein expression due to over-expression.

5. Quantitatively track specific domain production in C2C12 myoblasts in vivo during differentiation.

1.6 – Hypothesis

If the RMMG6 domain is over-expressed in developing C2C12 myoblasts then one should observe altered development of myotubes during myogenesis.

Chapter 2: Methods

All materials used within this study are listed in Appendix 1, along with the manufacturer's information.

2.1 – Bacterial and Mammalian Cultures

Bacterial clone pG3RMMG#6 contains a pGEX-3X vector that incorporates the immunogenic titin domain RMMG#6. It was cultivated using Luria-Bertani broth supplemented with Ampicillin (LB_{AMP} broth) (Appendix 2.5) and maintained on Luria-Bertani agar plates supplemented with Ampicillin (LB_{AMP} agar) (Appendix 2.8). pAcGFP1-C1 is a mammalian plasmid type that is propagated in *E. coli*. It possesses a gene that produces a green fluorescent protein (GFP), bacterial resistance to kanamycin, and ability for selection in mammalian cell cultures using neomycin. It was used as the fusion vector. pAcGFP1-C1 cell cultures were cultivated using Luria-Bertani broth supplemented with Kanamycin (LB_{KAN} broth) (Appendix 2.4) and maintained on Luria-Bertani agar plates supplemented with Kanamycin (LB_{KAN} agar) (Appendix 2.7). One Shot® TOP10 Chemically Competent *Escherichia coli* (Invitrogen) cells were used for bacterial cell transformation (genotype: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G). C2C12 *Mus* musculus myoblast cells were used for all mammalian cell transfections.

2.2 – Production of Chemically Competent Cells

3 ml Luria-Bertani (LB) (Appendix 2.3) was inoculated with a One Shot® TOP10 *E. coli* culture and incubated for 18 hours at 37 °C with agitation. That culture was then

added to 250 ml LB broth and again incubated at 37 °C with agitation to an O.D. (600 nm) ≤ 1.0 and immediately cooled on ice. The cells were centrifuged at 5000 x g for 5 minutes at 4 °C using a Sorvall® RC 5B PLUS Centrifuge and Sorvall SA-600 rotor. The supernatant was removed and the cells were re-suspended with 15 ml sterile 0.15 M NaCl (Appendix 2.9). Again the cells were pelleted in the same manner as before and the supernatant was removed. The cells were re-suspended in sterile transformation buffer (Appendix 2.10) and divided into 1.5 ml screw cap microcentrifuge tubes. Culture tubes were incubated on ice and at 4 °C for 20 hours then transferred to and stored at -80 °C until required.

2.3 – Bioinformatic Analysis of pAcGFP1-C1 and pG3RMMG#6

The pAcGFP1-C1 plasmid sequence was imported into the Restriction Mapper program. The output was analyzed to determine which enzyme would cut the plasmid only once to linearize it. Next, BVTech Plasmid: Plasmid Drawing Software for Windows was used to construct the theoretical fusion plasmid RMMG#6/pAcGFP1-C1. This theoretical plasmid sequence was again imported into the Restriction Mapper program to determine which enzyme would cut out the RMMG#6 sequence in such a way, only if the insert was incorporated in the proper orientation.

2.4 – Bacterial Plasmid Preparations

3 ml Luria-Bertani broth supplemented with Ampicillin (LB_{AMP} broth) (Appendix 2.5) and 3 ml Luria-Bertani broth supplemented with Kanamycin (LB_{KAN} broth) (Appendix 2.4) were inoculated with pG3RMMG#6 and pAcGFP1-C1 cultures

respectively, and incubated at 37 °C with agitation for 8 hours. Each 3 ml starter culture was diluted to 500 ml LB_{AMP} or LB_{KAN} broth and incubated again at 37 °C with agitation for 12 - 16 hours. Plasmid DNA was purified using the QIAGEN Plasmid Mini Kit, following the Maxi protocol from the manufacturer [18]. Purified DNA was re-suspended in 1 ml nuclease free water heated to 70 °C. DNA was quantified using the BioRad SmartSpec™ Plus spectrophotometer at absorbencies of 260 nm and 280 nm and stored at -20 °C.

2.4.1 – RMMG#6 DNA Preparations

An *EcoR*1 restriction enzyme digestion was performed on the purified RMMG#6 sample to remove the immunogenic titin domain from the pGEX-3X plasmid. The reaction mixture contained 600 ng of purified DNA, 4 µl *EcoR*I buffer (New England BioLabs) and 1 µl *EcoR*I enzyme (New England BioLabs) in a total volume of 40 µl. A control reaction was assembled with the same components, minus the enzyme. The mixtures were vortexed at a low speed and incubated at 37 °C for 1 hour. Reaction mixtures were heated to 65 °C for 30 minutes to heat inactivate the enzyme.

2.4.2 – pAcGFP1-C1 DNA Preparation

An *EcoR*I restriction enzyme digestion was completed on the pAcGFP1-C1 plasmid to achieve linearization. The reaction mixture contained 600 ng of purified DNA, 4 µl *EcoR*I Reaction buffer (10x) and 1 µl *EcoR*I enzyme in a total volume of 40 µl. Similarly, a second reaction was set up without the enzyme and was considered the

control. The mixture was incubated at 37 °C for 1 hour. Enzyme activity was inactivated by incubation at 65 °C for 30 minutes.

The digested DNA was treated with Calf Intestinal Phosphatase (CIP) to remove the 5' phosphate groups to prevent self-ligation. The reaction mixture included 20 µl previously restriction endonuclease treated pAcGFP1-C1 plasmid DNA, 3 µl 10X CIP buffer (New England BioLabs), 1 µl CIP (New England BioLabs), and 26 µl nuclease free water in a total volume of 50 µl. The mixture was incubated at 37 °C for 30 minutes.

2.4.3 – Electrophoretic Analysis of *EcoR*1 Digests

*EcoR*1 digested and undigested reaction products of both plasmids, pG3RMMG#6 and pAcGFP1-C1, were analyzed by agarose gel electrophoresis. Samples were prepared using, 10 µl digested or undigested plasmid DNA and 2 µl Amresco EZ-Vision Three DNA Dye and Buffer 6x. Each sample was loaded on a 2% agarose gel (Appendix 2.12), and run at 120 volts. Bands were visualized and photographed using a Stratgene EagleEye™ II camera and Stratgene EagleSight Software (Version 3.22).

2.5 – RMMG#6/pAcGFP-C1 Ligation and Transformation

Three separate ligation reactions were prepared to achieve an optimal number of clones. The first reaction mixture contained 4 µl pAcGFP1-C1 plasmid DNA (~24 ng), 4 µl RMMG#6 DNA (~128 ng), 1 µl 10x T4 DNA Ligase buffer (New England BioLabs), and 1 µl T4 DNA Ligase (New England BioLabs) (1:5.3 ratio of vector to insert). The second mix used 3 µl pAcGFP1-C1 (~18 ng) and 5 µl RMMG#6 (~160 ng), with similar volumes of buffer and ligase (1:8.9 ratio of vector to insert). The third reaction contained

2 µl pAcGFP1-C1 (~12 ng) and 6 µl RMMG#6 (~192 ng), with similar volumes of buffer and ligase (1:16 ratio of vector to insert). Each reaction mixture contained a total volume of 10 µl and was incubated for approximately 15 hours at 4 °C.

Each ligation mixture, along with the purified pAcGFP1-c1 plasmid, was used to transform previously made chemically competent One Shot® TOP10 *E. coli* cells. 2 µl of each ligation mix was added to 100 µl of cells and incubated on ice for 30 minutes. Cells were then transferred to a 42 °C water bath for 1 minute, followed by immediate incubation on ice for an additional 5 minutes. 900 µl LB broth (Appendix 2.3) was added to each tube and incubated with agitation at 37 °C for 1.5 hours. 50 µl and 200 µl of each transformation were plated on LB_{Kan} agar plates (Appendix 2.7) and incubated at 37 °C for 18 - 20 hours.

2.6 – Clone Screening

2.6.1 – Clone Screening Part 1: Pre-Screening

Colonies were spotted in a grid like manner on LB_{Kan} agar plates (Appendix 2.7) and incubated for another 18 - 20 hours at 37 °C. Due to the substantial number of clones in each transformation (184 total colonies), 19 test tubes, each containing 5 ml LB_{KAN} broth (Appendix 2.4), were inoculated with 10 clones apiece and incubated for 18 - 20 hours at 37 °C with agitation. **Table 2** lists each pre-screened sample, along with the corresponding clones used to inoculate each. Plasmid DNA was purified using the QIAGEN QIAprep® Spin Miniprep Kit, following the manufacturer's protocol [19] and eluted off the spin column using 60 µl nuclease free water, heated to 70 °C.

Table 2. List of pre-screened cultures and the clones incorporated within them.

Culture Number (Transformation Letter, sequential number)	Clones within Each Culture
E1	3, 4, 5, 6, 7, 8, 9, 10, 11, 12
E2	15, 16, 17, 18, 19, 20, 21, 22, 23, 24
E3	25, 26, 27, 28, 29, 30, 31, 32, 33, 34
E4	35, 36, 38, 39, 40, 41, 42, 43, 44, 45
E5	46, 47, 50, 51, 52, 53, 54, 55, 56, 57
E6	58, 59, 62, 63, 64, 65, 66, 67, 68, 69
E7	70, 71, 73, 74, 75, 76, 77, 78, 79, 80
E8	81, 82, 83, 84, 85, 86, 87, 88, 89, 90
E9	91, 92, 93, 94
F1	3, 4, 5, 6, 7, 8, 9, 10, 11, 12
F2	13, 14, 15, 16, 17, 18, 19, 20, 21, 22
F3	23, 24, 25, 26, 27, 28, 29, 30, 31, 32
F4	33, 34, 35, 36, 38, 39, 40, 41, 42, 43
F5	44, 45, 46, 47, 50, 51, 52, 53, 54, 55
G1	3, 4, 5, 6, 7, 8, 9, 10, 11, 12
G2	13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23
G3	24, 25, 26, 27, 28, 29, 30, 31, 32, 33
G4	34, 35, 36, 38, 39, 40, 41, 42, 43, 44
G5	45, 46, 47

Samples were digested with an *EcoR*1 restriction enzyme (300 ng Plasmid DNA, 2 µl 10 x *EcoR*1 Reaction Buffer, 0.5 µl *EcoR*1, and nuclease free water to a total volume of 20 µl) for 1 hour at 37 °C. A control reaction was set up for each sample in a similar way, except the *EcoR*1 enzyme was not added. After incubation, 3.5 µl Amresco EZ-Vision Three DNA Dye and Buffer 6x was added to each digest and control reaction. 12 µl of each digested sample, along with its respective control were loaded onto a 2 % agarose gel, along with a 100 bp molecular weight DNA ladder. Agarose gels were analyzed using the Stratagene EagleEye system.

2.6.2 – Clone Screening Part 2: *Eco*R1 Digestion

Two pre-screening cultures from Transformation F, F4 and F5, and two from Transformation G, G2 and G4, were selected for individual colony screening. Each clone was incubated for 18 - 20 hours in 3 ml LB_{Kan} broth at 37 °C with agitation. Plasmid DNA was purified using the QIAGEN QIAprep® Spin Miniprep Kit, following the manufacturer's protocol (QIAprep® Miniprep handbook, p22-23) and eluted off the spin column using 60 µl nuclease free water heated to 70 °C. Purified DNA samples were subjected to an *EcoR*I enzymatic digestion. Digestion and control reactions were, again, set up as previously described (Digest reactions: 300 ng Plasmid DNA, 2 µl 10 x *EcoR*1 Reaction Buffer, 0.5 µl *EcoR*1, and nuclease free water to a total volume of 20 µl; Control reactions: 300 ng Plasmid DNA, 2 µl 10 x *EcoR*1 Reaction Buffer, and nuclease free water to a total volume of 20 µl). Samples were incubated at 37 °C for 1 hour followed by the addition of 3.5 µl Amresco EZ-Vision Three DNA Dye and Buffer 6x. 12 µl of each sample were loaded on a 2 % agarose gel and analyzed using EagleEye system.

2.6.3 – Clone Screening Part 3: *Hind*III Digestion

Clones that exhibited a band at approximately 646 bp were then treated with a *Hind*III restriction endonuclease to determine if the titin fragment was inserted in the proper orientation. For each clone, digest reactions were set up using 300 ng plasmid DNA, 2 µl Buffer 2 (New England BioLabs), 0.5 µl *Hind*III (New England BioLabs), and nuclease free water in a total volume of 20 µl. A control reaction was also set up in the

same way except without the enzyme. Digestion samples and their controls were incubated at 37 °C for 1 hour followed by the addition of 3.5 µl Amresco EZ-Vision Three DNA Dye and Buffer 6x. Again, 12 µl of each sample were loaded on to a 2 % agarose gel and analyzed using the EagleEye system.

2.6.4 – RMMG#6 Insert DNA Sequencing

All clones positive for insert were sequenced using the Beckman Coulter GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit. Sequencing reactions were set up and processed according to the manufacturer's protocol [2]. Reactions included approximately 164 ng purified plasmid DNA, 2 µl primer (1.6 pmol), 8 µl DTCS Quick Start Kit (provided with kit), and sterile nuclease free water in a total volume of 20 µl. Each reaction was first processed in a BioRad iCycler thermal cycler according to the program shown in **Table 3**. The products were analyzed using the Beckman-Coulter CEQ^{TM} 2000XL automated sequencer.

The following primers were used for all clone sequencing reactions:

Forward (5' end) Primer: 5' - ACT TCG GCT TCG TGA CCG CC - 3'

Reverse (3' end) Primer: 5' - TCA GGG GGA GGT GTG GGA GG - 3'

Table 3. Thermo Cycler Program for the Amplification Step of the RMMG6 Sequencing Process.

Cycle Number	Number of Repeats	Temperature	Time
Cycle 1	1	96 °C	2 min
Cycle 2	35	96 °C 57 °C 60 °C	20 sec 20 sec 20 sec
Cycle 3	1	4 °C	∞

Sequences were evaluated using Genious software and BLASTn analysis. Sequences were also aligned with the known RMMG#6 sequence (EU428784). The three best sequences were selected and sequenced again in triplicate. Consensus sequences were constructed using the Genious software and analyzed using BLASTn analysis.

2.7 – Mammalian Cell Transfection

2.7.1 – C2C12 Culture Initiation

Stock C2C12 mouse muscle myoblast cultures, stored in liquid nitrogen, were initiated by thawing quickly in a 37 °C water bath. Next, they were transferred to a 50 ml sterile conical tube where 2 ml C2C12 Initial Growth Media (C2IGM) (Appendix 2.13), heated to 37 °C, were added. The culture was incubated at room temperature for 2 minutes. Again, 2 ml C2IGM was added to the culture and incubated at room temperature for 2 minutes. The total volume of the tube was then transferred to a 75 cm^2

Corning flask and another 15 ml C2IGM was added. The culture was incubated at 37 °C and 5 % CO_2 for 24 hours. Cultures were viewed using an Olympus LH 50A inverted phase contrast microscope to confirm cell attachment to the flask wall. Every 3 days following the culture inoculation, media was removed and 20 ml C2C12 Growth Media (C2GM) (Appendix 2.14) was added. Cultures continued to be incubated at 37 °C and 5 % CO_2.

2.7.2 – Kill Curve of Untransfected C2C12 Cultures

When each culture reached 80 – 90 % confluence, it was washed with 20 ml DMEM and trypsinized with 9 ml 1x trypsin (Gamma Irradiated 0.25% Porcine Trypsin (1:250) in HBSS with 0.1% EDTA-2Na, without Ca and Mg). The flask was incubated for 5 minutes at 37 °C and 5 % CO_2. 9 ml C2GM was added to the culture flask and the cell suspension was transferred to a 50 ml conical tube. 0.75 ml of the cell suspension was pipetted in to wells 1 – 12 of a 24 well cell culture plate (well surface area of 2 cm^2), as shown in **Table 4**. 0.75 ml C2GM was added to each well and the plate was incubated at 37 °C and 5 % CO_2 for 24 hours.

Every day, each well was photographed at 40 x and 100 x magnification using an Olympus LH 50A inverted phase contrast microscope and SPOT idea™ 5mp Color Mosaic camera. Every second day, media was removed by pipetting and the addition of media in each well was as followed: 1.5 ml C2GM was added to wells 1 through 4. 1.5 ml C2GM + 0.5 mg/ml Neomycin (Appendix 2.16) was added to well 5; 1.5 ml C2GM + 1.0 mg/ml Neomycin (Appendix 2.17) was added to well 6; 1.5 ml C2GM + 1.5 mg/ml

Neomycin (Appendix 2.18) was added to well 7; 1.5 ml C2GM + 2.0 mg/ml Neomycin (Appendix 2.19) was added to well 8; 1.5 ml C2GM + 2.5 mg/ml Neomycin (Appendix 2.20) was added to well 9; 1.5 ml C2GM + 3.0 mg/ml Neomycin (Appendix 2.21) was added to well 10; 1.5 ml C2GM + 3.5 mg/ml Neomycin (Appendix 2.22) was added to well 11; 1.5 ml C2GM + 4.0 mg/ml Neomycin (Appendix 2.23) was added to well 12, as shown in **Table 4**. The plate was then incubated at 37 °C and 5.0 % CO_2 for another 48 hours and the process was continued for a total of 8 days. Cell counts were taken for each well and graphically analyzed using Microsoft Excel (Version 14.0.6112.5000).

Table 4. Cell culture set up with initial cell culture volume and Neomycin supplemented media concentrations.

1 Initial cell culture volume = 0.75 µl [Neomycin] = 0 mg/ml	5 Initial cell culture volume = 0.75 µl [Neomycin] = 0.5mg/ml	9 Initial cell culture volume = 0.75 µl [Neomycin] = 2.5mg/ml
2 Initial cell culture volume = 0.75 µl [Neomycin] = 0mg/ml	6 Initial cell culture volume = 0.75 µl [Neomycin] = 1.0mg/ml	10 Initial cell culture volume = 0.75 µl [Neomycin] = 3.0mg/ml
3 Initial cell culture volume = 0.75 µl [Neomycin] = 0mg/ml	7 Initial cell culture volume = 0.75 µl [Neomycin] = 1.5mg/ml	11 Initial cell culture volume = 0.75 µl [Neomycin] = 3.5mg/ml
4 Initial cell culture volume = 0.75 µl [Neomycin] = 0mg/ml	8 Initial cell culture volume = 0.75 µl [Neomycin] = 2.0mg/ml	12 Initial cell culture volume = 0.75 µl [Neomycin] = 4.0mg/ml

2.7.3 – Kill Curve of Transfected C2C12 Cultures

A second kill curve was set up to determine which method of selection, using a range of neomycin, was most effective on transfected C2C12 cell cultures. The second kill curve was set up using 1 ml cell culture volumes portioned out into eight 60 mm^2 culture dishes with sterile microscope slide covers in the bottom of each dish. Cultures were incubated for 24 hours at 37 °C and 5 % CO_2. The ClonTech Xfect Transfection

Reagent was used to transfect C2C12 cells using 7.5 µg clone G35 plasmid DNA following the manufacturers protocol [28]. At 4 hours post-transfection, media was removed by aspiration and 1 ml C2GM was added. Culture dishes were incubated for 20 hours at 37 °C and 5 % CO_2.

At 24 hours post-transfection, media was again removed by aspiration. 1 ml of media was added to each culture dish. The application of the type of media is shown in **Table 5** and is as follows: Control A used C2GM changed daily for 4 days followed by the use of C2C12 Differentiation Media (C2DM) (Appendix 2.15) changed every second day for 8 days; Control B used C2DM changed every other day for 8 days; Dishes 1 – 3 used C2GM supplemented with 2.5 mg/ml, 2.75 mg/ml (Appendix 2.24), or 3.0 mg/ml Neomycin, respectively, changed daily for 4 days, then C2DM supplemented with 2.5 mg/ml (Appendix 2.25), 2.75 mg/ml (Appendix 2.26), or 3.0 mg/ml (Appendix 2.27) Neomycin, respectively, that was changed every second day for 8 days; Dishes 4 – 6 used C2DM supplemented with 2.5 mg/ml, 2.75 mg/ml, or 3.0 mg/ml Neomycin, respectively, changed every other day for 8 days. Each culture was photographed directly before each media change using an Olympus LH 50A inverted phase contrast microscope and SPOT idea™ 5mp Color camera. Cell counts were taken for each well and graphically analyzed using Microsoft Excel.

Table 5. **Second Kill Curve Cell Culture Set Up and Media Treatments.**

Culture Dish Number	Type of media	Number of Days	Additional Media	Number of Days
Control A	C2GM + 2.75mg/ml Neomycin	Daily media change for 4 days	C2DM + 2.75mg/ml Neomycin	Every second day media change for 8 days
Control B	C2DM + 2.75mg/ml Neomycin	Every second day media change for 8 days	N/A	N/A
1	C2GM + 2.5mg/ml Neomycin	Daily media change for 4 days	C2DM + 2.5mg/ml Neomycin	Every second day media change for 8 days
2	C2GM + 2.75mg/ml Neomycin	Daily media change for 4 days	C2DM + 2.75mg/ml Neomycin	Every second day media change for 8 days
3	C2GM + 3.0mg/ml Neomycin	Daily media change for 4 days	C2DM + 3.0mg/ml Neomycin	Every second day media change for 8 days
4	C2DM 2.5mg/ml Neomycin	Every second day media change for 8 days	N/A	N/A
5	C2DM + 2.75mg/ml Neomycin	Every second day media change for 8 days	N/A	N/A
6	C2DM 3.0mg/ml Neomycin	Every second day media change for 8 days	N/A	N/A

When each culture completed the predetermined number of media changes, each sample was washed with 1.5 ml 1x Tris Buffered Saline (TBS) (Appendix 2.28). Before aspiration of the TBS, the microscope side cover was removed using sterile tweezers and placed cell side down. Each slide was observed using an Olympus IX 51 Inverted Microscope using both bright field and fluorescent settings. Pictures were taken using a SPOT RT KE camera.

The microscope slide covers were then put back in their respective culture dish and the TBS was removed. Next, 200 µl of 1x SDS Sample Buffer (Appendix 2.32) was pipetted over the bottom of the culture dish to dissolve the cells. The liquid was then transferred to a microcentrifuge tube and centrifuged at 10,000 x g for 10 minutes at room temperature. Each sample was then heated to 80 - 100 °C for 3 minutes and stored at -20 °C. All samples were later run on a 10 % SDS-PAGE gel (Appendix 2.37) with a 6 % stacking gel (Appendix 2.38) and constant amperage of 20 mA. The gels were analyzed using a BioRad Pharos FX™ Plus Molecular Imager and BioRad Quantity One 1-D Analysis Software (Version 4.6.9).

2.7.4 – Overexpression of the Immunogenic Domain of Titin, RMMG#6, on C2C12 Cells

Primary C2C12 cultures were initiated in the same manner as previously described. The transfections of 28 culture dishes were completed using the ClonTech Xfect Transfection Reagent following the manufacture's protocol. 7.5 µg purified clone G35 plasmid DNA were used to transfect 14 culture dishes in the experimental group. 7.5 µg purified pAcGFP1-C1 plasmid DNA were used to transfect each of 14 dishes in the GPF control group. None of the dishes in the negative control group were transfected.

2.7.4.1 – Protein and Fluorescent Microscopy Sample Preparation

24 hours post transfection, media was removed by aspiration from the experimental group and GFP group culture dishes. 1 ml C2DM + 2.5 µg/ml Neomycin

was added to 12 culture dishes and then continued incubation at 37 °C and 5 % CO_2. The two cultures not subjected to media changes were either fixed for later use in fluorescent microscopy or used to obtain protein. To fix samples, media was removed and the cells were washed with 1 ml TBS. 1 ml 3.7 % formaldehyde (Appendix 2.29) was added to the dish and incubated at room temperature for 5 minutes. After removal of the formaldehyde, 1 ml TBS was added, the dish was sealed with parafilm and stored at 4 °C for later use. Protein samples were collected in the same manner as previously described (Methods: 7.3). This process was repeated every second day for 13 days. The negative control group was handled in a similar way except C2DM with no antibiotic selector supplementation was used.

2.7.4.2 – Protein and Fluorescent Microscopy Sample Analysis

20 µl of each protein sample were loaded on a 10 % SDS-PAGE gel (Appendix 2.37) with a 6 % stacking gel (Appendix 2.38). Gels were run at constant amperage of 20 mA. The gels were analyzed using a BioRad Pharos FX™ Plus Molecular Imager and BioRad Quantity One 1-D Analysis Software.

Fluorescent microscopy slides were prepared using 30 µl of buffered glycerol with anti-fade solution (Appendix 2.31) placed on a microscope slide. Previously fixed cell culture microscope slide covers were removed from each culture dish and placed cell side down on the microscope slide. Each slide was viewed first using a bright field profile setting and Nomarski/ DIC/ Hoffman image type within the SPOT Basic program. Slides were then observed using the Calcofluor profile and fluorescent image type.

2.8 - qPCR Quantitation of Endogenous RMMG#6 mRNA

C2C12 cultures were initiated in the same manner as previously described. The primary culture was passaged into four 25 cm^2 Corning Flasks and incubation was continued until cells were approximately 80 – 90 % confluent. C2GM was removed and C2DM was added to three of the 25 cm^2 Corning Flasks.

2.8.1 – Genomic DNA Purification

Genomic DNA was purified only from the C2C12 culture T = 12 days post differentiation initiation. A small scrape of cells was removed from the cell culture flask using a sterile plastic loop and transferred to a sterile microcentrifuge tube with 200 µl PBS and 20 µl proteinase K. 200 µl Buffer AL without added ethanol was added to the tube. The sample was mix thoroughly by vortexing and incubated at 56°C for 10 minutes. Next, 200 µl 100% ethanol was combined with the sample and again, mix thoroughly by vortexing. The mixture was then pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuge at 6000 x g for 1 minute. The flow through was discarded. The spin column was then placed in a new 2 ml collection tube and 500 µl Buffer AW1 was added. The solution was centrifuged for 1 minute at 6000 x g and again the flow through was discarded. The spin column was placed in a new 2 ml collection tube. 500 µl Buffer AW2 was pipetted into the spin column and centrifuged for 3 minutes at 20,000 x g to dry the DNeasy membrane. The flow through was discarded. To elute the DNA off the spin column it was placed in a clean 1.5 ml microcentrifuge tube and 200 µl Buffer AE was pipetted directly onto the membrane and

incubated at room temperature for 1 minute. Next the sample was centrifuged for 1 minute at 6000 x g. DNA was quantified using the BioRad SmartSpec™ Plus spectrophotometer at absorbencies of 260 nm and 280 nm.

2.8.2 – RNA Extraction

RNA Extractions were performed at time points 0, 4, 8, and 12 days post initiation of differentiation. For each culture, media was removed, and 1 ml Ribosol™ RNA Extraction Reagent was added for every 10 cm^2 culture dish area. Ribosol was pipetted over the cell culture to dissolve the cells. The solution was transferred to a 15 ml sterile conical tube and incubated at room temperature for 5 - 10 minutes. 200 µl of chloroform was added for every 1 ml of extraction reagent added and shaken for 15 second, followed by incubation at room temperature for 3 minutes. Each sample was centrifuged at 1,200 x g for 15 minutes at 4 °C. The upper aqueous phase was extracted and equally distributed into 1.5 ml microcentrifuge tubes. 0.5 ml isopropanol was added for each 1 ml of the extraction reagent initially used. Samples were incubated at room temperature for 15 minutes, and then centrifuged at 12,000 x g for 10 minutes at 4 °C. The supernatant was removed, leaving the pellet to be washed 1 time with 75 % ethanol prepared with sterile, RNase free water. 1 ml of ethanol wash was added for every 1 ml extraction reagent initially used. Samples were then vortexed briefly and centrifuged at 7,500 x g for 5 minutes at 4 °C. The RNA pellet was resuspended in 50 µl sterile RNase free water for every 10 cm^2 plate volume. Samples were incubated for 10 minutes at 55 - 60 °C. RNA was quantified using the BioRad SmartSpec™ Plus spectrophotometer at absorbencies of 260 nm and 280 nm.

2.8.3 – DNase Treatment of RNA

Each sample was set up in a 0.5 ml RNase free microcentrifuge tube, on ice, and included 1 µg RNA sample, 1 µl 10x reaction buffer with $MgCl_2$, 1 µl DNase I, and DECP-treated water to a total volume for 10 1 µl. Samples were incubated at 37 °C for 30 minutes. DNase I activity was inactivated by the addition of 1 µl 50 mM EDTA solution and incubation at 65 °C for 10 minutes. Samples were stored at -80 °C.

2.8.4 – cDNA Synthesis

cDNA synthesis was completed using the BioRad iScript™ Select cDNA Synthesis Kit according to the manufacturers protocol for gene-specific primers. Each reaction was set up in a 0.2 ml PCR tube and included: 4 µl 5x iScript select reaction mix, 2 µl of each gene specific primer (mTITIN2 Forward and Reverse and; Actin 2 Forward and Reverse) (10 µM), 2 µl GSP enhancer solution, 1 µg RNA sample, 1 µl iScript reverse transcriptase, and nuclease-free water to a volume of 20 µl. Reactions were mixed gently and incubated at 42 °C for 60 minutes. iScript reverse transcriptase activity was inactivated by incubation at 85 °C for 5 minutes. cDNA products were stored at -20 °C until needed.

2.8.5 – Gradient Polymerase Chain Reaction (Gradient PCR)

Gradient PCR reactions were set up to determine the most effective annealing temperatures for the *titin* and *actin* primer sets. Reactions were assembled as followed: 12.5 µl GoTaq® Green Master Mix 2x, 2 µl forward primer (10 µM), 2 µl reverse primer

(10 µM), 2 µl genomic DNA, and 4 µl nuclease free water. The PCR reaction was completed in a BioRad iCycler using the program shown in **Table 6**.

Table 6. Gradient PCR of genomic DNA. Genomic DNA was extracted from C2C12 cells at T = 12 days post differentiation initiation. *Gradient temperatures are as followed (°C): 50, 50.8, 52.1, 54, 56.2, 58, 59.3, 60.

Cycle Number	Number of Repeats	Temperature	Time
Cycle 1	1	95 °C	5 min
Cycle 2	35	95 °C Gradient Temp* 72 °C	30 sec 30 sec 1 min
Cycle 3	1	72 °C	7 min

The following primers were used for all PCR reactions:

mTitin 2:

Forward (5' end) Primer: 5' -TGT CTT TGA CTT GGA ACC CTC CAA - 3'

Reverse (3' end) Primer: 5' - TCT CAG TAC CAA TGA GGC GGC TTT - 3'

Actin 2:

Forward (5' end) Primer: 5' - AAC GTG CCC ATC TAT GAG GGC TAT - 3'

Reverse (3' end) Primer: 5' - TTT GAT GTC GCG CAC AAT CCT ACG - 3'

2.8.6 – Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

An RT-PCR reaction was set up for each time point using cDNA produced from C2C12 RNA samples to detect genomic DNA contamination. Reactions mixtures were assembled in 0.2 ml thin wall PCR tubes on ice as followed: 2 µl cDNA, 2 µl forward primer, 2 µl reverse primer, 12.5 µl GoTaq Green Master Mix, and 4 µl nuclease free water. The RT-PCR reaction was completed in a BioRad iCycler using the program shown in **Table 7**. Samples were analyzed on a 1.5 % agarose gel.

Table 7. PCR program for RT-PCR of C2C12 cDNA at T = 0, 4, 8, and 12 days post initiation of differentiation.

Cycle Number	Number of Repeats	Temperature	Time
Cycle 1	1	95 °C	5 min
Cycle 2	35	95 °C 55 °C 72 °C	30 sec 30 sec 1 min
Cycle 3	1	72 °C	7 min

2.8.7 - Quantitative Polymerase Chain Reaction (qPCR)

qPCR was used to determine the levels of expression of the immunogenic domain during cellular differentiation using the BioRad iQ SYBR Green Supermix Kit. Reactions were set up in a 96 well plate and included: 12.5 µl iQ SYBR Green Supermix, 5 µl each of forward and reverse primers (concentrations ranging from 20, 10, 5, and 2 µM per reaction), 2.5 µl cDNA template and nuclease-free water to a total

volume of 25 µl. All reactions were processed in a BioRad iCycler5 using the program shown in **Table 8**. Actin expression levels were used to normalize the titin mRNA levels.

Table 8. BioRad iCycler qPCR Program.

Cycle Number	Number of Repeats	Temperature	Time	Temperature Change	End Temperature
Cycle1	1	95 °C	3 min	-	-
Cycle 2	40	95 °C 60 °C	10 sec 30 sec	-	-
Cycle 3	81	60 °C	1 min	0.5 °C	95 °C
Cycle 4	1	4 °C	∞	-	-

Chapter 3: Results

3.1 – Fusion Plasmid Construction

The first goal of this study was to construct a plasmid that contains the RMMG#6 immunogenic titin domain and would produce this protein attached to a monitor for expression and localization of that domain. The vector of choice was pAcGFP1-C1. Analysis of the vector sequence was completed using Restriction Mapper. The pAcGFP1-C1 vector (**Figure 3 A**) was shown to contain one *EcoR*1 restriction site, at position 1359. This digestion would result in linearization of the plasmid. The pG3RMMG#6 plasmid (**Figure 3 B**) was also shown to contain two *EcoR*1 restriction sites allowing the RMMG#6 sequence to be completely removed from the pGEX3-3X plasmid.

The theoretical construction of the fusion plasmid RMMG#6/pAcGFP1-C1 is shown in **Figure 4**. Analysis using Restriction Mapper determined there is a *Hind*III restriction site following the GFP sequence (at position 1352) and also at position 635 of 646 within the RMMG#6 fragment (or position 2006 within the fusion plasmid). Treatment of the fusion plasmid with a *Hind*III enzyme would cut the majority of the RMMG#6 fragment out of the fusion plasmid if it was inserted in the proper orientation, yielding a DNA sequence of approximately 635 nucleotides in length. However, if the RMMG#6 sequence was inserted in a reverse orientation a sequence of 17 nucleotides would be removed from the plasmid.

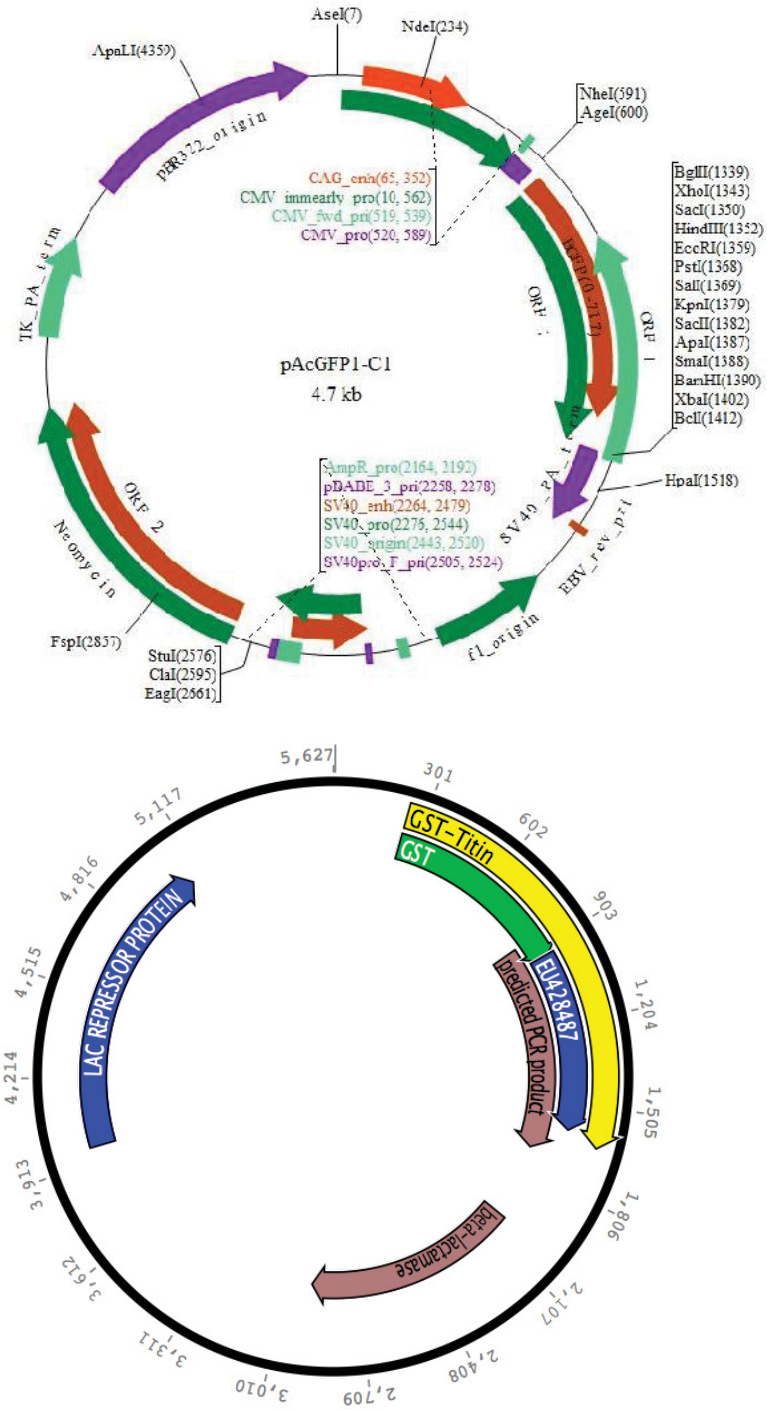


Figure 3. Map of Initial Plasmids. **A**. pAcGFP1-C1 plasmid incorporating a green fluorescent gene (613-1329), one *EcoR*1 restriction site (cuts before 1360), one *Hind*III restriction site (cuts before 1354), and a gene providing resistance to Kanamycin and Neomycin (2630-3418). **B**. pG3RMMG#6 incorporating the immunogenic titin domain RMMG#6 (945-1591), a GST gene (258-917), two *EcoR*1 restriction sites (cuts before 945 and 1591).

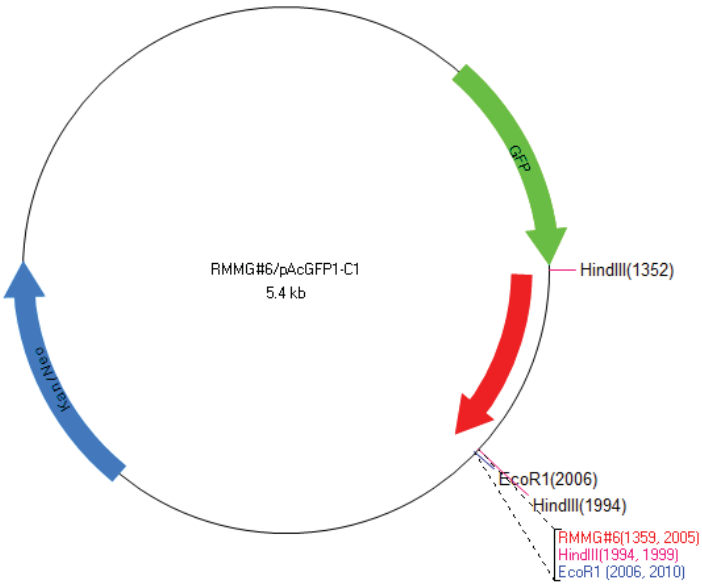


Figure 4. Theoretical construct of the RMMG#6/pAcGFP1-C1 fusion plasmid. Contains a GFP gene (613-1329), an inserted RMMG#6 sequence (1359-2005), two *Hind*III restriction sites (1352 and 1994), and two *EcoR*1 restriction sites (1359 and 2006).

3.1.1 – Bacterial Preparations

The construction process began with two *E. coli* cultures, each containing the pG3RMMG#6 or pAcGFP1-C1 plasmid. Plasmid DNA was purified and samples were analyzed by spectrophotometer at absorbances 260 nm and 280 nm. Purity was assessed by the 260/280 ratio and only samples within a range of 1.7 to 1.9 were used throughout the study.

An *EcoR*1 digestion was performed and analyzed using gel electrophoresis on a 2% agarose gel. The results are shown in **Figure 5**. Lanes 2 and 5 are the respective controls of the pG3RMMG#6 and pAcGFP1-C1 digestions. Multiple bands are seen in these lanes due to supercoiling of each plasmid. Lane 3 shows the digested pG3RMMG#6 plasmid and it contains two bands. The band above 3000 bp contains the linearized pGEX-3X plasmid while the band between the 600 and 700 bp region is the excised RMMG#6 immunogenic titin domain sequence with a length of approximately 646 bp. As a result of a single *EcoR*1 restriction site within the pAcGFP1-C1 plasmid, lane 4 contains only one band above 3000 bp.

Following further plasmid preparations, such as the treatment with CIP, three ligation reactions and transformations were completed. The increased number of ligation reactions was due to the multiple previous attempts, and failures, of the ligation and transformation process. Previously a 1:6 ratio of vector to insert was enforced, but due to a combination of factors these attempts failed. During the final attempt, each of the three reactions had a varied ratio of insert to vector. Ligation E had a ratio of 1:5.3, Ligation F had a ratio of 1:8.8 and Ligation G had a ratio of 1:16 vector to insert.

After 15 hours of incubation 2 µl of each ligation mixture were added to 100 µl aliquots of competent cells. A positive control that was comprised of One Shot TOP10 *E. coli* cells transformed with purified pAcGFP1-C1 plasmid, and a negative control of pure untransformed One Shot TOP10 *E. coli* culture, was also established. 50 µl and 200 µl volumes of each transformation and control were plated on LB_{KAN} agar plates and incubated for 16-18 hours at 37 °C. **Table 9** shows the total number of clones for each

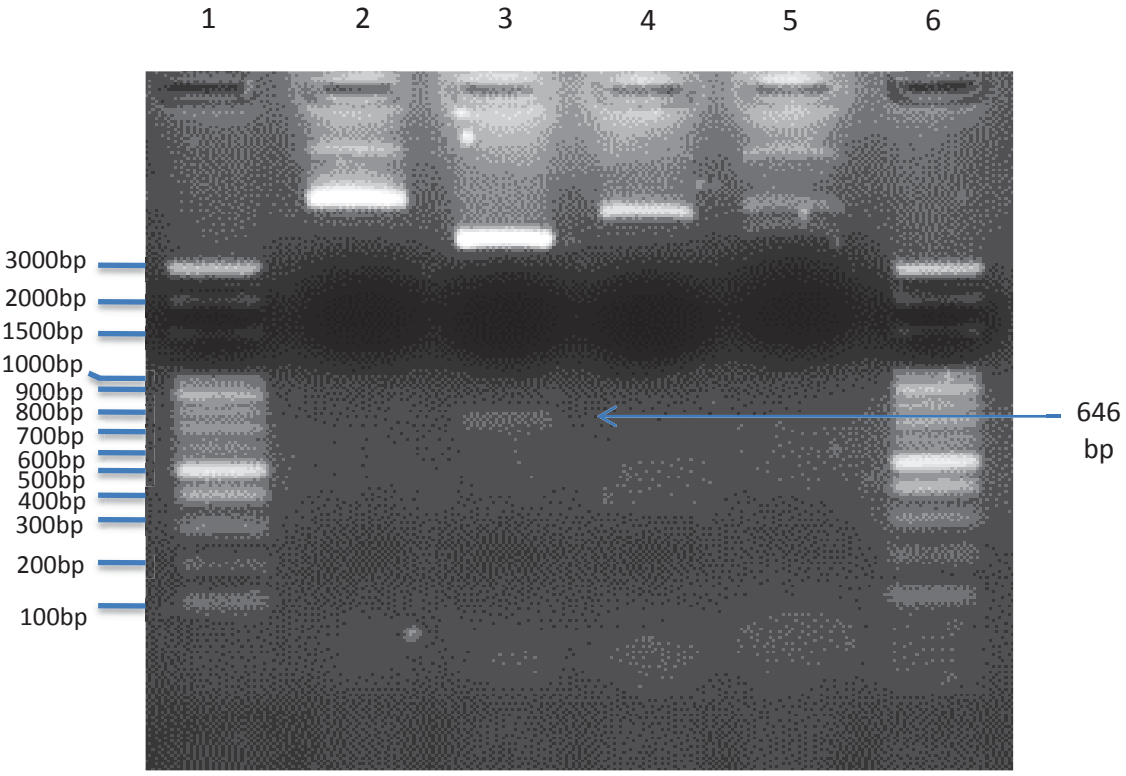


Figure 5. *EcoR*1 digested samples of RMMG#6 and pAcGFP1-C1. Lane 1 100 bp Molecular weight marker; Lane 2 RMMG#6 control; Lane 3 RMMG#6 digested; Lane 4 pAcGFP1-C1 digested; Lane 5 pAcGFP1-C1 control; Lane 5 Molecular weight marker (100 bp). 2% agarose gel. Band in Lane 3 at 646 bp is the immunogenic titin domain excised from the pGEX3 plasmid.

transformation and control group. The number of positive control clones confirms that the cells were competent to take up plasmid DNA, while the negative control group, having no viable clones, indicated that the competent cells alone do not possess a resistance to the antibiotic kanamycin.

Table 9. Number of clones resulting from each transformation.

Transformation Name	Number of clones
Transformation E	84
Transformation F	50
Transformation G	44
Positive Control	56
Negative Control	0

3.1.2 – Clone Screening

3.1.2.1 – Clone Screening Part 1: Pre-screening

Due to the large number of clones generated by the three transformations, a pre-screening method was employed. Groups of ten clones were used to inoculate LB_{KAN} broth and each culture was subjected to plasmid purification and *EcoR*1 digestion. Products of each digestion were analyzed on a 2 % agarose gel along with a respective control sample.

Figure 6 shows the analyzed gel of clone groups from Transformation E. An Amresco 100 bp molecular weight marker was used in lanes 1 and 12 in the top row and lanes 1 and 10 in the bottom row. None of the digested clone groups E1 through E5 (Top row: lanes 3, 5, 7, 9, and 11) or E6 through E9 (Bottom row: lanes 3, 5, 7, and 9) revealed

a band in the 600 - 700 bp region. The lack of a band within this region indicated these clones only contained a self-ligated pAcGFP1-C1 plasmid and therefor all 84 clones resulting from the Transformation E were discarded.

The analysis of clone groups F1, F2, F3, F4, and F5 from Transformation F are shown in **Figure 7**. An Amresco 100 bp molecular weight marker was used in lanes 1 and 12. Digested samples of each group of clones occupy lanes 3, 5, 7, 9, and 11. In each of these lanes a band within the 700 bp region can be seen. This confirmed that at least one in each group of ten clones possessed a vector that incorporated the immunogenic titin domain.

Analysis of Transformation G clone groups G1, G2, G3, G4, and G5 are shown in **Figure 8.** An Amresco 100 bp molecular weight marker was used in lanes 1 and 12. Each set of *EcoR*1 digested clones were loaded into lanes 3, 5, 7, 9, and 11. Lanes 5 and 9 each show a bright band at about 700 bp, revealing that at least one clone within each group was successfully transformed with an RMMG#6/pAcGFP1-C1 plasmid. Lanes 3 and 7 also show a band in this region, however, it is significantly fainter. There was no banding within this region in Lane 11 (group G5) and consequently the clones contained within that sample (#45, #46, and #47) were discarded.

3.1.2.2 – Clone Screening Part 2: *EcoR*1 Digestion

After elimination of close to half of the total number of clones due to pre-screening, individual colony screening was required. Clone groups F4 and F5 in Transformation F were selected for individual screening because they produced the

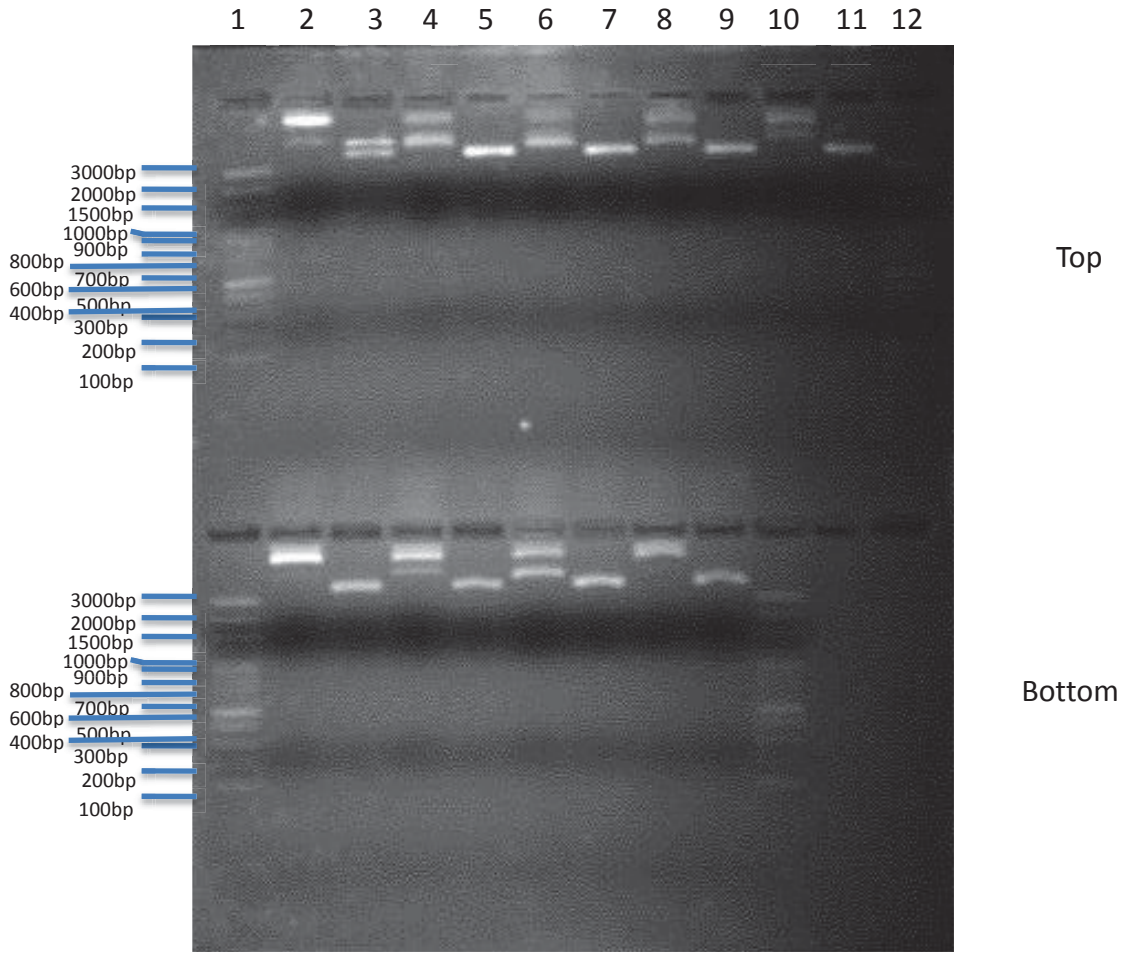


Figure 6. **Pre-screening of Transformation E of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid.** Each sample contained 10 clones to aid in quick elimination of multiple samples. Subjecting samples to an *EcoR*1 enzymatic digestion removes the RMMG#6 insert if it is present. Top Row: Lane 1 100bp molecular weight marker, Lane 2 E1 Control, Lane 3 E1 Digested, Lane 4 E2 Control, Lane 5 E2 Digested, Lane 6 E3 Control, Lane 7 E3 Digested, Lane 8 E4 Control, Lane 9 E4 Digested, Lane 10 E5 Control, Lane 11 E5 Digested, Lane 12 100bp molecular weight marker. Bottom Row: Lane 1 100bp molecular weight marker, Lane 2 E6 Control, Lane 3 E6 Digest, Lane 4 E7 Control, Lane 5 E7 Digest, Lane 6 E8 Control, Lane 7 E8 Digest, Lane 8 E9 Control, Lane 9 E9 Digest, Lane 10 100bp molecular weight marker. Banding at approximately 646 bp was not observed in any sample lanes revealing none of the clones contained a plasmid that included the RMMG#6 insert.

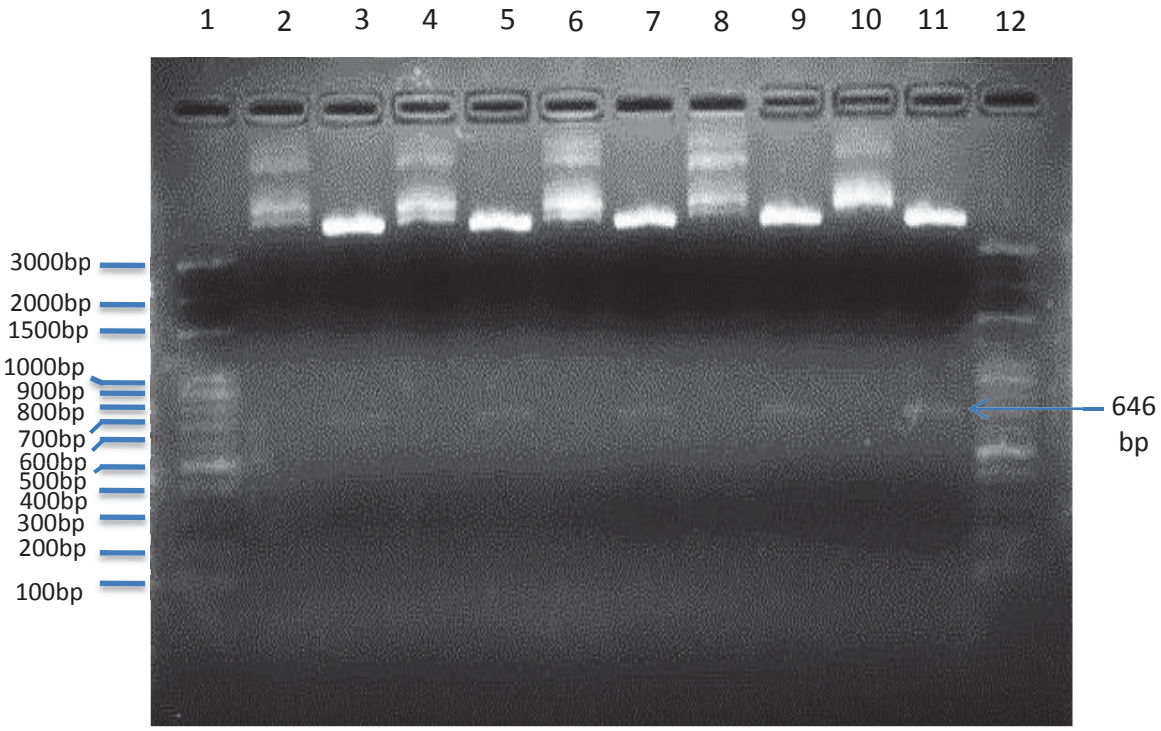


Figure 7. **Pre-screening of Transformation F of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid.** Each sample contained 10 clones to aid in quick elimination of multiple samples. Subjecting samples to an *EcoR*1 enzymatic digestion removes the RMMG#6 insert if it is present. Lane 1 100 bp molecular weight marker, Lane 2 F1 Control, Lane 3 F1 Digest, Lane 4 F2 Control, Lane 5 F2 Digest, Lane 6 F3 Control, Lane 7 F3 Digest, Lane 8 F4 Control, Lane 9 F4 Digest, Lane 10 F5 Control, Lane 11 F5 Digest, Lane 12 100 bp molecular weight marker. Banding at approximately 646 bp in Lanes 3, 5, 7, 9, and 11 revealed at least one clone within each group contained the RMMG#6 insert.

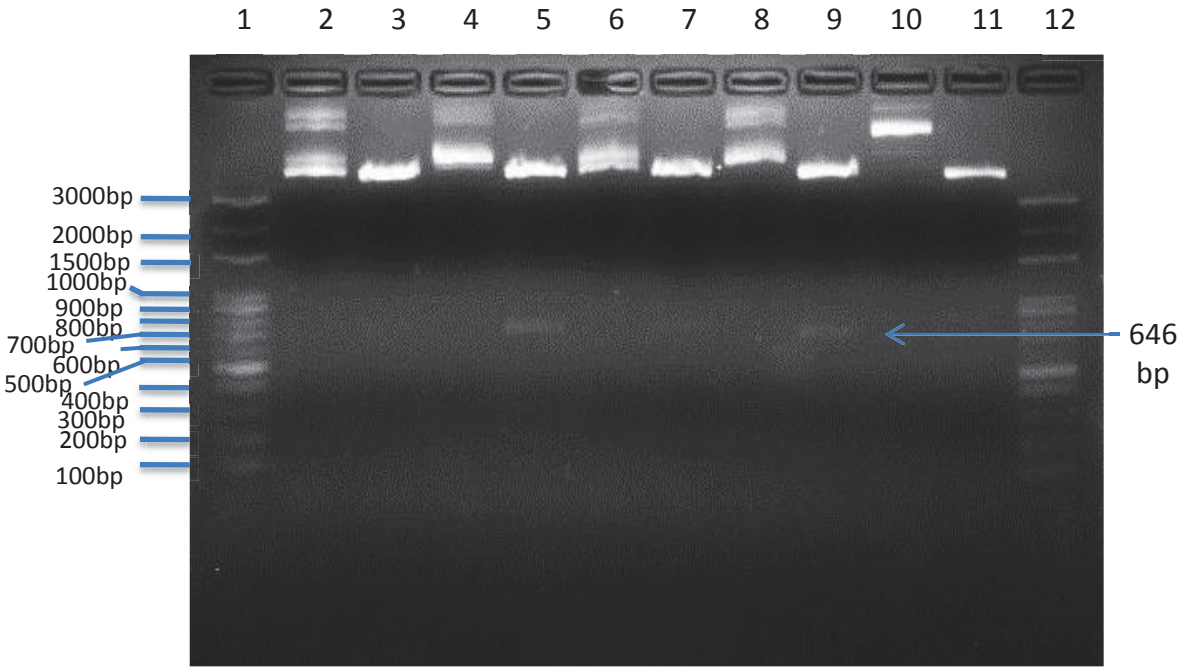


Figure 8. **Pre-screening of Transformation G of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid.** Each sample contained 10 clones to aid in quick elimination of multiple samples. Subjecting samples to an *EcoR*1 enzymatic digestion removes the RMMG#6 insert if it is present. Lane 1 Molecular weight marker 100 bp, Lane 2 G1 Control, Lane 3 G1 Digest, Lane 4 G2 Control, Lane 5 G2 Digest, Lane 6 G3 Control, Lane 7 G3 Digest, Lane 8 G4 Control, Lane 9 G4 Digest, Lane 10 G5 Control, Lane 11 G5 Digest, Lane 12 Molecular weight marker 100 bp. Lanes 3, 5, 7, and 9 showed banding at approximately 646 bp and confirmed at least one clone within each group contained the RMMG#6 insert.

brightest bands in the pre-screening gels (**Figure 7**). Each clone within these two groups were individually cultured and purified. An *EcoR*1 enzymatic digestion was performed and each of the control and digested samples were analyzed using agarose gel electrophoresis.

Figure A3.1 (Appendix 3) analyzed five clones, F33, F34, F35, F36, and F38 and revealed all contained an insert except clone F33. **Figure A3.2** (Appendix 3) analyzed another five clones, F39, F40, F41, F42, and F43. Clones within this set that were positive for insert were F39, F40 and F42. Clones F41 and F43 were removed from any further screening processes. **Figure A3.3** (Appendix 3) examined clones F45, F46, F47, F50, and F51. Faint banding was detected near the 700 bp range for clones F46 and F47 confirming these two contained an insert. **Figure A3.4** (Appendix 3) was the final gel that analyzed clones for Transformation F. Clones F44, F52, F53, and F55 all were positive for insert.

Next, two groups of clones (G2 and G4) from the Transformation G pre-screening were selected to be individually screened using the same process. Of the first group clone numbers: G13, G14, G15, G16, G17, G18, G19, G20, G21, G22, and G23 were individually cultured and purified. Each was subjected to an *EcoR*1 enzymatic digestion and analyzed using agarose gel electrophoresis. **Figure A3.4** (Appendix 3), **Figure A3.5** (Appendix 3) and **Figure A3.6** (Appendix 3) show the conclusions of this investigation. Seven clones out of the initial eleven revealed a band at the 700 bp region. Those clones were: G13, G14, G16, G17, G18, G21, and G22. Again, those clones that were lacking the 700 bp band were eliminated. The second group of clones that were individually

screened included G34, G35, G36, G38, G39, G40, G41, G42, G43, and G44. The resulting positive clones were shown in **Figure 9** and **Figure A3.7** (Appendix 3) (Clones G34, G35, G41, G42, and G44).

3.1.2.3 – Clone Screening Part 3: *Hind*III Digestion

All clones that were verified to have the titin immunogenic domain insert were then evaluated by enzymatic digestion with *Hind*III and agarose gel electrophoresis. As previously shown in Figure A. A. the pAcGFP1-C1 vector contains a *Hind*III site directly before the *EcoR*1 site that is separated by one nucleotide. The titin immunogenic domain (EU428784) also contains a *Hind*III site that cuts at position 635. If a clone possesses a vector with the insert in the proper orientation a band of 641 bp should be seen on an agarose gel. However, if the constructed vector contains the insert in a backwards orientation, a digestion with the *Hind*III enzyme would produce a fragment of approximately 17 nucleotides in length and would not be seen on an agarose gel. Thus using a *Hind*III enzyme as the third means of screening, it can be determined if the inserted sequence is in the proper orientation.

The remaining clones that were positive for insert were then screened using a *Hind*III digestion. DNA fragments were then analyzed by separation using agarose gel electrophoresis. **Figure A3.8** (Appendix 3) shows clones 34, 35, 36, 38, and 39 from Transformation F, all have a band in the 600 -700 bp region confirming all five clones to have a vector with an insert in the proper orientation**.** **Figure A3.9** (Appendix 3) displays samples F40, F41, F42, F44, and F46, also from Transformation F. Analysis of these

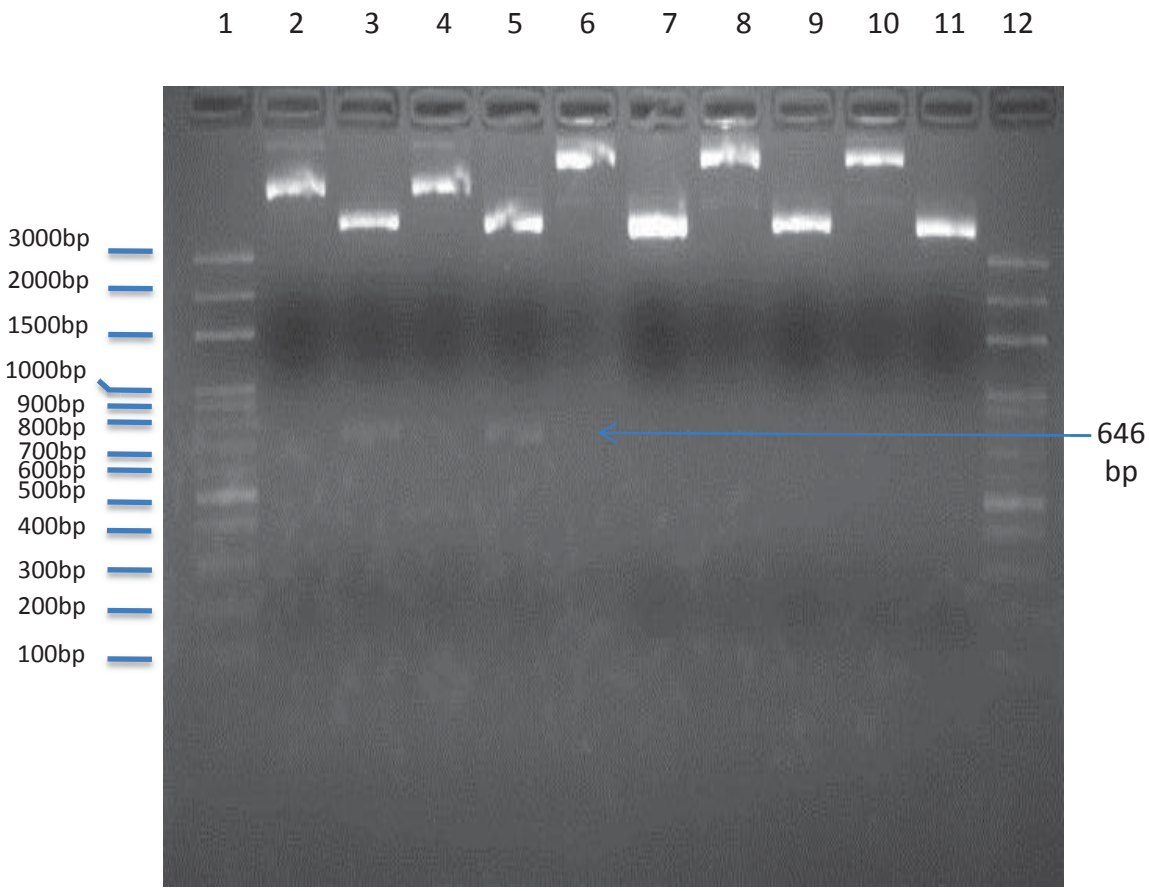


Figure 9. Screening Individual clones of Transformation G of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid. Subjecting samples to an *EcoR*1 enzymatic digestion removes the RMMG#6 insert if it is present. Lane 1 Molecular weight marker (100 bp), Lane 2 G34 Control, Lane 3 G34 Digested, Lane 4 G35 Control, Lane 5 G35 Digested, Lane 6 G36 Control, Lane 7 G36 Digested, Lane 8 G38 Control, Lane 9 G38 Digested, Lane 10 G39 Control, Lane 11 G39 Digested, Lane 12 Molecular weight marker (100 bp). Banding at approximately 646 bp in lanes 3 and 5 confirmed these clones contained a vector that incorporated the RMMG#6 insert.

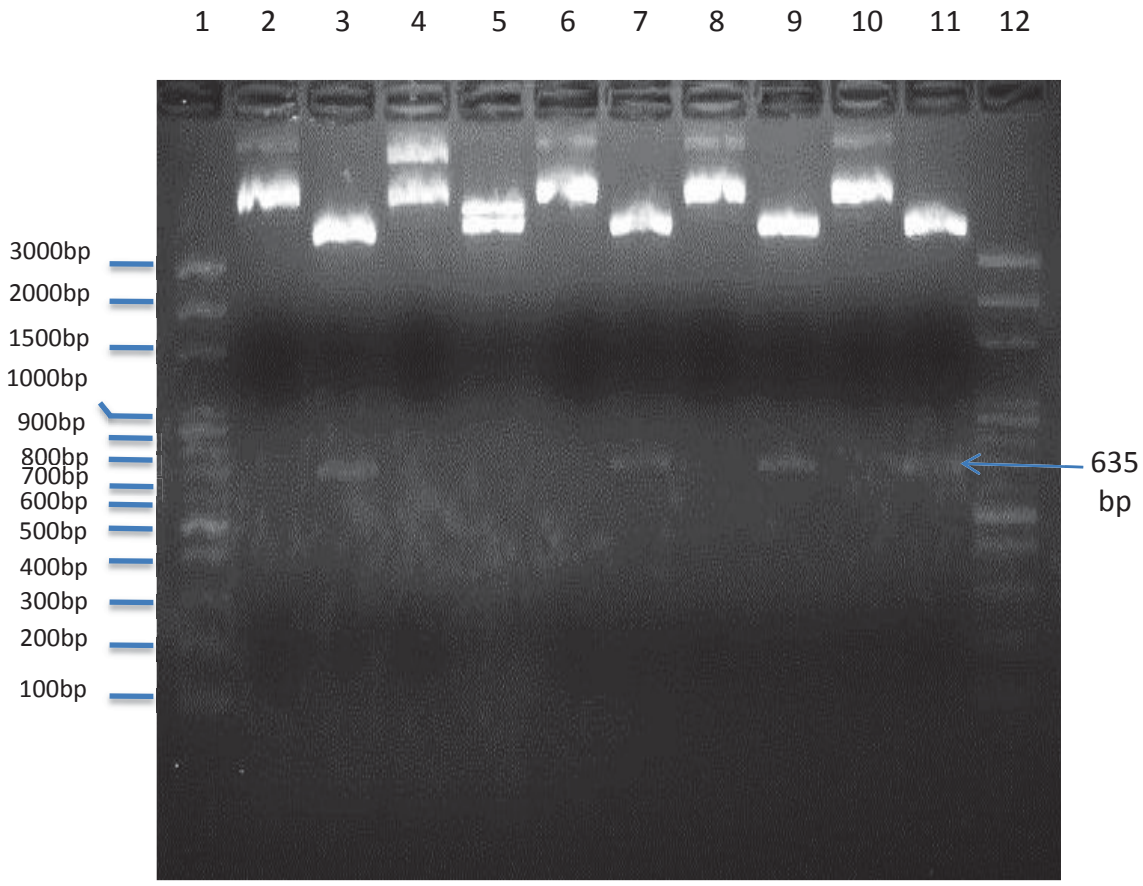


Figure 10. Screening Individual clones of Transformation F of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid using a *Hind*III enzymatic digestion. Subjecting samples to a *Hind*III enzymatic digestion removes a fragment that yields a band at approximately 635 bp if the RMMG#6 insert is in the proper orientation. Lane 1 Molecular weight marker (100 bp), Lane 2 G21 Control, Lane 3 G21 Digest, Lane 4 G22 agarose gel. However, if a vector contains an insert in a backwards orientation, digestion Control, Lane 5 G22 Digest, Lane 6 G34 Control, Lane 7 G34 Digest, Lane 8 G35 Control, Lane 9 G35 Digest, Lane 10 G41 Control, Lane 11 G41 Digest, Lane 12 Molecular weight marker (100 bp). Banding at approximately 635 bp in lanes 3, 7, 9, and 11 confirmed these clones contained a vector that incorporated the RMMG#6 insert and it was in the proper orientation.

samples show that only clones F41 and F44 have the titin fragment inserted appropriately. The other three clones were discarded. The final clones from Transformation F to be analyzed were clones F47, F52, F53, and F55. **Figure A3.10** (Appendix 3) shows that F47 and F52 contained the immunogenic titin domain oriented in the correct direction. The twelve final clones to be evaluated from Transformation G are depicted in **Figure A3.11** (Appendix 3), **Figure 10**, and **Figure A3.12** (Appendix 3). From these three figures eight of the twelve were confirmed to include the insert in the correct orientation.

In summation, a number of clones were removed from the study due to the screening process. There were however, a fair number of clones that had acquired an accurately constructed fusion plasmid. All 84 clones obtained from Transformation E were negative for insert. The total number of positive clones in Transformation F and Transformation G are unknown due to not all clones were screened. However 18 clones were screened from Transformation F and 21 from Transformation G and the complete results can be observed in **Table 10** and **Table 11**.

Table 10. **Transformation F colonies screened for insert in the proper orientation.** Screening methods included an *EcoR*1 digestion to determine if an insert is present and a *Hind*III digestion to determine if the insert was in the proper orientation. A (+) indicates a positive result (a band at approximately 646 or 635 bp respectively) determined from agarose gel electrophoresis. A (-) indicates a negative result (a lack of a band at approximately 646 or 635 bp respectively) after analysis using agarose gel electrophoresis.

	Clone Name	*EcoR*1 Result	*Hind*III Result
	F34	+	+
	F35	+	+
	F36	+	+
	F38	+	+
	F39	+	+
	F40	+	-
	F41	+	+
	F42	+	-
	F43	-	-
	F44	+	+
	F45	-	-
	F46	+	-
	F47	+	+
	F50	-	-
	F51	-	-
	F52	+	+
	F53	+	-
	F55	+	-
Total	**18**	**14**	**9**

Table 11. Transformation G colonies screened for insert in the proper orientation. Screening methods included an *EcoR*1 digestion to determine if an insert is present and a HindIII digestion to determine if the insert was in the proper orientation. A (+) indicates a positive result (a band at approximately 646 or 635 bp respectively) determined from agarose gel electrophoresis. A (-) indicates a negative result (a lack of a band at approximately 646 or 635 bp respectively) after analysis using agarose gel electrophoresis.

	Clone Name	*EcoR*1 Result	HindIII Result
	G13	+	-
	G14	+	+
	G15	-	-
	G16	+	-
	G17	+	-
	G18	+	+
	G19	-	-
	G20	-	-
	G21	+	+
	G22	+	-
	G23	-	-
	G34	+	+
	G35	+	+
	G36	-	-
	G38	-	-
	G39	-	-
	G40	-	-
	G41	+	+
	G42	+	+
	G43	-	-
	G44	+	+
Total	**21**	**12**	**8**

3.1.3 – DNA Sequencing

Preliminary sequences of 17 clones were analyzed using Genious software and BLASTn analysis. Sequences were compared to the known sequence of the titin immunogenic domain (EU 428784) (data not shown). Clones G14, G35, and G41 were selected for triplicate sequencing because they contained the most identities and fewest

gaps. The resulting triplicate sequences were again analyzed using Genious software program and consensus sequences were constructed. **Figure 11** shows the resulting consensus sequence and statistics for clone G35. **Figure A3.13** (Appendix 3) and **Figure A3.14** (Appendix 3) show each of the resulting consensus sequences and statistics for clones G14 and G41, respectively and can be found in Appendix 3. Those three sequences were then used to construct an overall consensus sequence which is shown in **Figure 12**.

A

```
1         10         20         30         40         50
|         |          |          |          |          |
CNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNACNCCTASGGCAT
KSATGAGCTGTMCAGTCCGMACTCANNGRTCTCSAGCTCARGCTKCGAAT
TCCCTAGCTCACAACCTAACNCAATGAGTCCTGCAAACTGACATNGGTTT
TCTCCAGAAGATGATGGAGGCTCTCCMATCACCAATTATGTCATTGAAAA
GCGTGAATCTGACCGCAGAGCATGGACCCCAGTGACATATACAGTTACCC
GACAAAATGCTACTGTCCAGGGTCTCATTCAAGGAAAAGCCTACTTTTTC
CGAATTGCGGCTGAAAATAGTATTGGCATGGGTCCATTTGTTGAGACATC
AGAGGCACTTGTTATCAGAGAGCCAATAACTGTACCAGAGCGTCCTGAAG
ACCTGGWNNAGTCAAAGAAGTTACTNAAAAATACTGTAACTTTGACTTGG
AATCCTCCTAAGTATGATGGTGGGTCAGAAATTATTAACTATGTCCTAGA
AAGTCGNRCTCATTGGGACTGAGAAGTTCCACAAAGTTACAAATGACAAC
TTGCTTAGCAGAAAATACACTGTTAAAGGCTTAAAAGAAGGTGATACCTA
TGAGTACCGTGTCAGTGCTGTCAACATTGTTGGACAAGGCAAACCATCAT
TNTTGCACNCAAACCAATTACTTGCAAGGNATGAGCTGGNCANCCCCCAA
CGCNTTCANCCTAGANNCTTCAGNAGANNTAAGNNCTCNACNGANTTCNG
ANGTTNNGGTGAANGCNNTTTTGNCCCTCACNNTGGNCCGNTCGTTTTNN
ACAAGGGSGNNAATTCNNTGCAGNNTCGACNNGGTACCNNNGSGGGCCCG
GGNATCCACCSNNNNNNMGAWAWCTRAKAATAANNNNCYAWACCTACMATT
GWAAGNYGSTTTTARWNNNCCMYYMYC
```

B

Length	927		
Frequencies:	#	%	%
A	237	25.6	29.9
C	188	20.3	23.7
G	171	18.4	21.6
T	197	21.3	24.8
K	3	0.3	
M	7	0.8	
N	101	10.9	
R	5	0.5	
S	7	0.8	
W	6	0.6	
Y	5	0.5	
GC	359	45.3	

Figure 11. Consensus sequence of clone G35 constructed using Genious software. **A.** The nucleotide sequence. **B.** The statistical output of the consensus sequence.

A

```
1         10         20         30         40         50
|         |          |          |          |          |
CCTCAAGNCNNCATNNNNNNNNNNNNNNNNCGGACKCTACNTTCGAGCTCA
AGCTTCGAATTCCCTAGCTCACAACCTAACCAATGAGTCCTGCAAACTGA
CATGGTTTTCTCCAGAAGATGATGGAGGCTCTCCAATCACCAATTATGTC
ATTGAAAAGCGTGAATCTGACCGCAGAGCATGGACCCCAGTGACATATAC
AGTTACCCGACAAAATGCTACTGTCCAGGGTCTCATTCAAGGAAAAGCCT
ACTTTTTCCGAATTGCGGCTGAAAATAGTATTGGCATGGGTCCATTTGTT
GAGACATCAGAGGCACTTGTTATCAGAGAGCCAATAACTGTACCAGAGCG
TCCTGAAGACCTGGAAGTCAAAGAAGTTACTAAAAATACTGTAACTTTGA
CTTGGAATCCTCCTAAGTATGATGGTGGGTCAGAAATTATTAACTATGTC
CTAGAAAGTCGGCTCATTGGGACTGAGAAGTTCCACAAAGTTACAAATGA
CAACTTGCTTAGCAGAAAATACACTGTTAAAGGCTTAAAAGAAGGTGATA
CCTATGAGTACCGTGTCAGTGCTGTCAACATTGTTGGACAAGGCAAACCA
TCATTTTGCACCAAACCAATTACTTGCAAGGATGAGCTGGGCACCCCCAA
CGCGTTCACCTAAGACTTCAGAGATAAGCTCACGATTCGAGTTGGTGAAG
CTTTTGCCCTCACTGGCCGTCGTTTTACAAGGGCGAAYTCTGCAGTCGAC
GGTACCGCGGGCCCNGGGATCCACCGGATCTAGAWGAACTGATMATAAWC
AGCCATACCTACAATTGTAAGNGGGTTTTACWNGTMYTATWY
```

B

Length	842		
Frequencies:	#	%	%
A	243	28.9	30.0
C	188	22.3	23.2
G	178	21.1	22.0
T	201	23.9	24.8
K	1	0.1	
N	22	2.6	
W	4	0.5	
Y	3	0.4	
M	2	0.2	
GC	366	45.2	

Figure 12. A consensus sequence of the total consensus sequence. The sequence was produced from the consensus sequences of clones G14, G35, and G41. **A.** The consensus sequence produced using the Genious program. **B.** The statistical output of the consensus sequence.

Each sequence was aligned with the known immunogenic titin domain sequence (EU 428784) using a 65 % similarity cost matrix, a global alignment (Needleman-Wunsch) alignment type, a gap open penalty of 12, and a gap extension penalty of 3. **Figure A3.15** (Appendix 3) shows the alignment of EU 428784 and clone G14. This pairing gave a pairwise % similarity of 76.2 % with 646 identical sites. The comparison of clone G35 with the known immunogenic titin domain (**Figure 13**) resulted in a pairwise % similarity of 68.8 % and 638 identical sites. The alignment of clone G41 and the known immunogenic titin domain sequence yielded a 74.1 % in a pairwise % similarity and 629 identical sites (Appendix 3, **Figure A3.16)**. Finally, the alignment of the consensus sequence of the consensus sequences with the known immunogenic domain sequence, shown in **Figure 14**, resulted in a pairwise % similarity of 76.7 % and 646 identical sites. The statistical results of each alignment are shown in **Table 12**.

Figure 13. Alignment of G35 Consensus sequence and titin immunogenic domain (EU 428784). Sequences were aligned using Genious software. Statistical analysis of the alignment gave 68.8% pairwise % similarity with 638/646 identity.

Figure 14. The alignment of the total consensus sequence and the known immunogenic titin domain (EU 428784). Consensus sequence constructed from the clones G14, G35, and G41. Sequences were aligned using Genious software. Statistical analysis of the alignment gave 76.7% pairwise % similarity with 646/646 identity.

Table 12. Statistical results of the alignment of each consensus sequence clone, G14, G35, G41 and Total Consensus sequence with the known titin immunogenic domain sequence (EU 428784). Analysis was completed using Genious software.

Alignment	Sequence Length (nt)	Pairwise % Similarity (%)	Identical Sites
EU428784	646	76.2	646
G14	848		
EU428784	646	68.8	638
G35	927		
EU428784	646	74.1	629
G41	849		
EU428784	646	76.7	646
Total Consensus	842		

BLASTn analysis was accomplished using each of the clone consensus sequences and the total consensus sequence. **Table 13** show seven sequences and the percent identities resulting from BLAST comparisons. As expected, each sequence shows a 96 % identity, or higher, to the known immunogenic titin domain sequence. Each clone also shares a 94% identity or higher to four *Homo sapiens* titin isoforms, N2-A, N2-B, transcript variant novex-1 and transcript variant novex-2. Furthermore, these sequences share an 88 to 89 % identity to the *Mus musculus* titin transcript variant N2-B and N2-A.

Table 13. BLASTn analysis of clone G14, G35, and G41 consensus sequences and the overall consensus sequence. Each clone is shown with the percent of identity to the sequences listed at the left of the table.

	G14	G35	G41	Total Consensus
Homo sapiens titin (TTN) mRNA, partial cds (EU428784.1)	99%	96%	96%	99%
Homo sapiens titin (TTN), transcript variant N2-A, mRNA (NM 133378.4)	99%	94%	96%	99%
Homo sapiens titin (TTN), transcript variant N2-B, mRNA (NM 003319.4)	99%	94%	96%	99%
Homo sapiens titin (TTN), transcript variant novex-2, (NM 133437.3)	99%	94%	96%	99%
Homo sapiens titin (TTN), transcript variant novex-1, mRNA (NM 133432.3)	99%	94%	96%	99%
Mus musculus titin (Ttn), transcript variant N2-B, mRNA (NM 028004.2)	89%	88%	88%	89%
Mus musculus titin (Ttn), transcript variant N2-A, mRNA (NM011652.3)	89%	88%	88%	89%

3.2 – Mammalian Cell Transfection

The second goal of this study was to transfect a mammalian cell culture with the constructed fusion plasmid, RMMG#6/pAcGFP1-C1. A C2C12 *Mus musculus* myoblast cell line was selected first, because titin is a major component in skeletal muscle cells.

The interest if the study focuses on the production and localization of titin during growth and development through the differentiation process. A second reason for employing the C2C12 cell line was because of the significant similarity between *Mus musculus* and *Homo sapiens* titin isoforms. Transfection of C2C12 cells with the constructed plasmid increased the concentration of the immunogenic titin domain during development and differentiation. Morphological changes were detected by microscopy and determination of titin domain-GFP fusion protein localization was detected while utilizing the GFP tracker.

3.2.1 – Kill Curve of Untransfected C2C12 Cultures

Cell counts were determined for each well per day to determine which concentration of Neomycin was most effective in killing untransfected C2C12 cultures. **Figure 15** shows the effects of increasing concentrations of neomycin on C2C12 cultures after two days of treatment. The difference in growth of the culture shown in **Figure 15** part D (treatment with 2.0 mg/ml neomycin) and part E (treatment with 2.5 mg/ml neomycin) is drastic. **Figure 16** shows the cell count and graph for each concentration during the treatment period. Both figures agree that, within this range of neomycin concentrations, 2.5 mg/ml is the minimum concentration that has a profound effect on the growth of untransfected C2C12 cells.

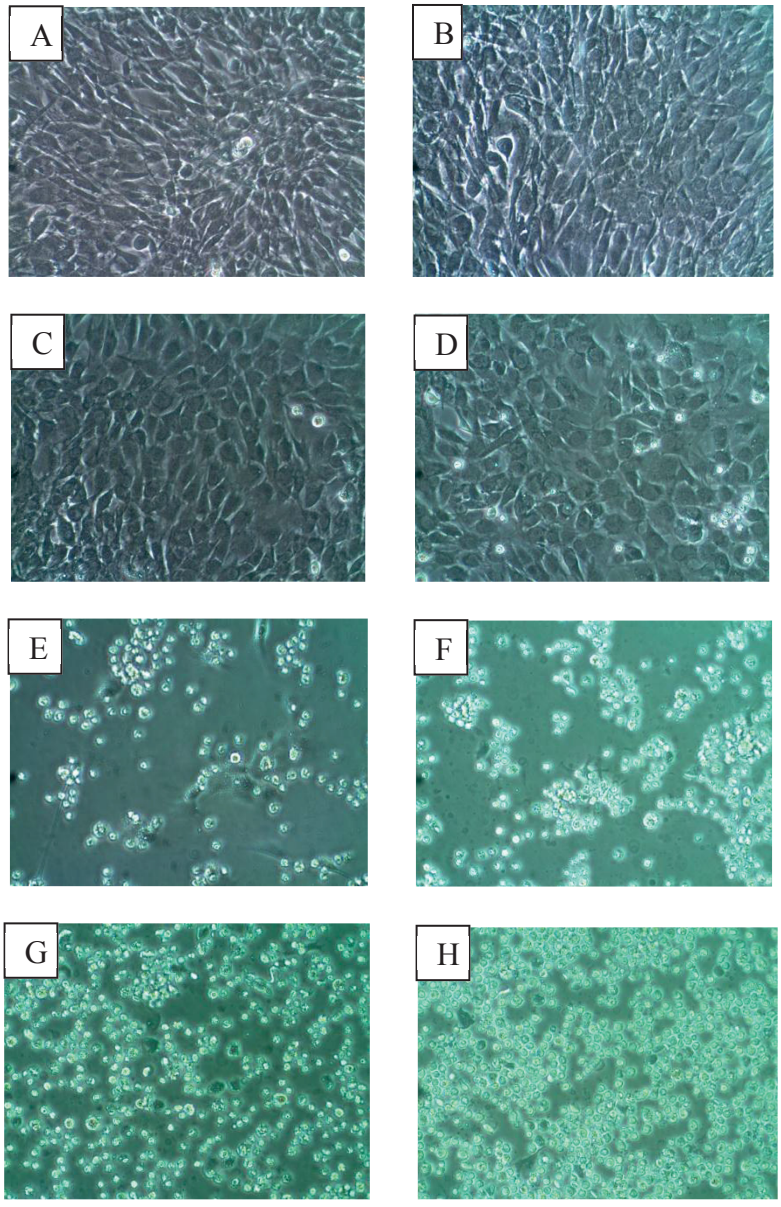
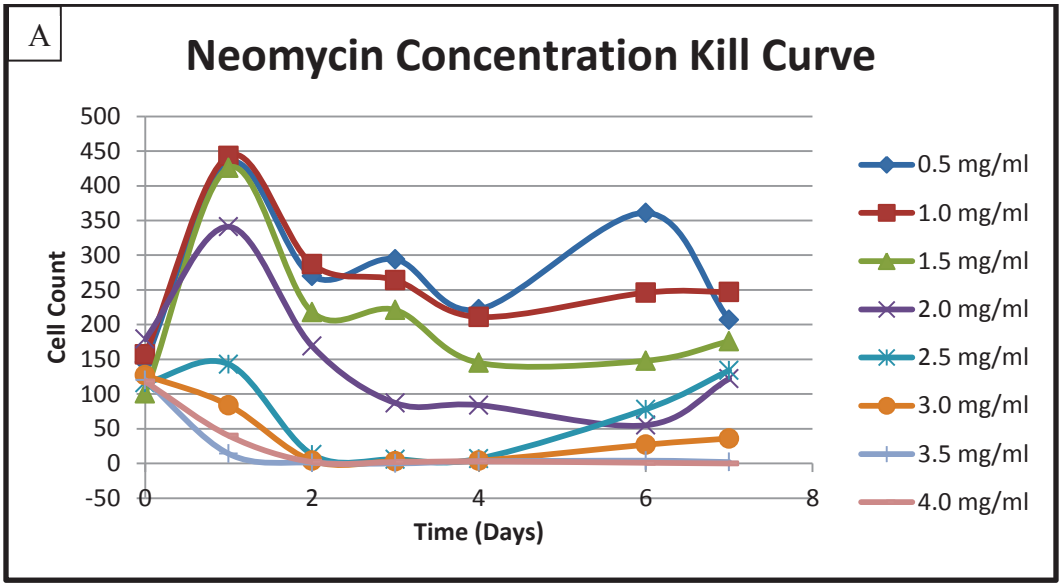


Figure 15. C2C12 cultures treated with varying neomycin concentrations at time = 2 days. A. Culture treated with C2GM + 0.5 mg/ml Neomycin. **B.** Culture treated with C2GM + 1.0 mg/ml Neomycin. **C.** Culture treated with C2GM + 1.5 mg/ml Neomycin. **D.** Culture treated with C2GM + 2.0 mg/ml Neomycin. **E.** Culture treated with C2GM + 2.5 mg/ml Neomycin. **F.** Culture treated with C2GM + 3.0 mg/ml Neomycin. **G.** Culture treated with C2GM + 3.5 mg/m Neomycin. **H.** Culture treated with C2GM + 4.0 mg/ml Neomycin.

A



B

Neomycin Concentration	Time (Days)						
	0	1	2	3	4	6	7
0.5	146	433	270	294	222	361	207
1	157	443	287	264	211	246	247
1.5	101	426	218	221	145	148	176
2	179	341	169	87	84	55	122
2.5	116	143	13	6	7	78	134
3	127	84	4	3	4	27	36
3.5	120	14	1	0	3	4	2
4	118	40	2	2	3	1	0

Figure 16. Kill Curve of Untransfected C2C12 cultures. A. The graph of C2C12 cultures treated with varying concentrations of neomycin over a seven day period. **B.** Table of C2C12 cell counts from each culture.

3.2.2 – Kill Curve of Transfected C2C12 cultures

Cell counts for each culture were taken on a daily basis and evaluated using Microsoft Excel. **Figure 17** illustrates the size of each culture throughout selection with varying neomycin concentrations and growth time lines. **Figure 17 A.** depicts three cultures (1, 2, and 3) treated with neomycin concentration (2.5, 2.75, and 3.0 mg/ml respectively). These cultures were first subjected to selection using C2GM supplemented with their respective neomycin concentrations for three days, followed by the induction and continuation of differentiation for another 13 days. Cell concentrations of each culture increase throughout the treatment with C2GM. Only after media was switched to C2DM with specified neomycin concentrations did the concentrations of each culture begin to decrease. The decrease was due to the lack of growth factors allowing continued proliferation and the continuation of selection.

Figure 17 B. depicts similar cultures treated with the same concentrations of neomycin, however, they were immediately induced into differentiation. Treatment with C2DM continued for eight days. Culture 4 was exposed to C2DM + 2.5 mg/ml neomycin for eight days following transfection. Culture 5 was treated with C2DM + 2.75 mg/ml neomycin for eight days following transfection. Data for Culture 6 (immediate differentiation with C2DM + 3.0 mg/ml neomycin) was excluded due to mold contamination on day 2 of the treatment. Cultures show an immediate decrease in cell concentration following the introduction of the differentiation media which is again primarily due to the lack of growth factors.

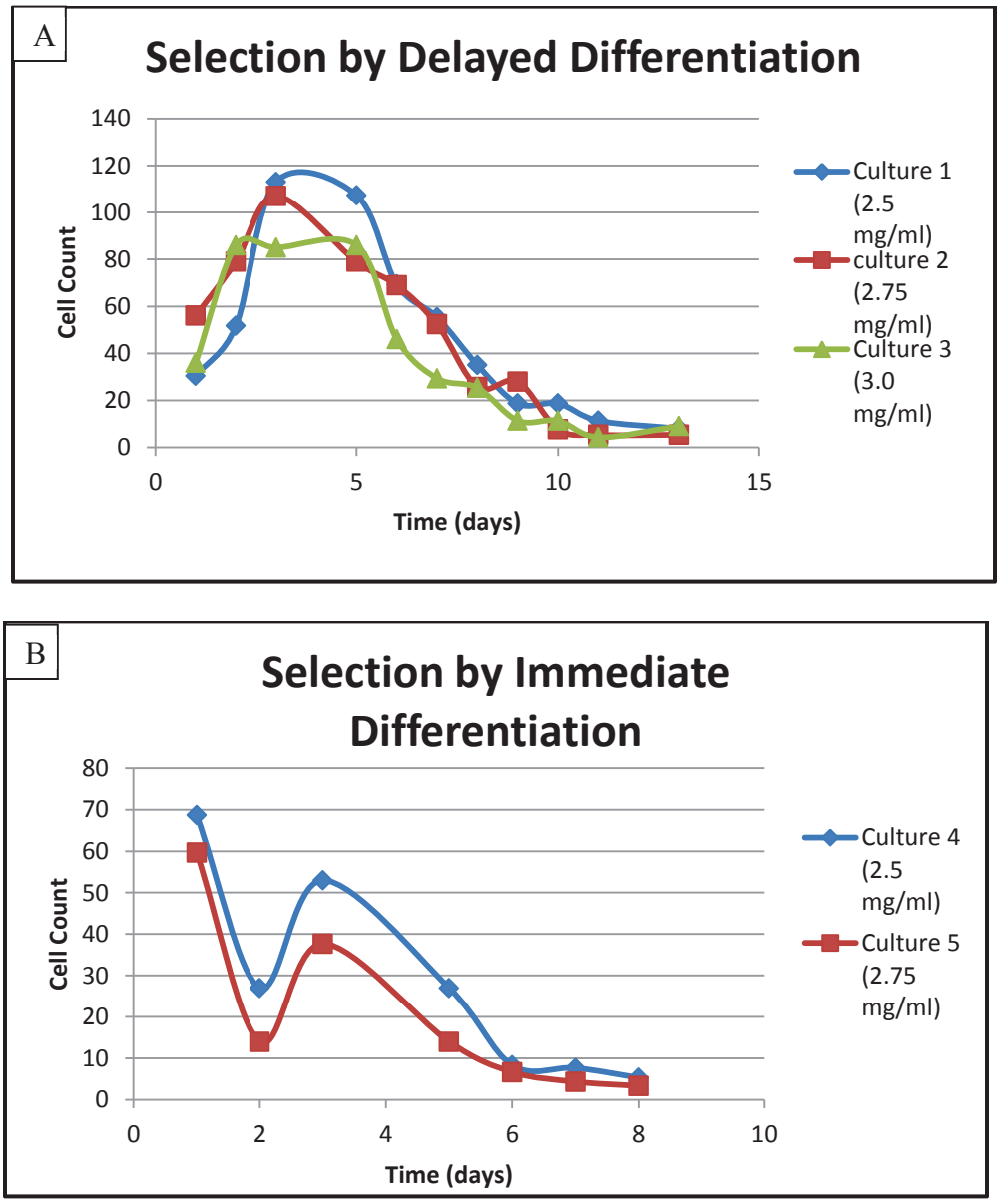


Figure 17. Selection for C2C12 Cells Transfected with Recombinant Plasmid. A. Cultures 1, 2, and 3, treated with increasing neomycin concentrations (2.5 mg/ml, 2.75 mg/ml, and 3.0 mg/ml respectively). Cultures were first treated with C2GM supplemented with respective neomycin concentrations for 3 days, followed by 8 days of treatment with C2DM including the designated concentration of neomycin. **B.** Cultures 4 and 5 were immediately induced into differentiation by the addition of C2DM with 2.5 mg/ml or 2.75 mg/ml neomycin and continued for 8 days.

Each of these cultures were also analyzed using fluorescent microscopy (data not shown) and SDS-PAGE, shown in **Figure 18**. Both cultures 4 and 5, that were subjected to immediate differentiation revealed a dark band in the region of approximately 66 KDa. This is the estimated size of the fusion protein product.

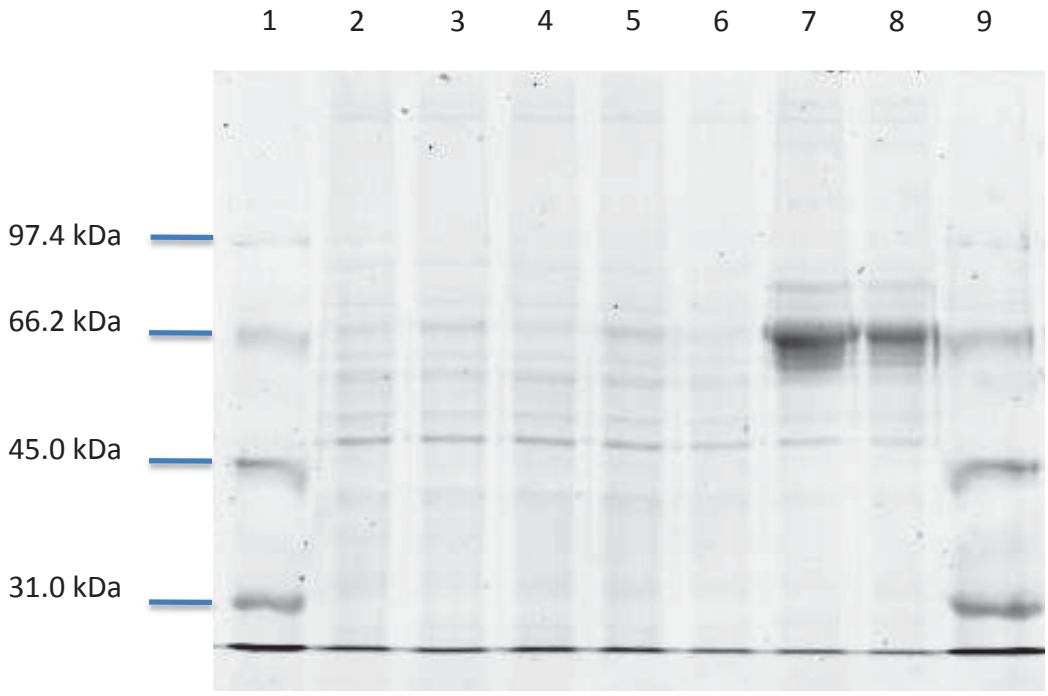


Figure 18. SDS-PAGE gel of transfected C2C12 cell cultures. Lane 1. Protein molecular weight marker. Lane 2. Control culture 1 treated for 3 days with C2GM followed by 8 days of C2DM. Lane 3. Control culture 2 treated for 8 days with C2DM. Lane 4. Culture 1, treated with C2GM + 2.5 mg/ml neomycin for 3 days followed by treatment with C2DM + 2.5 mg/ml for 8 days. Lane 5. Culture 2, treated with C2GM + 2.75 mg/ml neomycin for 3 days followed by treatment with C2DM + 2.75 mg/ml for 8 days. Lane 6. Culture 3, treated with C2GM + 3.0 mg/ml neomycin for 3 days followed by treatment with C2DM + 3.0 mg/ml for 8 days. Lane 7. Culture 4, treated with C2DM + 2.5 mg/ml neomycin for 8 days. Lane 8. Culture 5, treated with C2DM + 2.75 mg/ml neomycin for 8 days. Lane 9. Protein molecular weight marker.

3.3 – Overexpression of the Immunogenic Domain of Titin, RMMG#6, on C2C12 Cells

Three groups of C2C12 were formed including an experimental group (those transfected with clone G35 RMMG#6/pAcGFP1-C1 fusion plasmid), a GFP positive control group (C2C12 transfected with the pAcGFP1-C1 plasmid), and a negative control group (C2C12 cells not transfected). Experimental and GFP positive control group were immediately induced into differentiation and developed in C2DM + 2.5 mg/ml. Control group cultures were also immediately induced into differentiation and developed in C2DM media. Protein samples and microscopy slides were prepared every other day post differentiation initiation.

3.3.1 – Determination of Morphological Differences Through Microscopy

Figure 19, **Figure 20**, **Figure 21**, **Figure 22**, **Figure 23**, **Figure 24**, **Figure 25** depict bright field and fluorescent microscopy images. Each figure shows negative control, experimental and GFP positive control groups at time points 1, 3, 5, 7, 9, 11, and 13 days. **Figure 19** shows a culture from each group at 1 day post differentiation initiation. The cells in each picture look morphologically similar and have a subtle background fluorescents.

Figure 20, cultures at 3 days post differentiation initiation reveal minimal spotty fluorescents in the experimental group. These fluorescent spots localized within the regions between the nucleus and the outer edges of the cell. Cells contained within the GFP group show a similar pattern of limited fluorescents. There are a few cells that show

an exponentially increased concentration of fluorescents surrounding the nucleus. Control group cells maintain the minimal overall fluorescents shown in each group at T = 1 day.

Figure 21. Control group cells still have minimal background fluorescents. Experimental group fluorescent photographs show numerous fluorescent spotting, however those spots that are obviously not localized within a cell are not considered to be significant. When compared to the bright field photographs, these spots seem to be dead cells or miscellaneous debris expelled from the cell. GFP positive control group cells again show an increased number of fluorescent spotting localized very close to the nucleus with some migrating outward toward the edges of the cell.

Figure 22. Control group cells still show minimal background fluorescents. There is one cell producing more fluorescents that the others at the bottom right corner. Due to it being smaller and more round, it is most likely a cell that has died. Dead cells seem to produce something that gives off more fluorescents than any normal healthy living cell. Experimental group cells at T = 5 days post differentiation show an increase in fluorescence throughout the cell but mainly localizing around the nucleus. GFP positive control group reveal increased concentration and less defined fluorescent spotting still centered around the nucleus.

Figure 23. Control group cells continue to have the same minimal background fluorescents. Experimental group cells have numerous well defined fluorescent spots localized around the nucleus with some expanding out to the edges of the cell. GFP

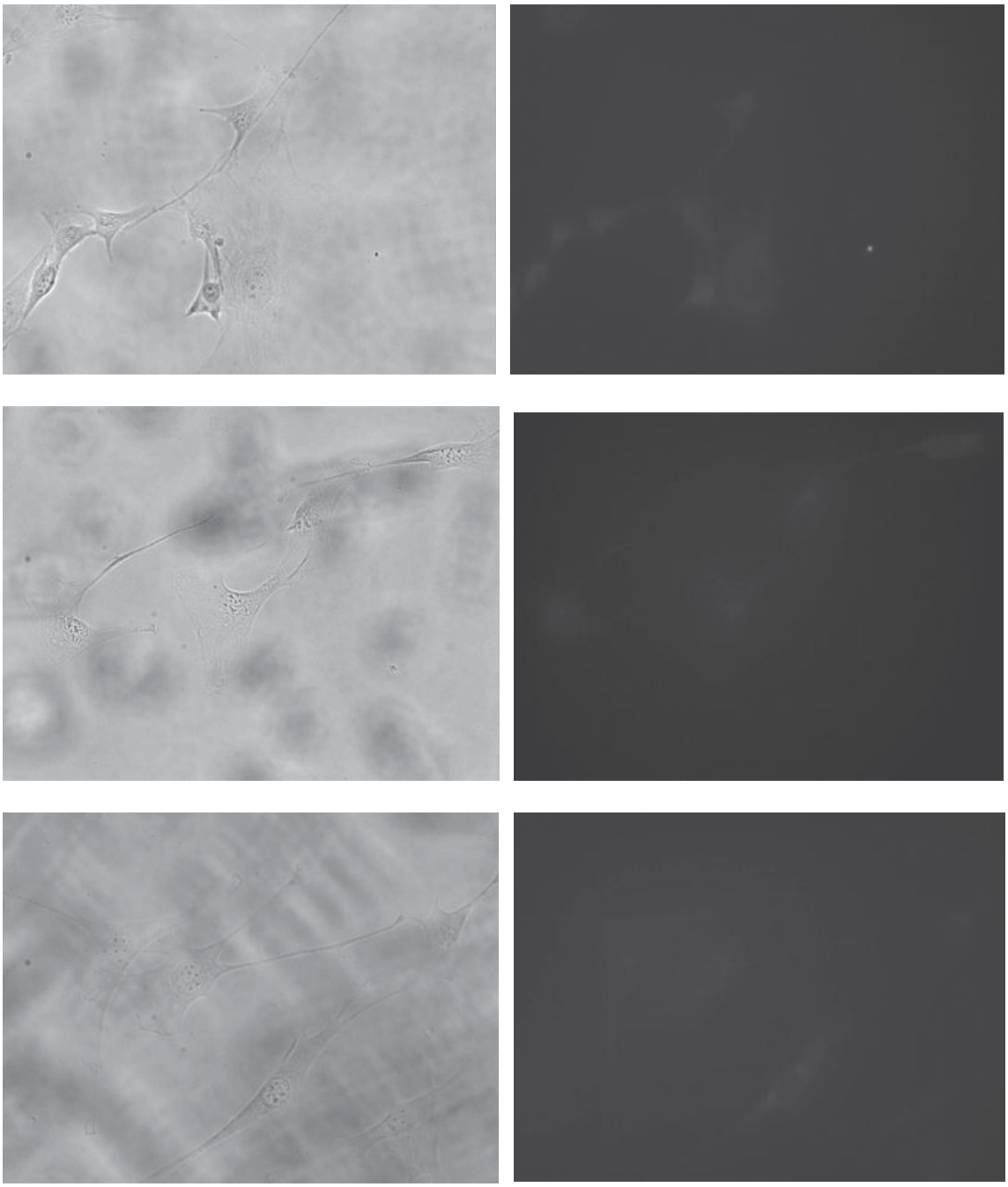


Figure 19. Bright field and fluorescent microscopy images of Control, Experimental, and GFP group cultures at T = 1 day post differentiation initiation. **Top** Control at T = 1 day post differentiation initiation, **Middle** Experimental at T = 1 day post differentiation initiation, **Bottom** GFP at T = 1 day post differentiation initiation.

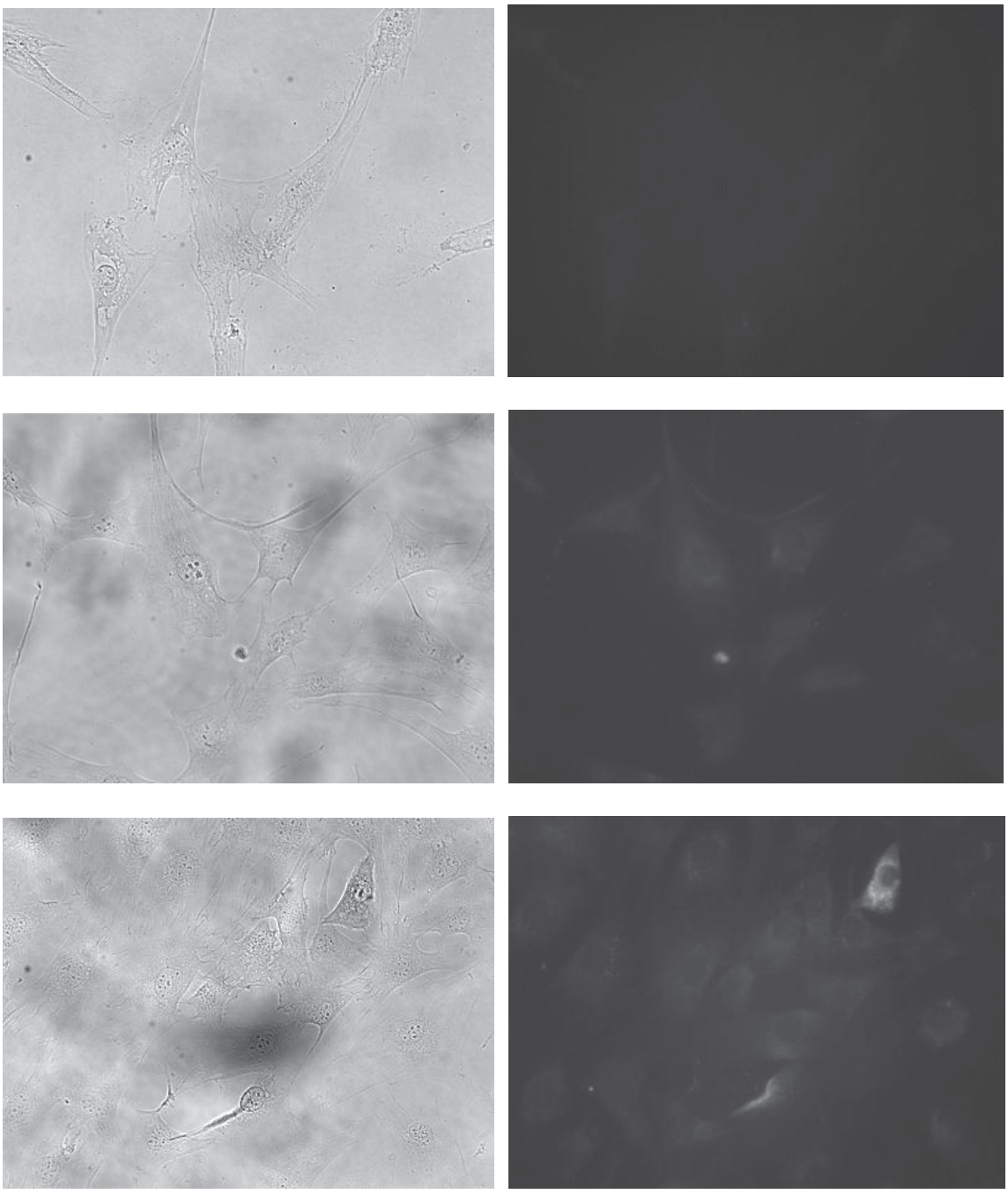


Figure 20. Bright field and fluorescent microscopy images of Control, Experimental, and GFP group cultures at T = 3 days post differentiation initiation. Top Control at T = 3 days post differentiation initiation, **Middle** Experimental at T = 3 days post differentiation initiation, **Bottom** GFP at T = 3days post differentiation initiation

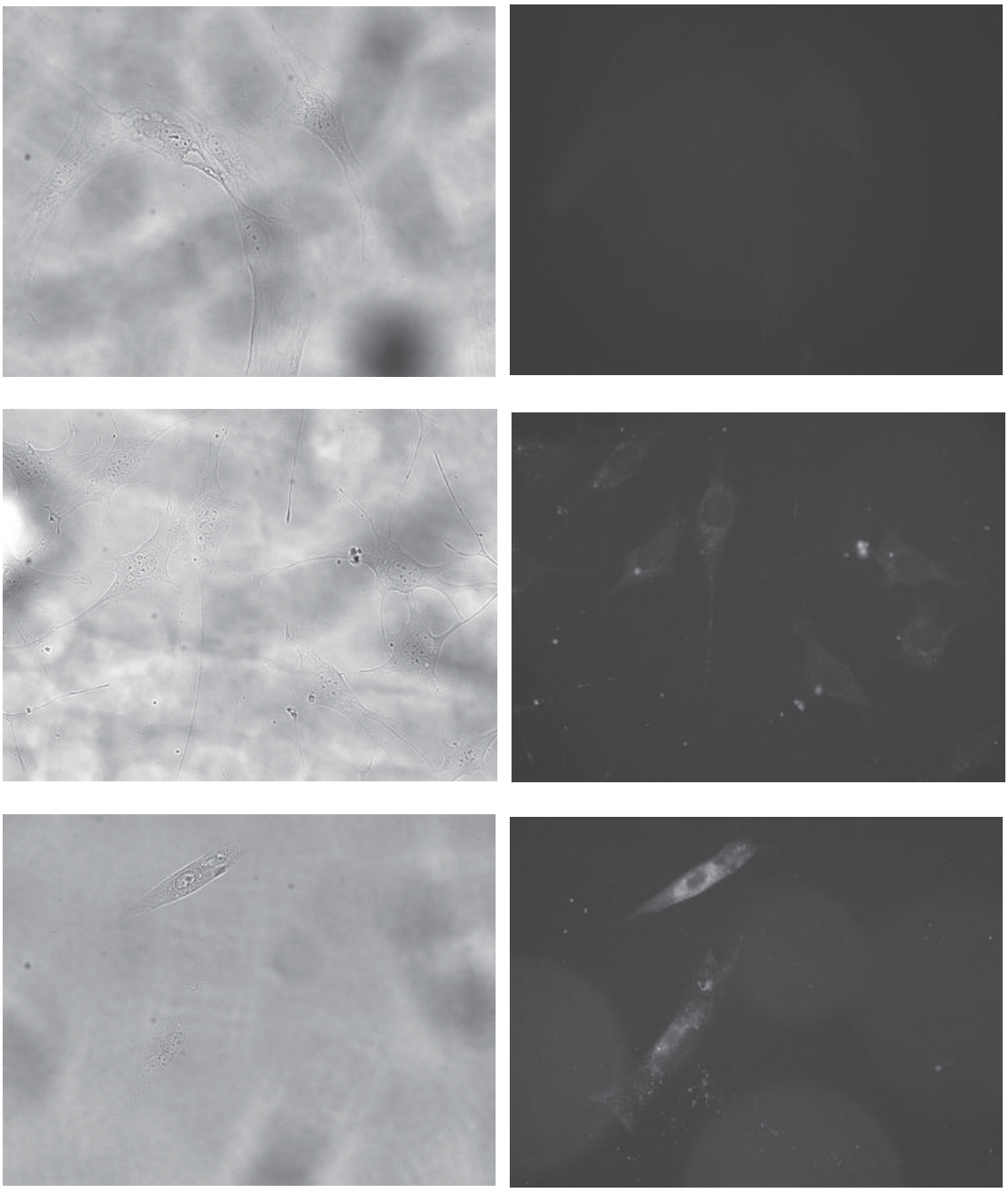


Figure 21. Bright field and fluorescent microscopy images of Control, Experimental, and GFP group cultures at T = 5 days post differentiation initiation. Top Control at T = 5 days post differentiation initiation, **Middle** Experimental at T = 5 days post differentiation initiation, **Bottom** GFP at T = 5 days post differentiation initiation.

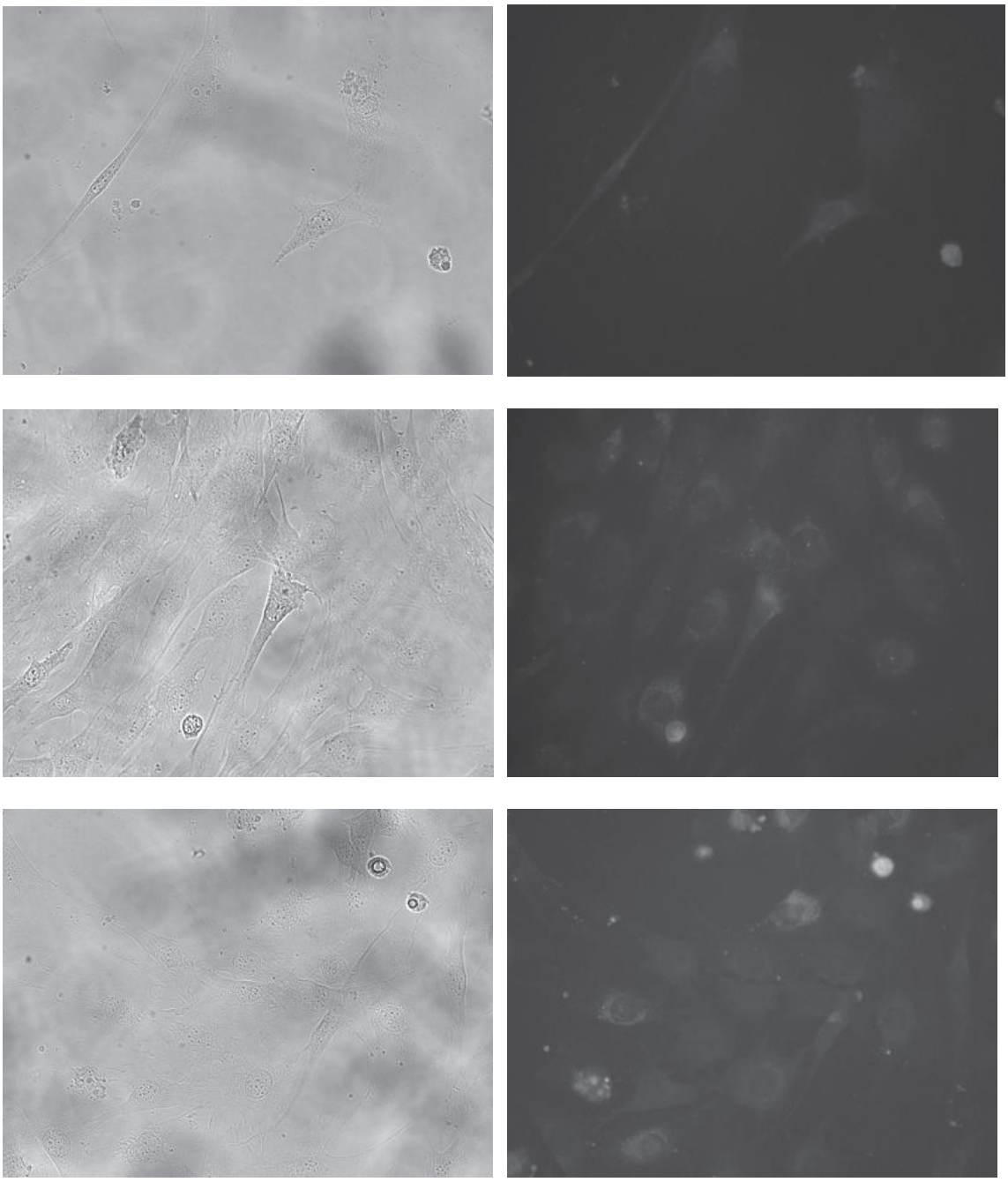


Figure 22. Bright field and fluorescent microscopy images of Control, Experimental, and GFP group cultures at T = 7 days post differentiation initiation. Top Control at T = 7 days post differentiation initiation, **Middle** Experimental at T = 7 days post differentiation initiation, **Bottom** GFP at T = 7 days post differentiation initiation.

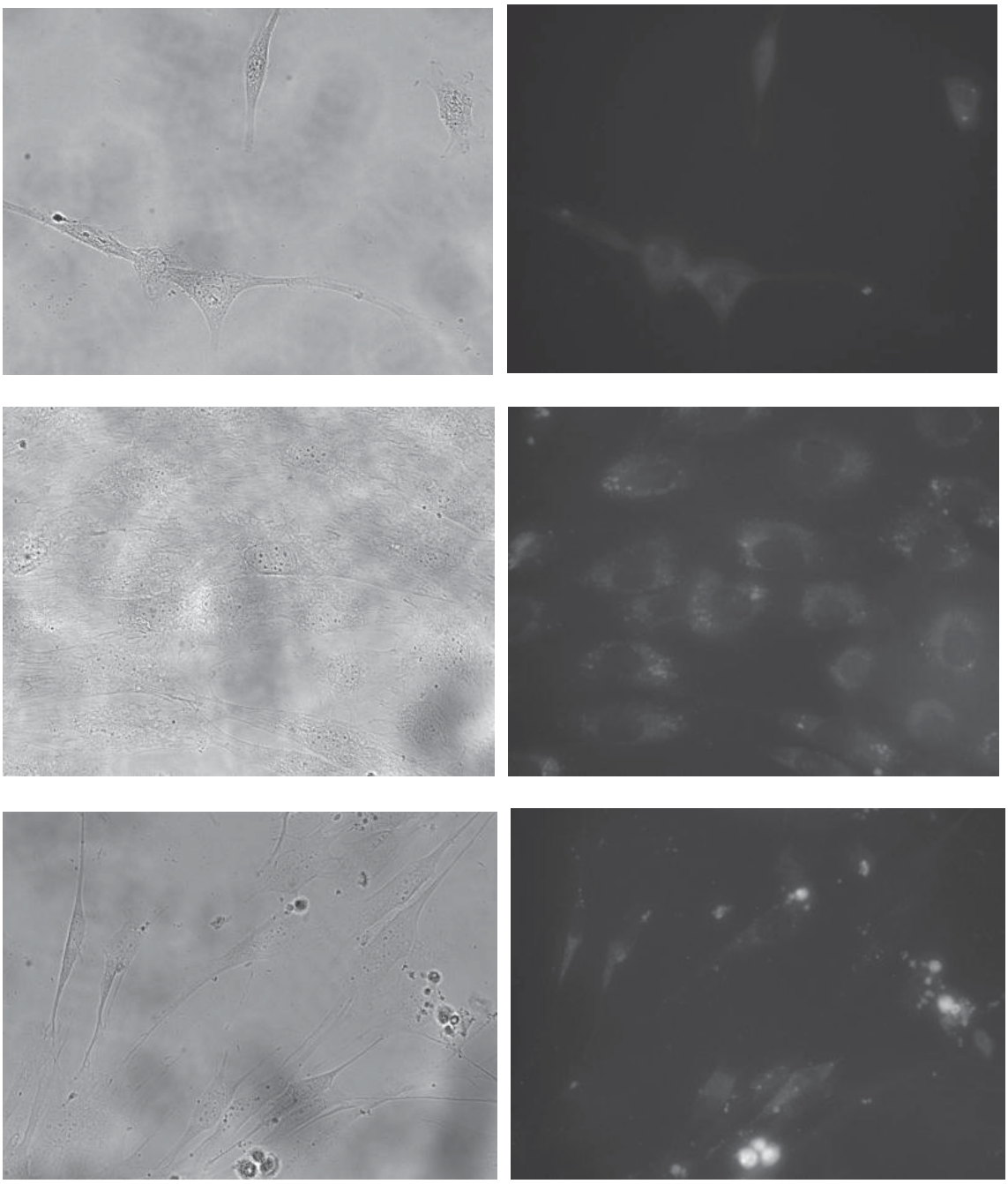


Figure 23. Bright field and fluorescent microscopy images of Control, Experimental, and GFP group cultures at T = 9 days post differentiation initiation. Top Control at T = 9 days post differentiation initiation, **Middle** Experimental at T = 9 days post differentiation initiation, **Bottom** GFP at T = 9 days post differentiation initiation.

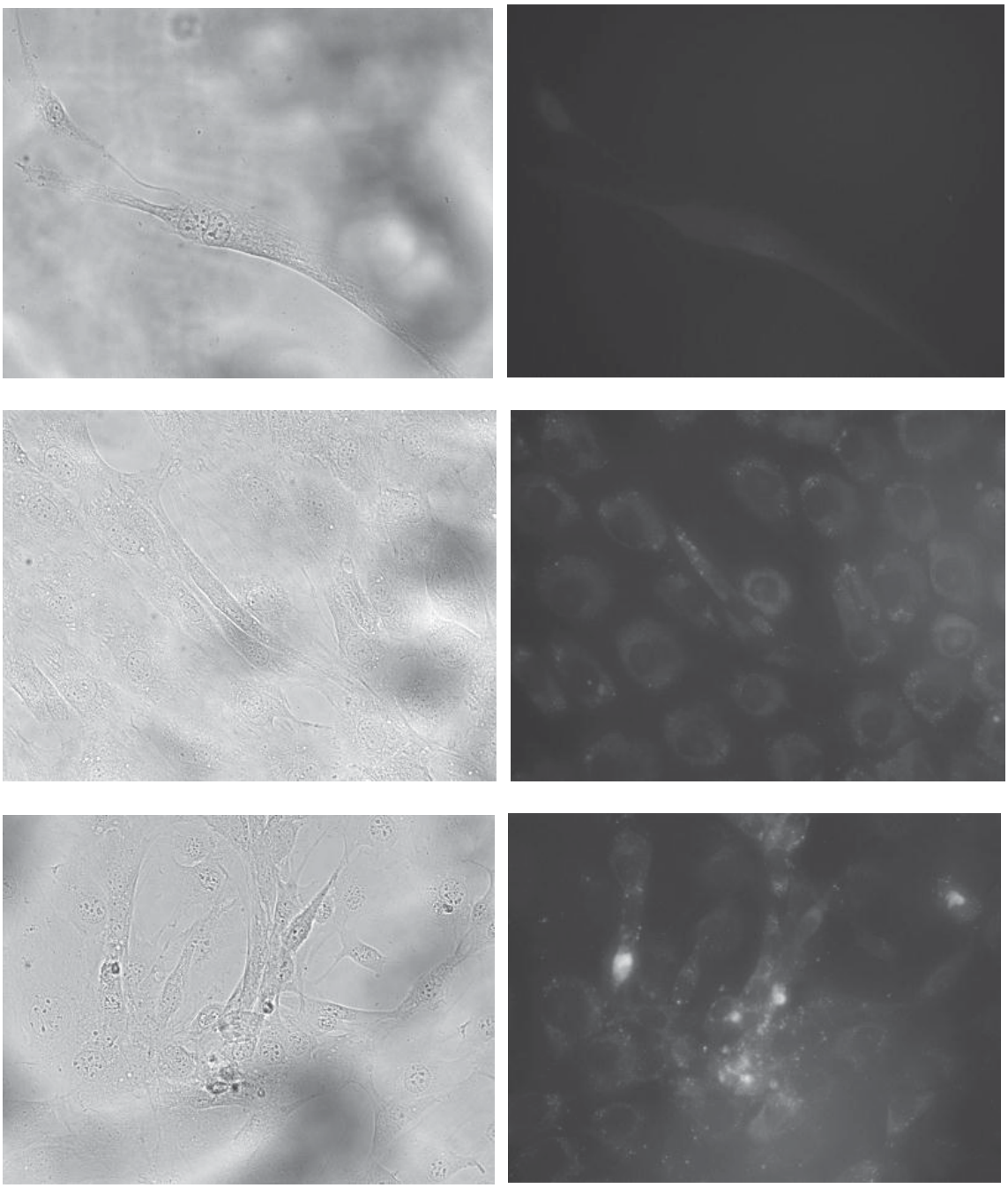


Figure 24. Bright field and fluorescent microscopy images of Control, Experimental, and GFP group cultures at T = 11 days post differentiation initiation. Top Control at T = 11 days post differentiation initiation, **Middle** Experimental at T = 11 days post differentiation initiation, **Bottom** GFP at T = 11 days post differentiation initiation.

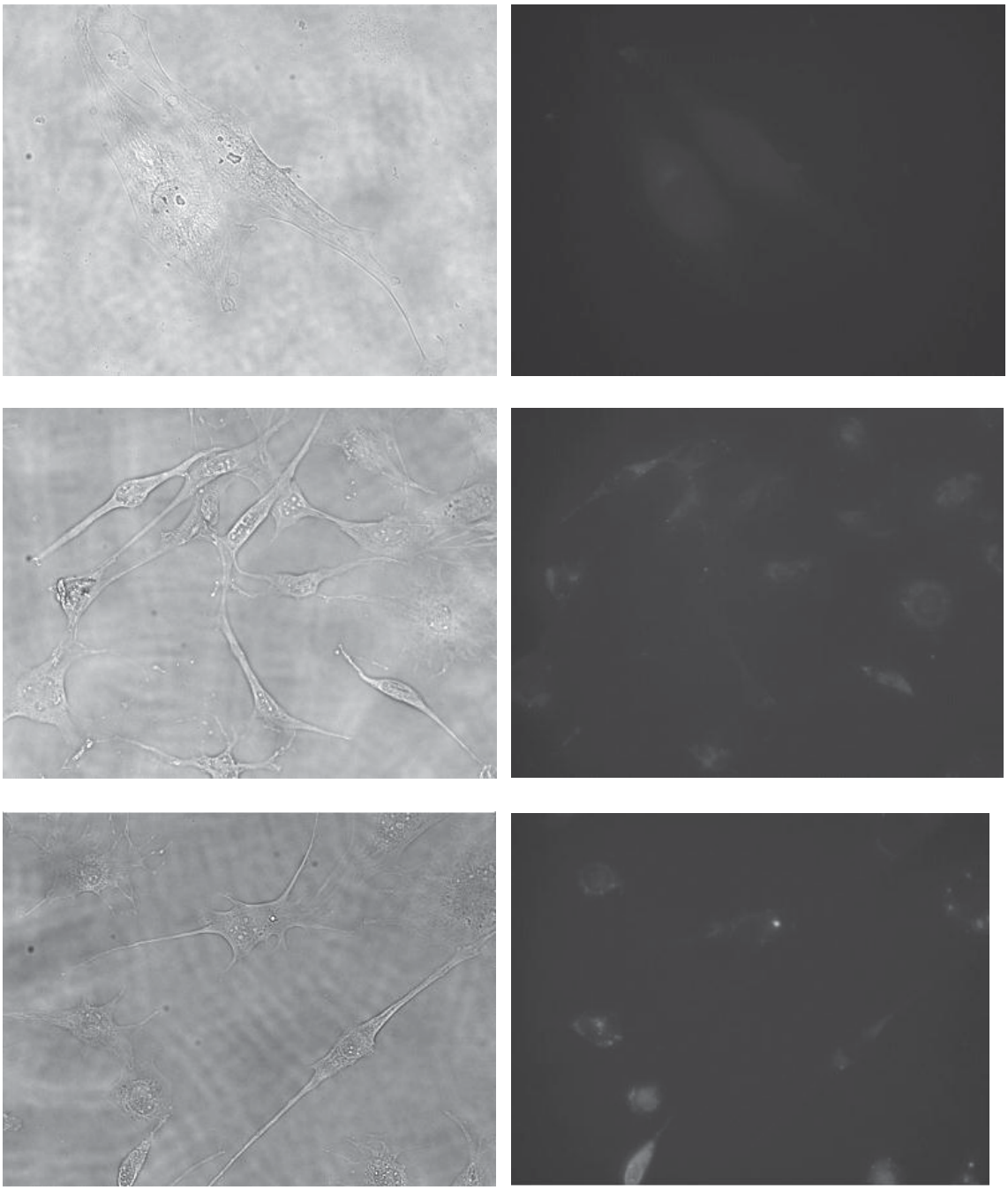


Figure 25. Bright field and fluorescent microscopy images of Control, Experimental, and GFP group cultures at T = 13 days post differentiation initiation. Top Control at T = 13 days post differentiation initiation, **Middle** Experimental at T = 13 days post differentiation initiation, **Bottom** GFP at T = 13 days post differentiation initiation.

positive control group have a hazy darker fluorescents localized around the nucleus. When comparing the bright field images of the experimental and GFP groups, the morphological features show the experimental group cells to be more round and less elongated than the GFP cells.

Figure 24. The control group still maintains a minimal amount of background fluorescents. Experimental group cells possess a more round morphology with a negligible elongation, like that of a cell at 11 days post differentiation initiation. Fluorescent microscopy images reveal spotting localized more toward the edges of the cell than closer towards the nucleus. GFP positive control group cultures show fluorescent spotting that does not seem to localize at any specific location within the cell.

In **Figure 25** the control group continues to maintain the same background fluorescents. The experimental group shows a cloudy fluorescents localized around the nucleus with minimal defined spotting sporadically throughout the cell. GFP positive group cells show a dark cloud of fluorescents surrounding the nuclei of some cells, with minimal defined fluorescent spotting in one of the six cells pictured.

3.3.2 – Time Trial Protein Analysis

SDS-PAGE gels were imaged and analyzed using the BioRad Pharos FX™ Plus Molecular Imager and BioRad Quantity One 1-D Analysis Software. **Figure 26** shows each of the imaged SDS-PAGE gels. 20 µl of each sample were loaded on each gel. Due

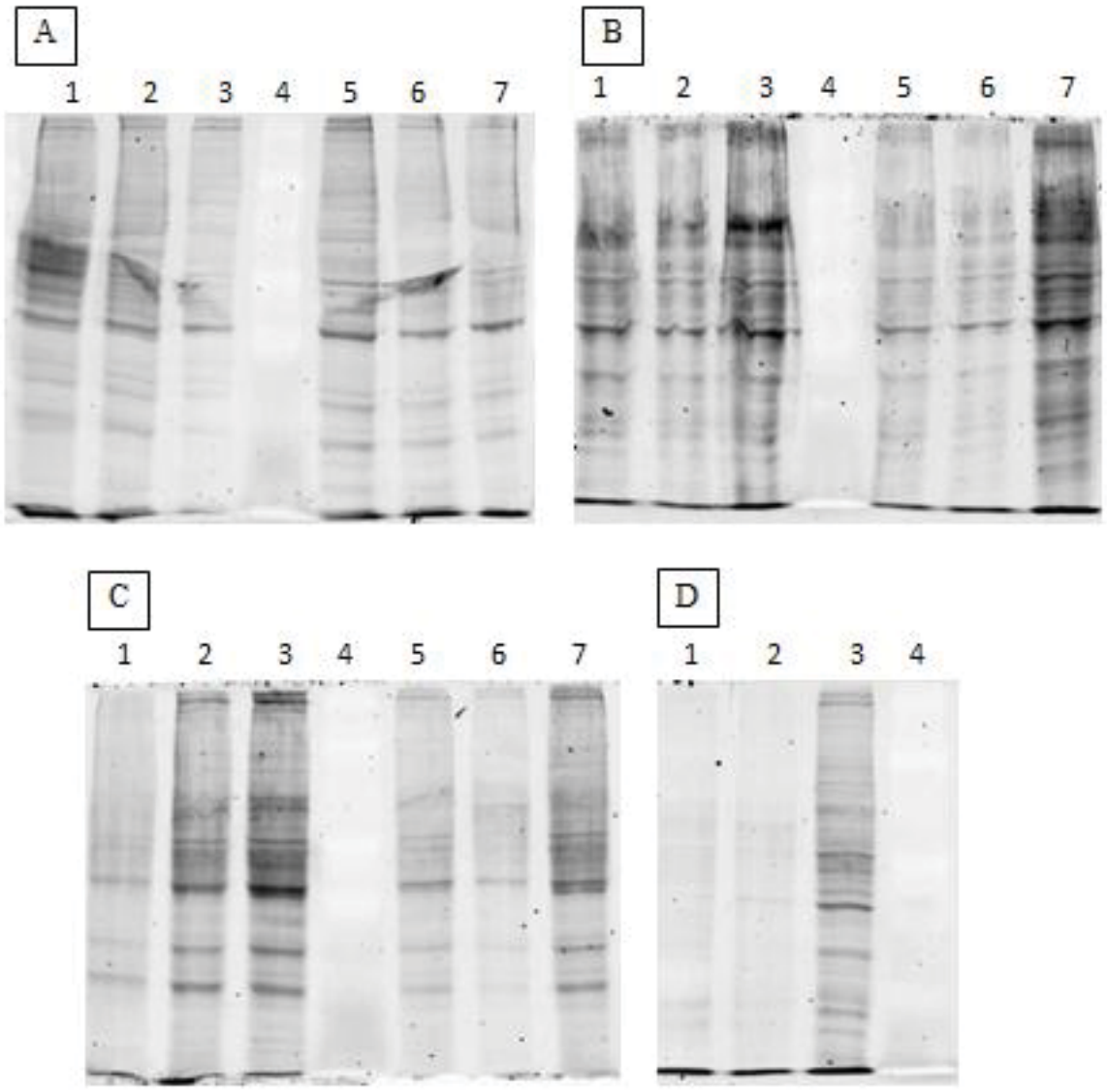


Figure 26. SDS-PAGE analysis of transfected and stock C2C12 cultures at time points 1, 3, 5, 7, 9, 11, and 13 days post differentiation. **A** Lane 1 Control group at 1 day. Lane 2 Experimental group at 1 day. Lane 3 GFP group at 1 day. Lane 4 Protein molecular weight marker. Lane 5 Control group at 3 days. Lane 6 Experimental group at 3 days. Lane 7 GFP group at 3 days. . **B** Lane 1 Control group at 5 days. Lane 2 Experimental group at 5 days. Lane 3 GFP group at 5 days. Lane 4 Protein molecular weight marker. Lane 5 Control group at 7 days. Lane 6 Experimental group at 7 days. Lane 7 GFP group at 7 days. . **C** Lane 1 Control group at 9 days. Lane 2 Experimental group at 9 days. Lane 3 GFP group at 9 days. Lane 4 Protein molecular weight marker. Lane 5 Control group at 11 days. Lane 6 Experimental group at 11 days. Lane 7 GFP group at 11 days. **D** Lane 1 Control group at 13 days. Lane 2 Experimental group at 13 days. Lane 3 GFP group at 13 days.

to the potential difference in concentration of each sample, no substantial conclusions about significant band intensity or appearance or lack of banding can be determined. Furthermore, due to the lack of visibility of the protein molecular weight standard, analysis of molecular weights of various protein bands are also unable to be concluded.

3.4 – Polymerase Chain Reaction Analysis

3.4.1 – Evaluation of Titin and Actin Primer Sets

The genomic DNA (gDNA) sample was used to assess titin and actin primer function at over a range of annealing temperatures. **Figure 27** shows the resulting analysis of the gradient PCR reactions analyzed on a 2 % agarose gel. The PCR products for both actin and titin reactions resulted in the expected band sizes.

3.4.2 – cDNA Purity Assessment

Next, to test the purity of both primer sets and the four cDNA samples, RT-PCR reactions were completed and analyzed on a 1.5 % agarose gel. In the top gel of **Figure 28** lanes 1 through 8 show the results of the amplification of C2C12 cDNA samples at time points 0, 4, 8, and 12 days post differentiation initiation using the actin primer set. The lane labeled gDNA is the RT-PCR result of the genomic DNA. The band in this lane when compared to those in lanes 1 through 8 show a size difference in PCR product of 57 bp. This suggests the gDNA contains an intron that the cDNA does not. This information also indicates there is no gDNA contamination in any of the cDNA samples. Additional confirmation of an intron is shown in **Figure 29** where each the cDNA and

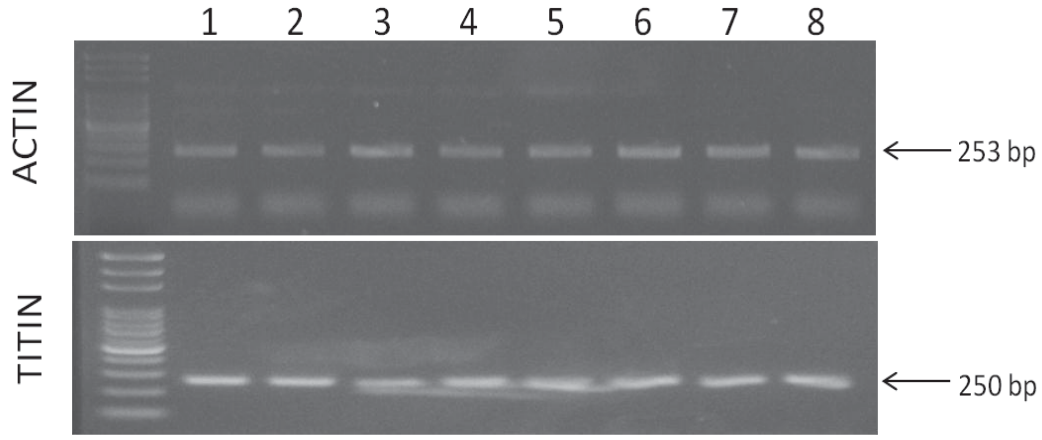


Figure 27: Gradient PCR using actin and titin primers to amplify from mouse c2c12 gDNA. Lane 1 is the PCR reaction at the annealing temperature of 50 °C. Lane 2: 50.8 °C. Lane 3: 52.1 °C. Lane 4: 54 °C. Lane 5: 65.2 °C. Lane 6: 58 °C. Lane 7: 59.3 °C. Lane 8: 60 °C.

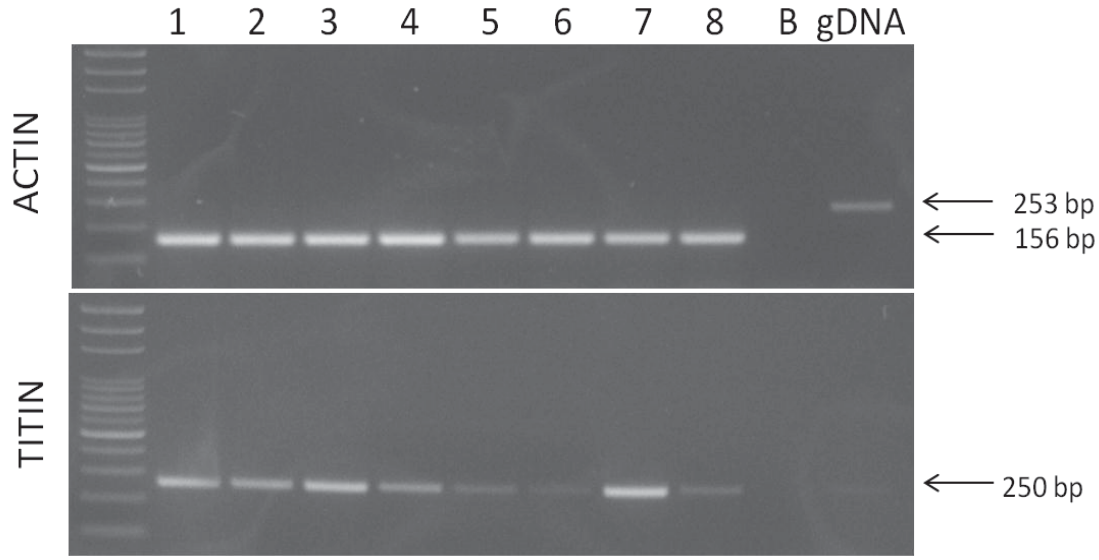


Figure 28: RT-PCR of actin and titin from mouse c2c12 cDNA. Lane 1 and 2: T=0 cDNA, Lane 3 and 4: T=4 cDNA, lane 5 and 6: T=8 cDNA, Lane 7 and 8: T=12 cDNA, B: Blank control reaction, gDNA: gDNA control reaction.

gDNA sequences are compared. Due to the size difference between cDNA and gDNA RT-PCR products and no visible band in the blank lane, it can be confirmed that all cDNA samples and the primer are uncontaminated.

The bottom gel in **Figure 28** do not show a size difference between cDNA and gDNA RT-PCR products. BLAST analysis (shown in **Figure 30**) of the available cDNA and gDNA sequences for the titin amplicon further confirm that there is no intron present.

```
Seq_1  1     AACGTGCCCATCTATGAGGGCTATgccctgccacacgccatcatgcgtctggacctggcc  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1     AACGTGCCCATCTATGAGGGCTATgccctgccacacgccatcatgcgtctggacctggcc  60

Seq_1  61    ggtcgcgacctcactgactacctgatgaaaatcctcactgagcgtggctattccttcgtg  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    ggtcgcgacctcactgactacctgatgaaaatcctcactgagcgtggctattccttcgtg  120

Seq_1  121   accacag-------------------------------------------------------  127
             |||||||
Seq_2  121   accacaggtgcgtgcttccaatgcccgctgagggagggcgggcagagggtgagcacacgc  180

Seq_1  128   ----------------------------------------------ctgaaCGTGAGATTGT  143
                                                           ||||||||||||||||
Seq_2  181   cccaggccttgccctgaggccccccactgcttttgctcctgcagctgaaCGTGAGATTGT  240

Seq_1  144   GCGCGACATCAAA  156
             |||||||||||||
Seq_2  241   GCGCGACATCAAA  253
```

Figure 29. Comparison of actin amplicons from C2C12 cDNA and gDNA. (cDNA, Seq 1 and gDNA, Seq 2). The highlighted region is the probable intron sequence.

```
Seq_1  1     gacctgaggacctggaagtcaaagaagtcactaaaaatactgtgtctttgacttggaacc  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1     gacctgaggacctggaagtCAAAGAAGTCACTAAAAATACTGTGTCTTTGACTTGGAACC  60

Seq_1  61    ctcccaagtatgatggcgggtcagaaattataaactatgtcctagaaagccgcctcattg  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  61    CTCCCAAGTATGATGGCGGGTCAGAAATTATAAACTATGTCCTAGAAAGCCGCCTCATTG  120

Seq_1  121   gtactgagaagttccacaaagttacaaatgacaacctgcttagcaggaaatacactgtga  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  121   GTACTGAGAAGTTCCACAAAGTTACAAATGACAACCTGCTTAGCAGGAAATACACTGTGA  180

Seq_1  181   aaggcttaaaagaaggcgatacatatgagtaccgcgtcagtgccgtcaatatcgttggac  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  181   AAGGCTTAAAAGAAGGCGATACATATGAGTACCGCGTCAGTGCCGTCAATatcgttggac  240

Seq_1  241   aaggcaagcc  250
             ||||||||||
Seq_2  241   aaggcaagcc  250
```

Figure 30. Comparison of titin amplicons from C2C12 cDNA and gDNA. (cDNA, Seq 1 and gDNA, Seq 2).

3.4.3 – Quantitative Polymerase Chain Reaction Parameter Analysis

qPCR reactions were completed using various cDNA and primer concentrations. **Figure 31** shows the amplification curves and standard graphs of C2C12 cDNA at T = 0 for the actin primer set. These results reveal an annealing temperature of 60 °C and a primer concentration of 200 nM are the most favorable specifications for the qPCR reaction. Using the same varying primer and cDNA concentrations, an optimal setting could not be found for the titin primer set. The results of the amplification curves and standard graphs for the titin primer set is illustrated in **Figure 32.** It is shown that all settings for this set of primers yield poor results. BLAST analysis of the titin primers are shown in **Figure 33** and they reveal a less than perfect match. It can be concluded that, due to the precision required for the qPCR technique, these primers will never yield significant results.

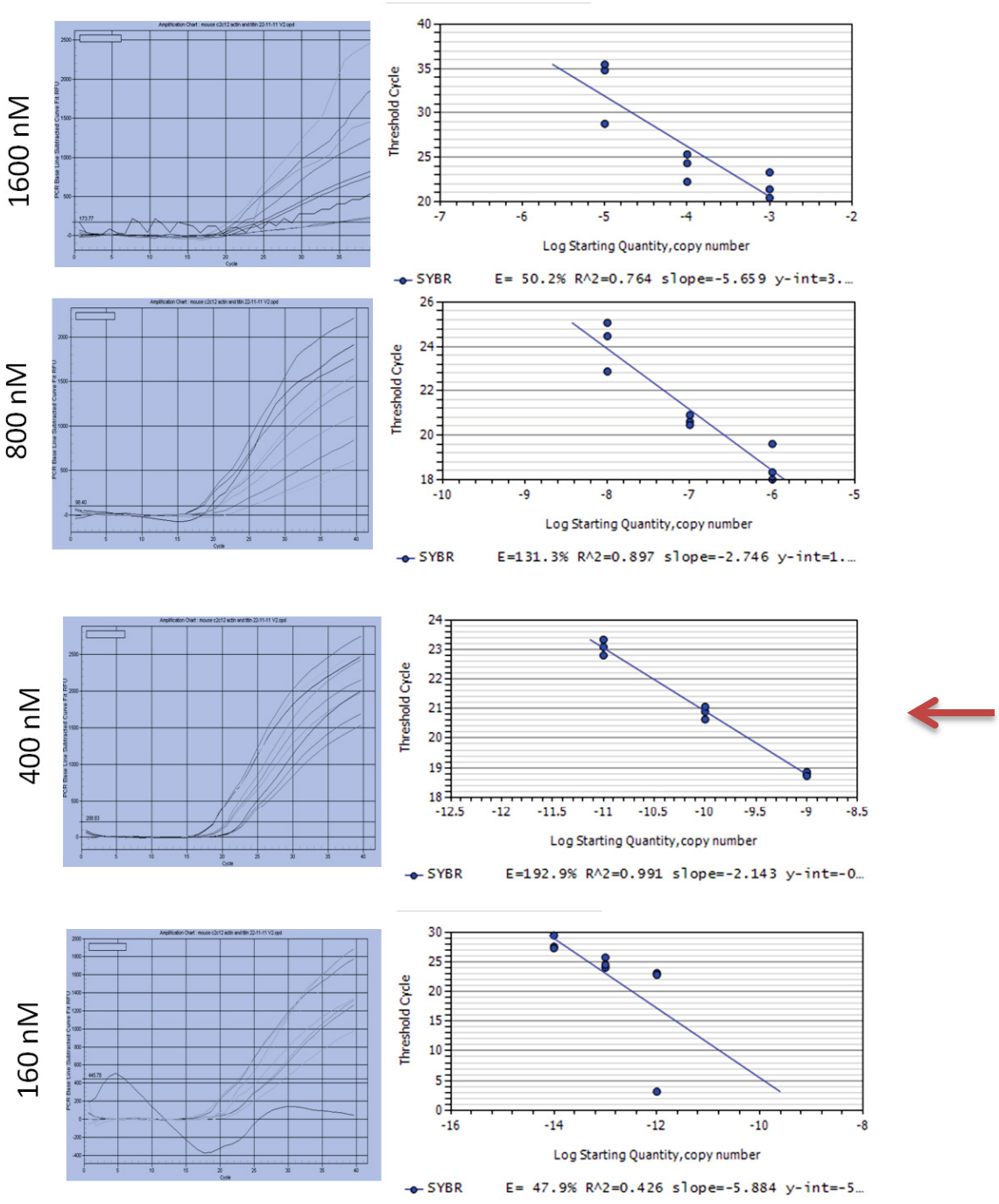


Figure 31: Standard curves in the amplification of actin. The optimum reaction is observed when primers at a 400 nM concentration are used. Graph indicated by red arrow

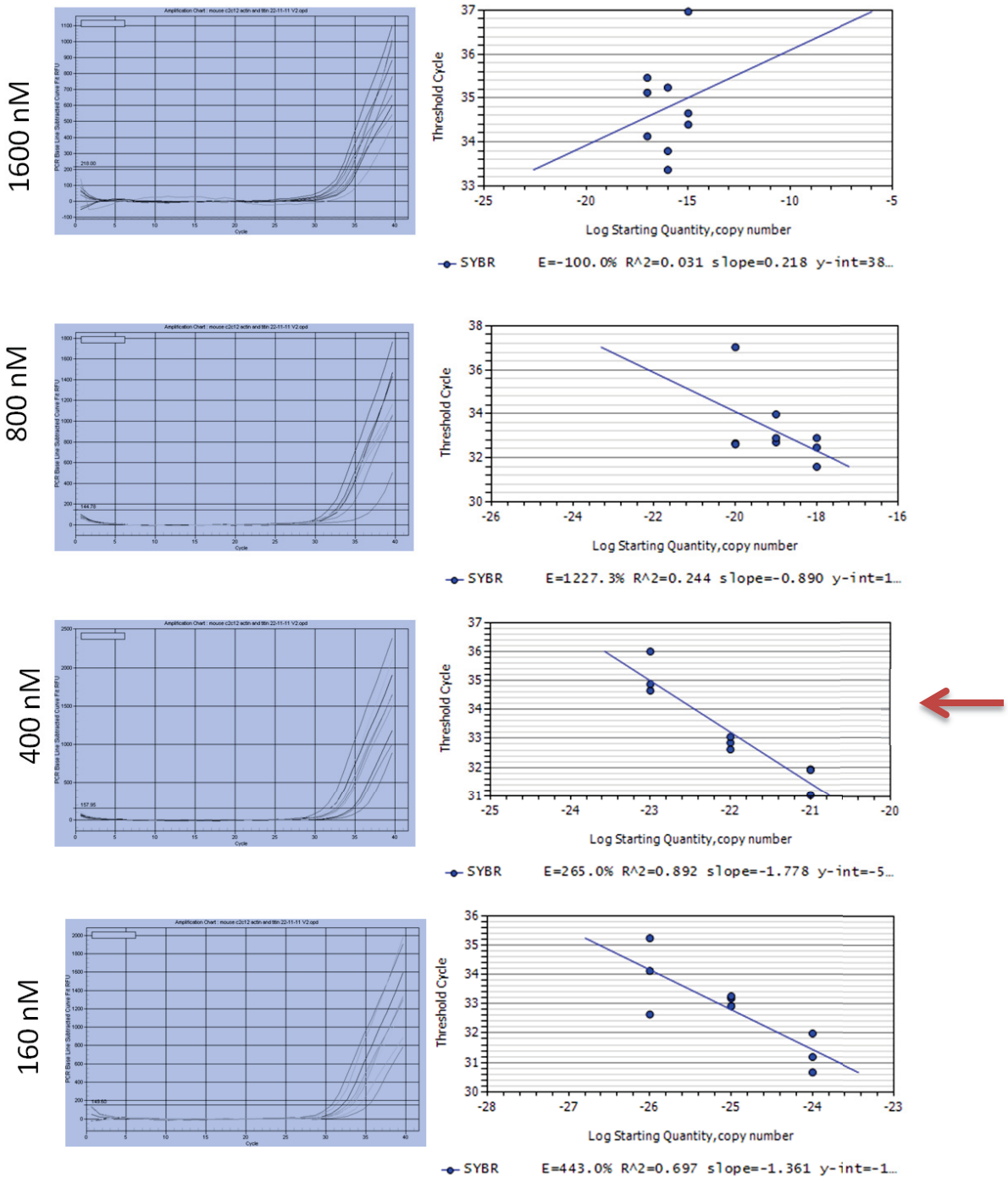


Figure 32: Standard curves in the amplification of titin. All amplification curves show these PCR parameters to be sub-optimal. The best reaction is observed using primers at a concentration of 400 nM, as indicated by the red arrow.

```
TITIN FORWARD GENE
Similarity : 17/19 (89.47 %)

Seq_1  1     CGTCCTGAAGACCTGGAAGT  20
              | ||||| ||||||||||
Seq_2  1     -gacctgaggacctggaagt  19
TITIN REVERSE GENE
Similarity : 18/20 (90.00 %)

Seq_1  1     GGTTTGCCTTGTCCAACAAT  20
             || |||||||||||||| ||
Seq_2  1     ggcttgccttgtccaacgat  20
```

Figure 33. BLAST analysis of titin primers and the *titin* sequence. (Primer, Seq 1; *titin*, Seq 2)

Chapter 4: Discussion

Titin is a gigantic protein, composed of 240 compactly folded domains, found within the sarcomere of skeletal muscle cells. Much research has been completed in determining the roles of titin such as it functions as a "shock absorber" of the sarcomere and prevents damage from the stress of muscle cell stretching [3], due in part to its elastic nature [8]. It is responsible for structure, it plays a role in signaling processes throughout the muscle cell, and mutations result diseases grouped together as titinopathies [13], however this gigantic protein still remains a mystery. Further characterization of titin and its role in cell structure and function is essential in understanding rippling muscle disease and its most interesting symptom, the progression of electrically silent rolling contractions along the length of a muscle induced by tapping or stretching.

The main goal of this research was to determine the effects of overexpression of the immunogenic titin domain on growth and differentiation of C2C12 cells. This research gives preliminary results that substantiate that this method of studying the immunogenic titin domain can lead to a significant understanding of the role of specific titin domains in muscle development and function. We conclude that these preliminary results are reproducible and the method of overexpression can be used to identify the role of the RMMG#6 immunogenic titin domain. Also, in the future, these methods can be employed to determine the functions of other domains along the titin protein.

4.1 – Plasmid Construction/Colony Screening:

We successfully constructed a plasmid that will produce the titin immunogenic domain in frame and attached to a GFP tracker protein shown by DNA sequencing, and Genious software and BLASTn analysis. The results of the analysis of clone sequences using Genious software show pairwise similarity between 68.8 and 76.2%. The reason the number is as low as it is, is because part of the clone sequence includes portions of the GFP plasmid. This percent would increase/improve provided the GFP plasmid sequences are removed from the sequence. Thus, these methods of plasmid construction can be used to successfully construct other fusion plasmids using other domains of interest within the titin protein. This plasmid makes a suitable vehicle for transfection of mammalian cells.

4.2 – Morphological analysis

The question of the role of the RMMG#6 domain in muscle development and function would be answered by analysis of transfection effects on C2C12 morphology. Preliminary results (not shown) revealed a difference in morphological features between control group and those transfected with the recombinant plasmid. Control cells moved through the differentiation process as expected, with elongation and fusion of cells. The recombinant cultures maintained a small single nucleated appearance.

Final time trial analysis gave similar results. Analysis of control groups at each time point revealed that a small number of cells within each culture progressed through the differentiation process. These cultures did not have as many cells that differentiated

as we have normally seen. This could be due to the cultures not being as confluent as they should have been. This experiment was conducted in such a manner that made it possible to look at the morphological aspects of individual cells. This experimental design has one caveat: if concentrations are low and cells are not close to one another, fusion of the cells cannot take place. Future experiments in which transfection will be carried out at sequential time points during myogenesis should allow us to determine the domain specific effects and the cell density effects.

The cell cultures within the experimental group did not show signs of cell fusion and forming multinucleated cell structures. The majority of the cells within these cultures, even when in a group of cells that were close to each other (**Figure HH and JJ**) did not show features of fusion. This is potentially due to the small concentration of cells resulting from the transfection/selection process. Few cells showed elongation but most remained small and round, a similar feature of those cells still in the cell replication process. Again for the purposes of this preliminary experimental process, we felt it was important to view cells on an individual basis.

GFP group cultures also did not show basic features of cell culture differentiation. The cells did show some elongation, more than the experimental group, however there was a lack of cell fusion.

4.3 – Fluorescent Microscopy:

Expression of either GFP–fusion protein or GFP alone, were confirmed in C2C12 cells by fluorescence microscopy. The control group showed minimal background

fluorescents. While using this group as a comparison for the experimental and GFP groups, this hazy fluorescents was disregarded. The GFP and experimental groups show an increase in fluorescents throughout the time points taken, beginning with a definite increase by day 3 post differentiation initiation. Each had a different pattern of fluorescent spotting. The GFP cells, with more elongation of the cell, show a localization of fluorescents (GFP protein) closely oriented around the nucleus throughout the first week of differentiation, followed by dispersal of these spots throughout the cell within the last week. The experimental group tended to have fluorescent localization throughout the cell, with no fluorescents within the nucleus. Further analysis and experimentation need to be done in order to optimize the information gained by this type of observation.

4.4 – SDS-PAGE Protein Analysis

Fusion protein expression was also studied at the biochemical level. The levels of expression in the preliminary studies proved to be highly variable. The protein samples obtained from all groups and time points were loaded onto an SDS-PAGE gel in equal volumes. The preliminary gels in **Figure LL** show a drastic range of concentrations between each sample. Due to this, we cannot confirm or dispute increases or decreases in various protein products between the three groups or seven time points. Another issue is the fact that the control and experimental group trials were completed at a separate time than the GFP group. Each initial culture used to set up these groups were passaged the same number of times before transfection, however the GFP group was allowed to proliferate for an extended period of time because they were transfected before they reached 80% confluence. This extra incubation period may have an effect on the

development and final protein concentrations obtained from each culture. Perhaps other techniques can be applied to more accurately measure fusion protein expression.

4.5 – Quantitative Polymerase Chain Reaction

Work has been initiated in our lab to follow expression by quantitative polymerase chain reaction. Methods and settings for obtaining substantial qPCR results for *actin* production during differentiation of C2C12 cells have been determined. Identification of effective setting for the quantification of the immunogenic titin domain in vivo has proven to be unsuccessful. The titin primer sets currently in use are not 100 % complimentary to the titin sequence of interest and therefor multiple adjustments to the qPCR settings have failed to produce any meaningful results. Despite the current impediment we believe that this method will be an advantageous method in furthering the understanding of the role of the RMMG#6 domain. Once the optimizations of primer and qPCR conditions have been determined, this approach shows great promise in studying expression of titin domain specific fusion protein expression.

4.6 – Future Research:

Preliminary results show promise of a successful method of analysis of the immunogenic titin domain, however, this research must be repeated to ensure reproducibility. Reproducibility will require more standardization of experimental conditions and more stringent control of the culture environment. During the morphological and fluorescent analysis of transformed C2C12 cells, cultures need to be complete at the same time. Before transfection, cell concentrations must be at least 80 %

confluent and this confluency must be maintained throughout the differentiation process. SDS-PAGE analysis of the protein samples obtained needs to be repeated using equal concentrations of each sample and another method of staining needs to be employed to ensure visibility of the protein standards. Finally, qPCR methods require additional modifications to ensure the quantification of only the domain of interest within this massive gene.

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Appendix I - Materials

Cell Cultures and Plasmids

Product Name	Manufacturer (City, State)
C2C12	American Type Culture Collection(ATCC) (Manassas, VA)
One Shot TOP10 Chemically Competant	Invitrogen (Carlsbad, CA)
pAcGFP1-C1	Clontech Laboratories, Inc. Produced by Takara Biotechnology (Mountain View, CA)
pGEX-3X vector	GE Healthcare Services (Piscataway, NJ)

Chemicals and Reagents

Product Name	Manufacturer (City, State)
25X TAE Buffer	Amresco (Solon, OH)
2-mercaptoethanol	Sigma Alderich (St. Louis, MO)
40% Acrylamide	Amresco (Solon, OH)
Agar	Fisher Scientific (Pittsburg, PA)
Agarose I Tablets, 500mg	Amresco (Solon, OH)
Ammonium Persulfate	BioRad (Hercules, CA)
Ampicillian	Sigma Alderich (St. Louis, MO)
Amresco EZ-VisionThree DNA Dye and Buffer 6x	Amresco (Solon, OH)
Antibiotic Antimycotic Solution 100X (10000 units Pennicillin, 10 mg Streptomycin, 25 ug Amphotericin B per ml)	Sigma Alderich (St. Louis, MO)
Calcium Chloride ($CaCl_2$)	Fisher Scientific (Pittsburg, PA)
Chloroform	Amresco (Solon, OH)

Chemicals and Reagents (Continued)

Product Name	Manufacturer (City, State)
DNA MW Marker, 100 bp Ladder	Amresco (Solon, OH)
Dulbecco's Modified Eagle's Media (with 1000mg glucose/L, L-glutamine, $NaHCO_3$, and pyridoxine, HCl)	Amresco (Solon, OH)
Ethanol	Amresco (Solon, OH)
Ethylenediaminetetraacetic acid (EDTA)	Fisher Scientific (Pittsburg, PA)
Formaldehyde	Fisher Scientific (Pittsburg, PA)
Glacial Acetic Acid	PharmCo-AAPER (Brookfield, CT)
Glucose	Amresco (Solon, OH)
Glycerol	Amresco (Solon, OH)
Glycine	Amresco (Solon, OH)
High Fetal Bovine Serum	Invitrogen (Carlsbad, CA)
Hydrochloric Acid (HCl)	Fisher Scientific (Pittsburg, PA)
Isopropanol	Amresco (Solon, OH)
Kanamycin	Sigma Alderich (St. Louis, MO)
Magnesium Chloride Anhydrous ($MgCl_2 \cdot 6H2O$)	J T Baker Reagent Chemicals (Phillipsburg, NJ)
Magnesium Sulfate ($MgSO_4$)	Amresco (Solon, OH)
Methanol	Amresco (Solon, OH)
Neomycin	Amresco (Solon, OH)
n-Propyl Gallate	Sigma Alderich (St. Louis, MO)
Nuclease Free Water	Amresco (Solon, OH)

Chemicals and Reagents (Continued)

Product Name	Manufacturer (City, State)
Potassium Chloride (KCl)	Amresco (Solon, OH)
Potassium Phosphate Monobasic Crystal	Fisher Scientific (Pittsburg, PA)
Sodium Acetate (NaOAc)	Fisher Scientific (Pittsburg, PA)
Sodium Chloride (NaCl)	Amresco (Solon, OH)
Sodium Phosphate Dibasic Anhydrous	Fisher Scientific (Pittsburg, PA)
Tetramethylethylenediamine (TEMED)	Sigma Alderich (St. Louis, MO)
Tris	Fisher Scientific (Pittsburg, PA)
Tris-HCl	Amresco (Solon, OH)
Trypsin 1x [Gamma Irradiated 0.25% Porcine Trypsin (1:250) in HBSS with 0.1% EDTA-2Na, without Ca and Mg]	Sigma Alderich (St. Louis, MO)
Tryptone	Amresco (Solon, OH)
TWEEN20	Amresco (Solon, OH)
Yeast Extract	Amresco (Solon, OH)

Kits, Enzymes, and Buffers

Product Name	Manufacturer (City, State)
10x CIP Buffer	New England BioLabs (Ipswich, MA)
10x DNase I Reaction Buffer	Invitrogen (Carlsbad, CA)
10X Reaction Buffer with MgCl2	Fisher Scientific (Pittsburg, PA)
10x T4 Buffer	New England BioLabs (Ipswich, MA)

Kits, Enzymes, and Buffers (Continued)

Product Name	Manufacturer (City, State)
Beckman Coulter GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit	Beckman Coulter, Inc. (Fullerton, CA)
BioRad iScript™ Select cDNA Synthesis Kit	BioRad (Hercules, CA)
Buffer 2	New England BioLabs (Ipswich, MA)
Calf Intestinal Phosphatase (CIP)	New England BioLabs (Ipswich, MA)
Clontech Xfect Transfection Reagent	Clontech Laboratories, Inc. Produced by Takara Biotechnology (Moutainview, CA)
DEPC-treated Water	Fisher Scientific (Pittsburgh, PA)
DNase I, Amp Grade 1 U/µl	Invitrogen (Carlsbad, CA)
DNase I, RNase-free	Fisher Scientific (Pittsburg, PA)
EcoR1 Reaction buffer (10x)	New England BioLabs (Ipswich, MA)
EcoR1 enzyme	New England BioLabs (Ipswich, MA)
GoTaq® Green Master Mix	Promega Corporation (Madison, WI)
***Hind*III**	New England BioLabs (Ipswich, MA)
iQ™ SYBR® Green Supermix	BioRad (Herculese, CA)
iScript™ Select cDNA Synthesis Kit	BioRad (Herculese, CA)
QIAGEN DNeasy Blood & Tissue Kit	Qiagen Laboratories (Valencia, CA)
QIAGEN Plasmid Mini Kit	Qiagen Laboratories (Valencia, CA)
QIAGEN QIAprep® Spin Miniprep Kit	Qiagen Laboratories (Valencia, CA)
Ribosol™ RNA Extraction Reagent	Amresco (Solon, OH)
T4 DNA Ligase	New England BioLabs (Ipswich, MA)

Equipment and Software

Product Name	Manufacturer (City, State)
Beckman Coulter CEQ™ 2000XL automated sequencer	Beckman Coulter (Brea, CA)
BioRad iCycler	BioRad (Hercules, CA)
BioRad Mini PROTEAN® 3 System	BioRad (Hercules, CA)
BioRad Pharos FX™ Plus Molecular Imager (#170-9460)	BioRad (Hercules, CA)
BioRad Power PAC 1000	BioRad (Hercules, CA)
BioRad SmartSpec™ Plus spectrophotometer	BioRad (Hercules, CA)
BioRad Quantity One 1-D Analysis Software (Version 4.6.9 Build 030)	BioRad (Hercules, Ca)
CentriVap DNA Concentrator (CAT # 7970010)	LabConCo® Corporation (Kansas City, MO)
Fisher BioTech Electrophoresis Systems 312nm Variable Intensity Transilluminator FBTIV-816	Fisher Scientific (Pittsburg, PA)
Fotodyne Incorporated electrophoresis chamber	Fotodyne Incorporated (Heartland, WI)
Fotodyne Power Supply Line Voltage 115VAC 50-60Hz	Fotodyne Incorporated (Heartland, WI)
Galaxy 16D VWR 37001-300	VWR International (Buffalo Grove, IL)
Genious Pro 3.5.4 (Build 2007-11-22 11:27; Java Version 1.5.0_30-b03-389-9M3425) © 2005-2007	Biomatters Ltd. (Auckland, New Zeland)
Olympus IX 51 Inverted Microscope	Olympus America (Center Valley, PA)
Olympus LH 50A Inverted Phase Contrast Microscope	Olympus America (Center Valley, PA)
Sorvall® RC 5B PLUS Centrifuge Rotor: Sorvall SA-600	Sorvall Products (Newtown, CT)
SPOT idea™ 5mp Color Mosaic (260461)	SPOT Imaging Solutions/ Diagnostic Instruments Inc. (Sterling Heights, MI)

Appendix 2 – Solutions and Media

Bacterial Culture Growth and Storage

2.1 1000x Kanamycin Stock

3 g	Kanamycin
100 ml	Deionized water

Kanamycin was dissolved in 100 ml deionized water. 1.5 ml aliquots were transferred to 1.8 ml microcentrifuge tubes and stored at -20° C.

2.2 1000x Ampicillin Stock

5 g	Ampicillin
100 ml	Deionized water

Ampicillin was dissolved in 100 ml deionized water. 1.5 ml aliquots were transferred to 1.8 ml microcentrifuge tubes and stored at -20 °C.

2.3 Luria-Bertani (LB) Broth (500 ml)

5.0 g	Tryptone
2.5 g	Yeast
2.5 g	NaCl
	Deionized water

Tryptone, yeast, and NaCl were combined with 400 ml deionized water. The solution was adjusted to a total volume of 500 ml with deionized water and sterilized by autoclaving at 120 °C for 20 minutes. Media was stored at 4 °C.

2.4 LB-Kanamycin (LB_{KAN}) Broth (500 ml)

500 ml	LB Broth, Sterile
500 µl	1000x Kanamycin

LB broth was prepared as previously mentioned (Appendix 2.3). When media was cool 500 µl of 1000x Kanamycin Stock (Appendix 2.1) was added. Media was stored at 4 °C.

2.5 LB-Ampicillin (LB_{AMP}) Broth (500 ml)

500 ml	LB Broth, Sterile
500 µl	1000x Ampicillin

LB broth was prepared as previously mentioned (Appendix 2.3). When media was cool 500 µl of 1000x Ampicillin Stock (Appendix 2.2) was added. Media was stored at 4 °C.

2.6 LB Agar (500 ml)

5.0 g	Tryptone
2.5 g	NaCl
2.5 g	Yeast
10 g	Agar
	Deionized water

Tryptone, NaCl, yeast, and agar were combined in 400 ml deionized water. The final volume was adjusted to 500 ml with deionized water and sterilized by autoclaving at 120 °C for 20 minutes. When the liquid cooled it was poured into sterile 100 mm x 15 mm polystyrene petri dished in a sterile hood. Plates were stored at 4 °C.

2.7 LB_{KAN} Agar (500 ml)

5.0 g	Tryptone
2.5 g	NaCl
2.5 g	Yeast
10 g	Agar
	Deionized water
500 µl	1000x Kanamycin

Tryptone, NaCl, yeast, and agar were combined in 400 ml deionized water. The final volume was adjusted to 500 ml with deionized water and sterilized by autoclaving at 120 °C for 20 minutes. When the solution cooled, 500 µl of 1000x kanamycin stock (Appendix 2.1) was added and the liquid was poured into sterile 100 mm x 15 mm polystyrene petri dished in a sterile hood. Plates were stored at 4 °C.

2.8 LB_{AMP} Agar (500 ml)

5.0 g	Tryptone
2.5 g	NaCl
2.5 g	Yeast
10 g	Agar
	Deionized water
500 µl	Ampicillin

Tryptone, NaCl, yeast, and agar were combined in 400 ml deionized water. The final volume was adjusted to 500 m; with deionized water and sterilized by autoclaving at 120 °C for 20 minutes. When the solution cooled, 500 µl of 1000x ampicillin stock (Appendix 2.2) was added and the liquid was poured into sterile 100 mm x 15 mm polystyrene petri dished in a sterile hood. Plates were stored at 4 °C.

Competent Cell Protocol

2.9 Sterile 0.15M NaCl

0.44 g	Sodium Chloride (NaCl)
50.0 ml	Deionized water

NaCl was added to 50 ml deionized water then sterilized by autoclaving at 120 °C for 20 minutes. The solution was stored at 4 °C.

2.10 Sterile Transformation Buffer (1 L)

(15% glycerol (v/v) 0.5M $CaCl_2$, 10mM Tris-HCl pH 8.0, and 10mM $MgCl_2$)

15.0 ml	Glycerol
1.47 g	Calcium Chloride ($CaCl_2$)
1.0 ml	Tris-HCl
0.20 g	Magnesium Chloride Anhydrous ($MgCl_2 \cdot 6H_2O$)
	Deionized water

Glycerol, $CaCl_2$, tris-HCl, and $MgCl_2 \cdot 6H_2O$ were added to 1 L deionized water, then sterilized by autoclaving at 120 °C for 20 minutes. The solution was stored at 4 °C.

Agarose Gel Electrophoresis

2.11 1x Tris-acetate-EDTA (TAE) Buffer

40 ml	25x TAE Buffer
960 ml	Deionized water

25x TAE buffer was added to deionized water and stored at room temperature.

2.12 2% Agarose gel

100 ml	1x TAE Buffer
2 g	Agarose I tablets (500 mg)

Agarose I tablets were dissolved in 1x TAE buffer (Appendix 2.11) and microwaved on high for 1 – 2 minutes. When liquid cooled slightly it was poured into an electrophoresis gel tray, a comb was inserted, and was allowed to solidify.

C2C12 Culture

2.13 C2C12 Initial Growth Media (C2IGM)

[DMEM +20% FBS +1% Antibiotic/Antimycotic]

79.0 ml	Dulbecco's Modified Eagle's Media (DMEM) (with 1000mg glucose/L, L-glutamine, $NaHCO_3$, and pyridoxine HCL)
20.0 ml	Fetal Bovine Serum (FBS)
1.0 ml	Antibiotic Antimycotic Solution 100x [10000 units Penicillin, 10 mg Streptomycin, 25 µg Amphotericin B per ml]

DMEM, FBS and Antibiotic/ Antimycotic solution were combined and filter sterilized. Media was stored at 4 °C.

2.14 C2C12 Growth Media (C2GM)

[DMEM +10% FBS +1% Antibiotic/Antimycotic]

500 ml	DMEM
55.0 ml	FBS
5.5 ml	Antibiotic Antimycotic Solution 100x [10000 units Penicillin, 10 mg Streptomycin, 25 µg Amphotericin B per ml]

DMEM, FBS and Antibiotic/ Antimycotic solution were combined and filter sterilized. Media was stored at 4 °C.

2.15 C2C12 Differentiation Media (C2DM)

[DMEM +1% FBS +1% Antibiotic/Antimycotic]

500 ml	DMEM
5.5 ml	FBS
5.5 ml	Antibiotic Antimycotic Solution 100x [10000 units Penicillin, 10 mg Streptomycin, 25 µg Amphotericin B per ml]

DMEM, FBS and Antibiotic/ Antimycotic solution were combined and filter sterilized. Media was stored at 4 °C.

2.16 C2C12 Growth Media + 0.5 mg/ml Neomycin

14.25 ml	C2GM
0.75 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2GM (Appendix 2.23) and stored at 4 °C.

2.17 C2C12 Growth Media + 1.0 mg/ml Neomycin

13.5 ml	C2GM
1.5 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2GM (Appendix 2.23) and stored at 4 °C.

2.18 C2C12 Growth Media + 1.5 mg/ml Neomycin

12.75 ml	C2GM
2.25 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2GM (Appendix 2.23) and stored at 4 °C.

2.19 C2C12 Growth Media + 2.0 mg/ml Neomycin

12.0 ml	C2GM
3.0 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2GM (Appendix 2.23) and stored at 4 °C.

2.20 C2C12 Growth Media + 2.5 mg/ml Neomycin

11.25 ml	C2GM
3.75 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2GM (Appendix 2.23) and stored at 4 °C.

2.21 C2C12 Growth Media + 3.0 mg/ml Neomycin

10.5 ml	C2GM
4.5 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2GM (Appendix 2.23) and stored at 4 °C.

2.22 C2C12 Growth Media + 3.5 mg/ml Neomycin

9.75 ml	C2GM
5.25 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2GM (Appendix 2.23) and stored at 4 °C.

2.23 C2C12 Growth Media + 4.0 mg/ml Neomycin

9.0 ml	C2GM
6.0 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2GM (Appendix 2.23) and stored at 4 °C.

2.24 C2C12 Growth Media + 2.75 mg/ml Neomycin

10.875 ml	C2GM
4.125 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2GM (Appendix 2.23) and stored at 4 °C.

2.25 C2C12 Differentiation Media + 2.5 mg/ml Neomycin

11.25 ml	C2DM
3.75 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2DM (Appendix 2.24) and stored at 4 °C.

2.26 C2C12 Differentiation Media + 2.75 mg/ml Neomycin

14.5 ml	C2DM
5.5 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2DM (Appendix 2.24) and stored at 4 °C.

2.27 C2C12 Differentiation Media + 3.0 mg/ml Neomycin

10.5 ml	C2DM
4.5 ml	Neomycin (10 mg/ml)

Neomycin was added to sterile C2DM (Appendix 2.24) and stored at 4 °C.

2.28 10x Tris Buffered Saline (10x TBS Buffer) (1 L)

24.22 g	Tris
87.66 g	NaCl
800 ml	Deionized water

Tris and NaCl were dissolved in 800 ml deionized water. The solution was titrated to pH to 7.3 and then diluted to 1 L with deionized water. Buffer was diluted to 1x prior to use and stored at room temperature.

2.29 3.7% Formaldehyde Fixing Solution

36 ml	1 x TBS
4 ml	37% Formaldehyde

37 % Formaldehyde was added to 1x TBS (Appendix 2.20) and stored at room temperature.

2.30 10 mM Phosphate Buffered Solution (PBS) (pH 7.4 with TWEEN20)

0.26 g	Potassium Phosphate Monobasic Crystal (KH_2PO_4)
2.17 g	Sodium Phosphate Dibasic Anhydrous ($Na_2HPO_4 \cdot 7H_2O$)
8.71 g	NaCl
	Deionized water
0.5 ml	TWEEN20

KH_2PO_4, $Na_2HPO_4 \cdot 7H_2O$, and NaCl were dissolved in 800 ml deionized water. The solution was titrated to pH 7.4 and adjusted to a total volume of 1 L. 0.5 ml TWEEN20 was added and stored at room temperature.

2.31 Buffered Glycerol with Anti-Fade Solution

[90% Glycerol (v/v), 10% PBS (v/v), 2% n-propyl gallate]

22.5 ml	Glycerol
2.5 ml	PBS
0.5 g	n-propyl gallate

Glycerol, PBS and n-propyl gallate were combined in a 50 ml conical tube. The tube was wrapped in foil and gently rocked on a shaker until the n-propyl gallate was dissolved. The solution was stored at 4 °C.

SDS-PAGE

2.32 1x Sodium Dodecyl Sulfate Sample Buffer (1x SDS Sample Buffer)

12.5 g	Glycerol
0.76 g	Tris
5.00 ml	2-mercaptoethanol
2.30 g	Sodium dodecyl sulfate (SDS)
12.3 ml	0.5M Hydrochloric Acid (HCl)
71.0 ml	Deionized water

Glycerol, Tris, 2-mercaptoethanol, SDS, and HCl were added to approximately 71 ml deionized water. The buffer was titrated to the optimal pH (6.8) and the final volume was adjusted to 100 ml. The buffer was stored at 4 °C.

2.33 10x Running Buffer Recipe (10x TGS Buffer) (1 L)

30 g	Tris
144.0 g	Glycine
10 g	SDS
	Deionized water

Tris, Glycine, and SDS were dissolved in deionized water. The pH was titrated to 8.3 and the final volume was adjusted to 1L with deionized water. The running buffer was diluted to 1x before use and stored at room temperature.

2.34 Resolving Gel Buffer (500 ml)

90.85 g	Tris
2.0 g	SDS
450 ml	Deionized water

Tris, and SDS were dissolved in 450 ml deionized water. The buffer was then titrated to pH 8.8 and diluted to 500 ml with deionized water and stored at 4 °C.

2.35 Stacking Gel Buffer (200 ml)

12.1 g	Tris
0.8 g	SDS
180 ml	Deionized water

Tris, and SDS were dissolved in 180 ml deionized water. The buffer was then titrated to pH 6.8 and diluted to 200 ml with deionized water and stored at 4 °C.

2.36 10% Ammonium Persulfate

1.0 g	Ammonium persulfate
10 ml	Deionized water

Ammonium persulfate was dissolved in 10 ml deionized water and stored at 4 °C.

2.37 10% SDS-PAGE Resolving gel

49.0 ml	Deionized water
25.0 ml	40% acrylamide
25.0 ml	Resolving Gel Buffer
1.0 ml	10% ammonium persulfate
0.04 ml	TEMED

40% acrylamide, Resolving Gel Buffer (Appendix 2.15), and 10% ammonium persulfate (Appendix 2.17) were added to 49.0 ml deionized water. TEMED was added to the solution immediately prior to injection in to the gel casting cassette. Gels were allowed to set at room temperature until firm (30 - 60 minutes). Gels were stored in a plastic container between paper towels moistened with deionized water at 4 °C.

2.38 6% SDS-PAGE Stacking Gel

2.9 ml	Deionized water
0.75 ml	40% acrylamide
1.3 ml	Stacking Gel Buffer
0.05 ml	10% ammonium persulfate
0.004 ml	TEMED

40% acrylamide, Stacking Gel Buffer (Appendix 2.16), and 10% ammonium persulfate (Appendix 2.17) were added to 2.9 ml deionized water. TEMED was added immediately prior to pipetting onto a previously cast running gel (Appendix 2.18). A comb was inserted and the stacking gel was allowed to solidify (15 - 30 minutes).

2.39 De-stain Solution

100 ml	Glacial Acetic Acid
150 ml	Methanol
750 ml	Deionized water

Glacial acetic acid, methanol and deionized water were combined and stored at room temperature.

Appendix 3 – Supplementary Figures

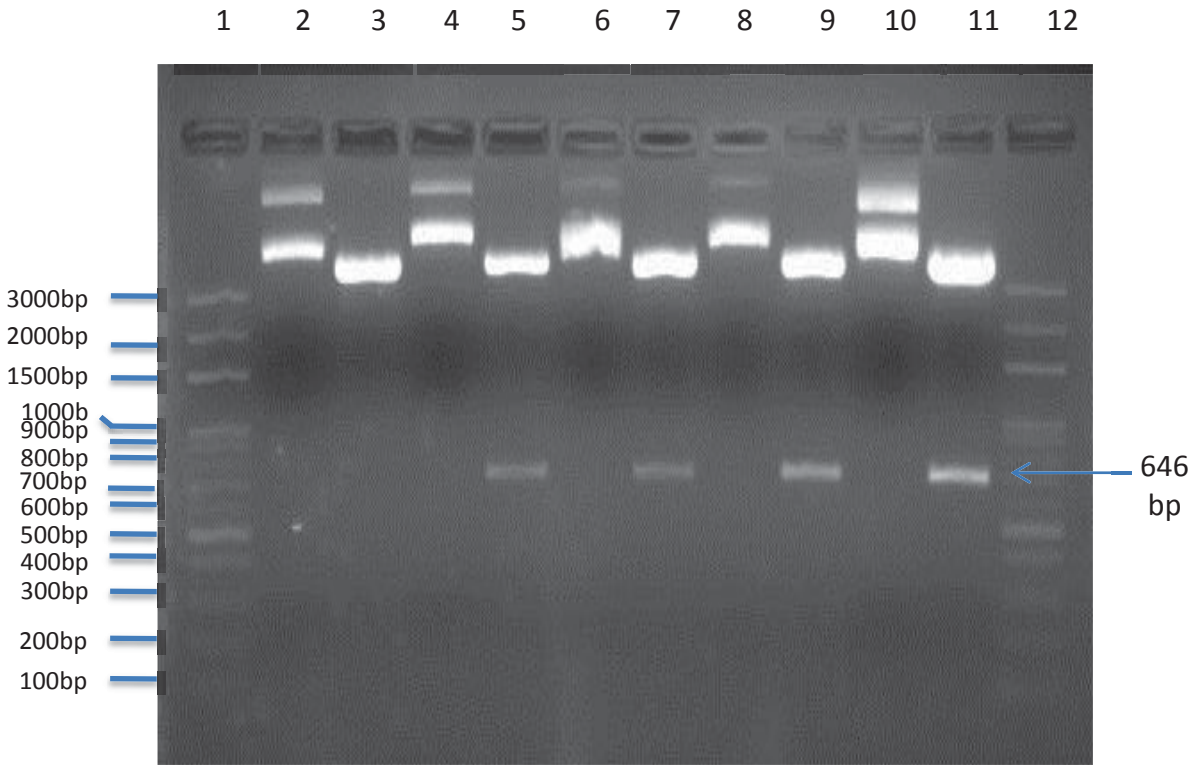

Figure A3.1. **Screening Individual clones (33, 34, 35, 36, 38) of Transformation F of One Shot TOP 10 *E. coli* with RMMG#6/pAcGFP1-C1 plasmid.** Subjecting samples to an *EcoR*1 enzymatic digestion removes the RMMG#6 insert if it is present. Lane 1 Molecular weight marker 100bp, Lane 2 F33 Control, Lane 3 F33 Digested, Lane 4 F34 Control, Lane 5 F34 Digested, Lane 6 F35 Control, Lane 7 F35 Digested, Lane 8 F36 Control, Lane 9 F36 Digested, Lane 10 F38 Control, Lane 11 F38 Digested, Lane 12 Molecular weight marker (100bp). Banding at approximately 646 bp in lanes 5, 7, 9, and 11 confirmed these clones contained a vector that incorporated the RMMG#6 insert.

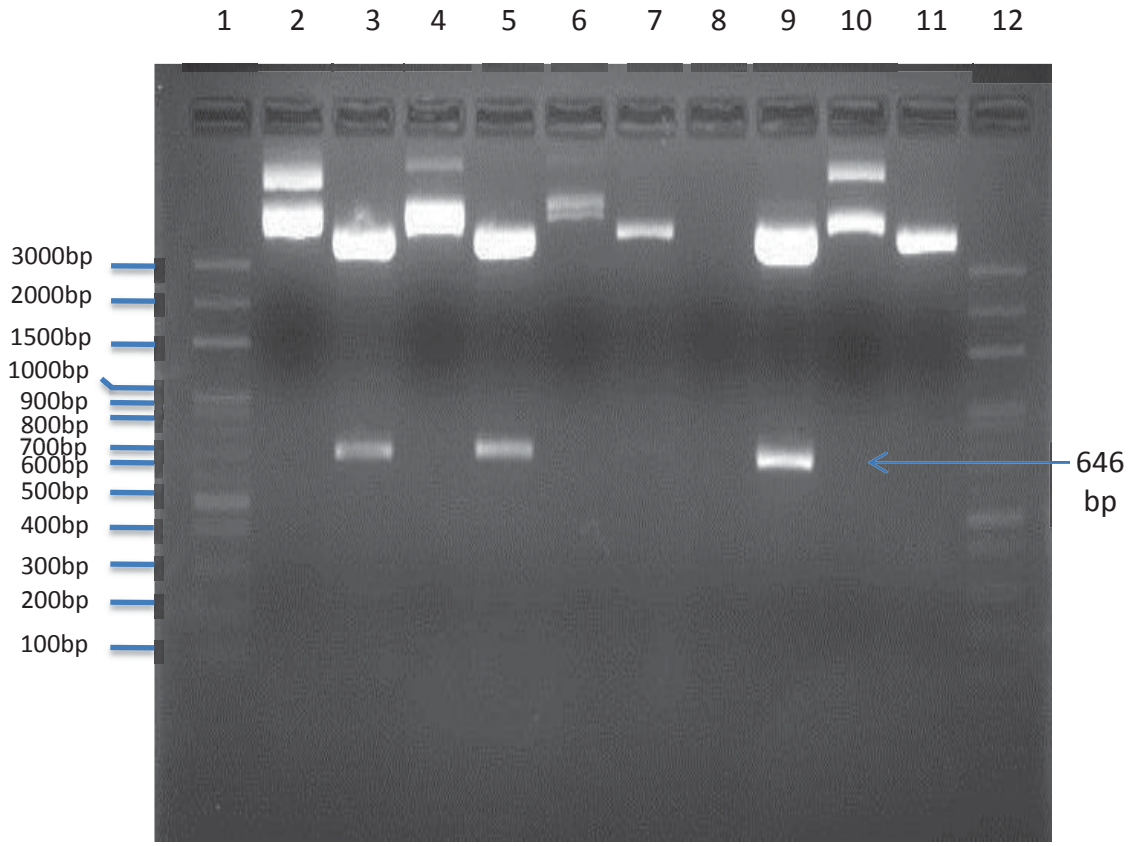

Figure A3.2. Screening Individual clones (39, 40, 41, 42, 43) of Transformation F of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid. Subjecting samples to an *EcoR*1 enzymatic digestion removes the RMMG#6 insert if it is present. Lane 1 Molecular weight marker (100bp), Lane 2 F39 Control, Lane 3 F39 Digested, Lane 4 F40 Control, Lane 5 F40 Digested, Lane 6 F41 Control, Lane 7 F41 Digested, Lane 8 F42 Control, Lane 9 F42 Digested, Lane 10 F43 Control, Lane 11 F43 Digested, Lane 12 Molecular w marker (100bp). Banding at approximately 646 bp in lanes 3, 5, 7, and 9 confirmed these clones contained a vector that incorporated the RMMG#6 insert.

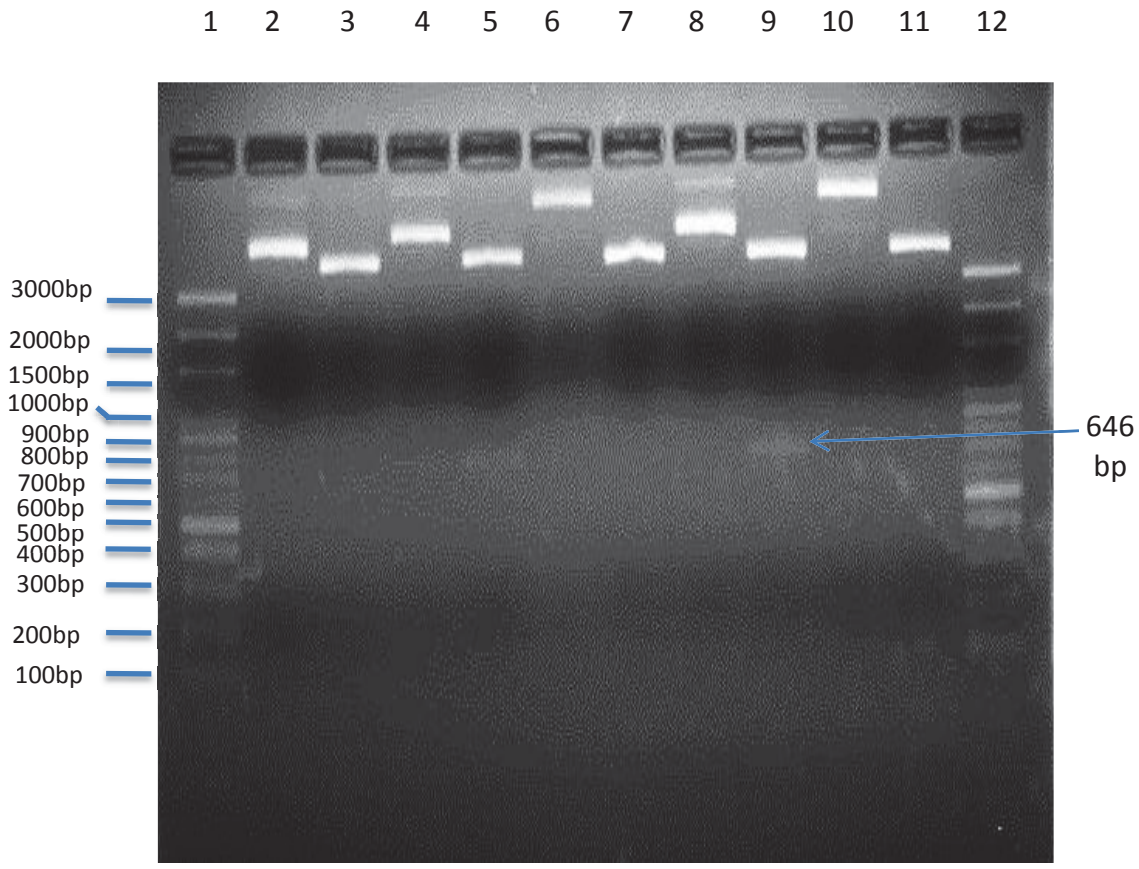

Figure A3.3. Screening Individual clones (45, 46, 47, 50, 51) of Transformation F of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid. Subjecting samples to an *EcoR*1 enzymatic digestion removes the RMMG#6 insert if it is present. Lane 1 Molecular weight marker (100 bp), Lane 2 F50 Control, Lane 3 F50 Digested, Lane 4 F47 Control, Lane 5 F47 Digested, Lane 6 F45 Control, Lane 7 F45 Digested, Lane 8 F46 Control, Lane 9 F46 Digested, Lane 10 F51 Control, Lane 11 F51 Digested, Lane 12 Molecular weight Marker (100 bp). Banding at approximately 646 bp in lanes 5 and 9 confirmed these clones contained a vector that incorporated the RMMG#6 insert.

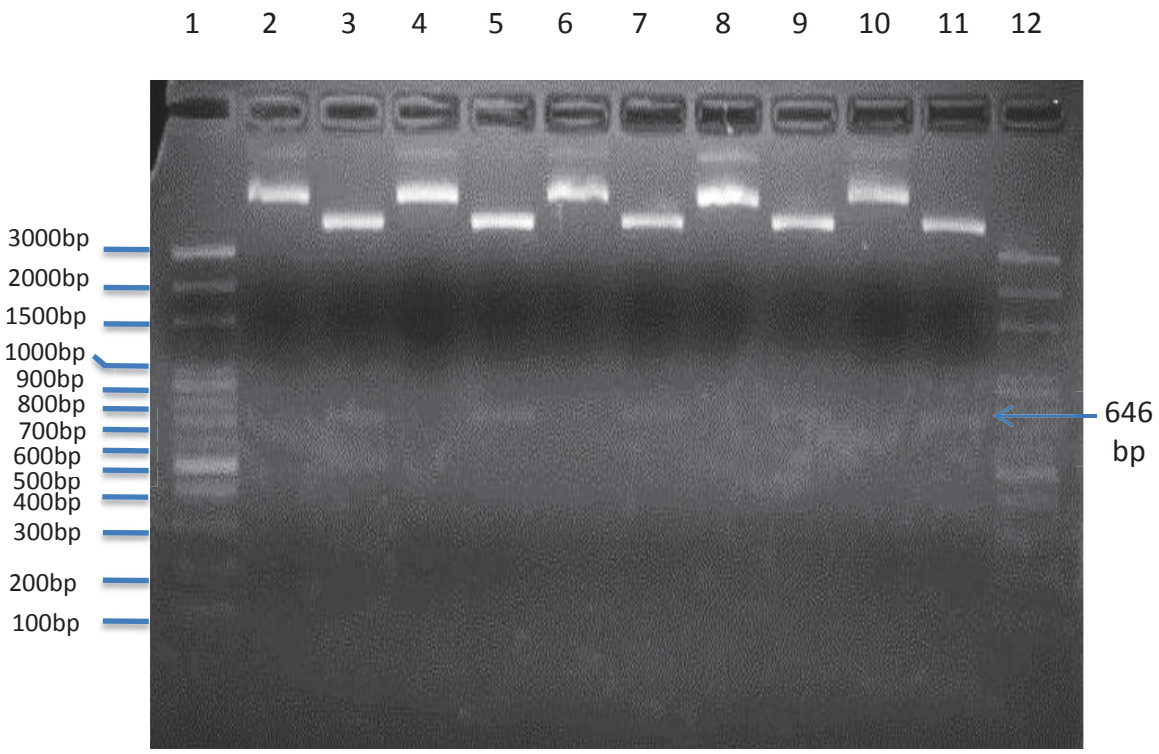

Figure A3.4. Screening Individual clones (F: 44, 52, 53, 55, G:17) of Transformation F and Transformation G of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid. Subjecting samples to an *EcoR*1 enzymatic digestion removes the RMMG#6 insert if it is present. Lane 1 Molecular weight marker (100 bp), Lane 2 F44 Control, Lane 3 F44 Digested, Lane 4 F53 Control, Lane 5 F53 Digested, Lane 6 F55 Control, Lane 7 F55 Digested, Lane 8 F52 Control, Lane 9 F52 Digested, Lane 10 G17 Control, Lane 11 G17 Digested, Lane 12 Molecular weight marker (100 bp). Banding at approximately 646 bp in lanes 3, 5, 7, 9, and 11 confirmed these clones contained a vector that incorporated the RMMG#6 insert.

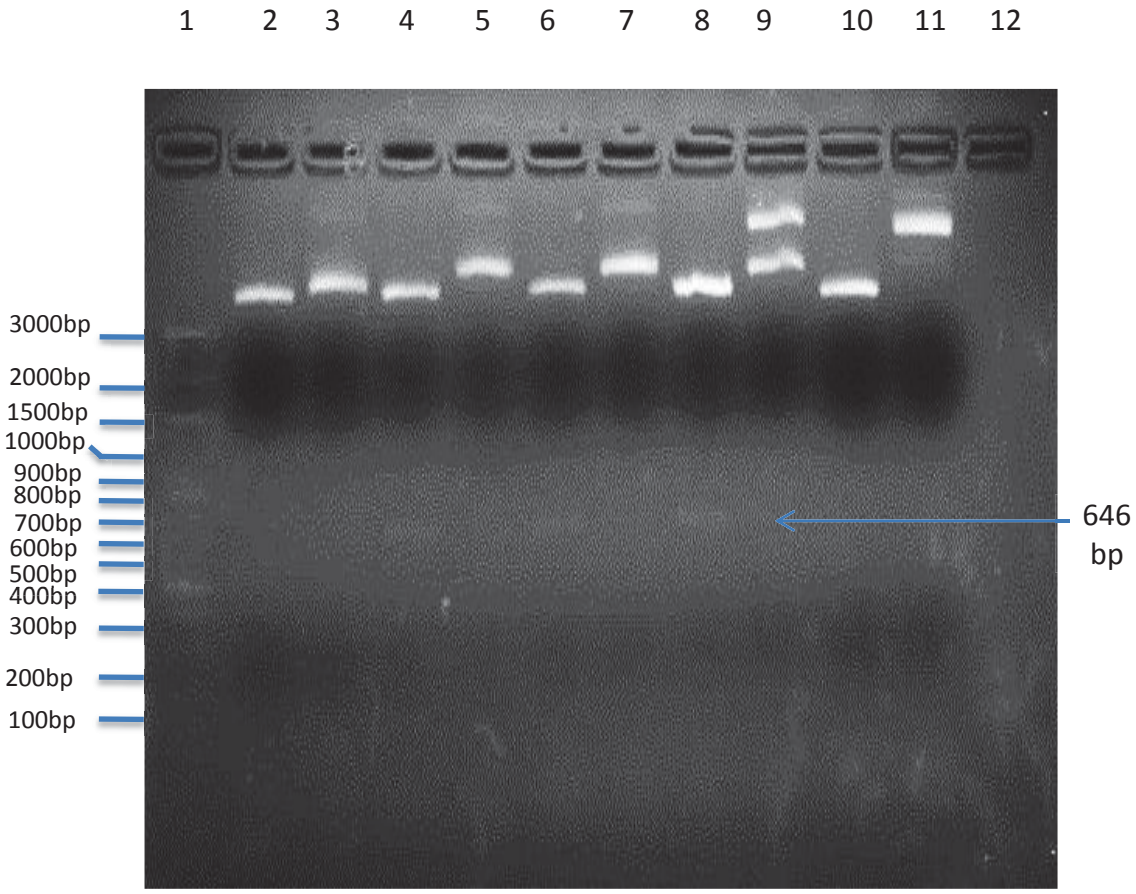

Figure A3.5. Screening Individual clones (13, 14, 15, 16, 23) of Transformation G of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid. Subjecting samples to an *EcoR*1 enzymatic digestion removes the RMMG#6 insert if it is present. Lane 1 Molecular weight marker (100 bp), Lane 2 G15 Digested, Lane 3 G15 Control, Lane 4 G13 Digested, Lane 5 G13 Control, Lane 6 G14 Digested, Lane 7 G14 Control, Lane 8 G16 Digested, Lane 9 G16 Control, Lane 10 G23 Digested, Lane 11 G23 Control, Lane 11 Blank. Banding at approximately 646 bp in lanes 4, 6, and 8 confirmed these clones contained a vector that incorporated the RMMG#6 insert.

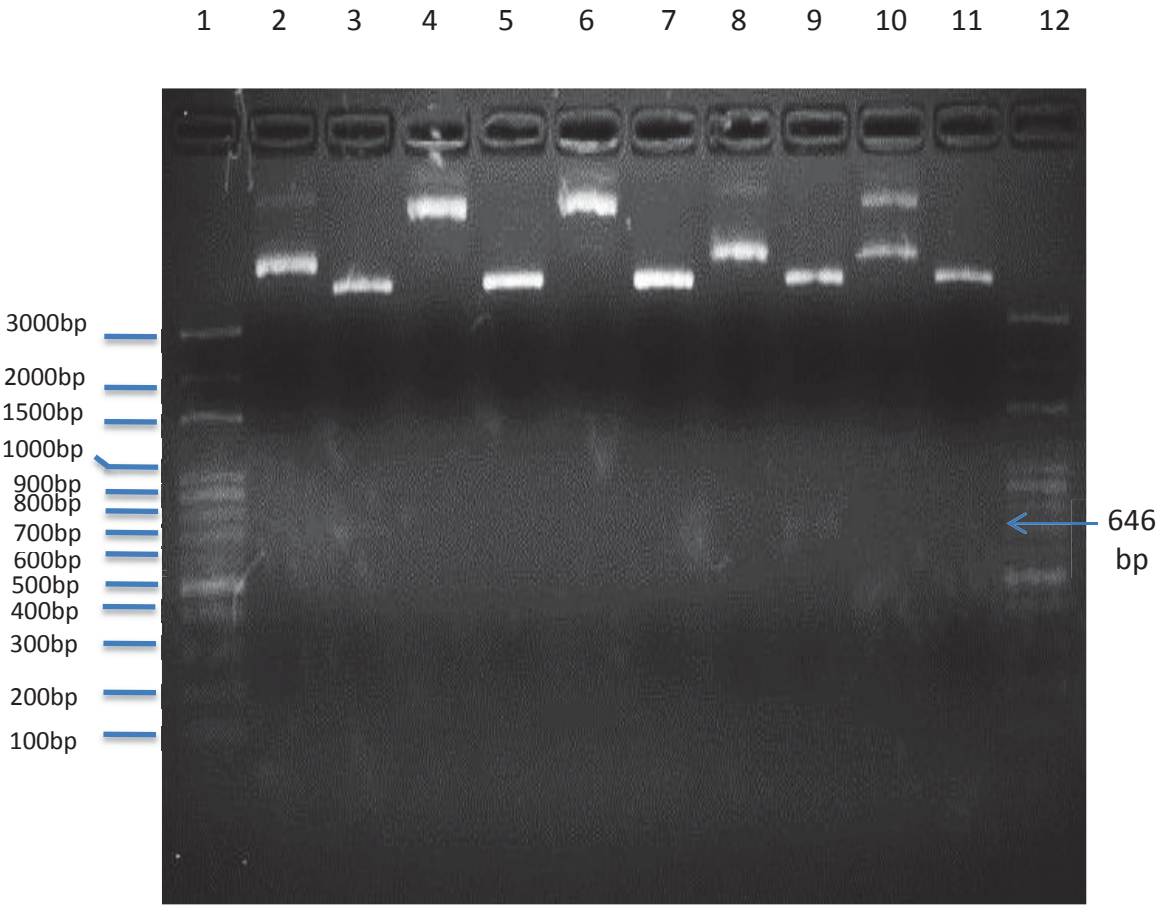

Figure A3.6. Screening Individual clones (18, 19, 20, 21, 22) of Transformation G of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid. Subjecting samples to an *EcoR*1 enzymatic digestion removes the RMMG#6 insert if it is present. Lane 1 Molecular weight marker (100 bp), Lane 2 G18 Control, Lane 3 G18 Digested, Lane 4 G19 Control, Lane 5 G19 Digested, Lane 6 G20 Control, Lane 7 G20 Digested, Lane 8 G21 Control, Lane 9 G21 Digested, Lane 10 G22 Control, Lane 11 G22 Digested, Lane 12 Molecular weight marker (100 bp). Banding at approximately 646 bp in lanes 3, 9, and 11 confirmed these clones contained a vector that incorporated the RMMG#6 insert.

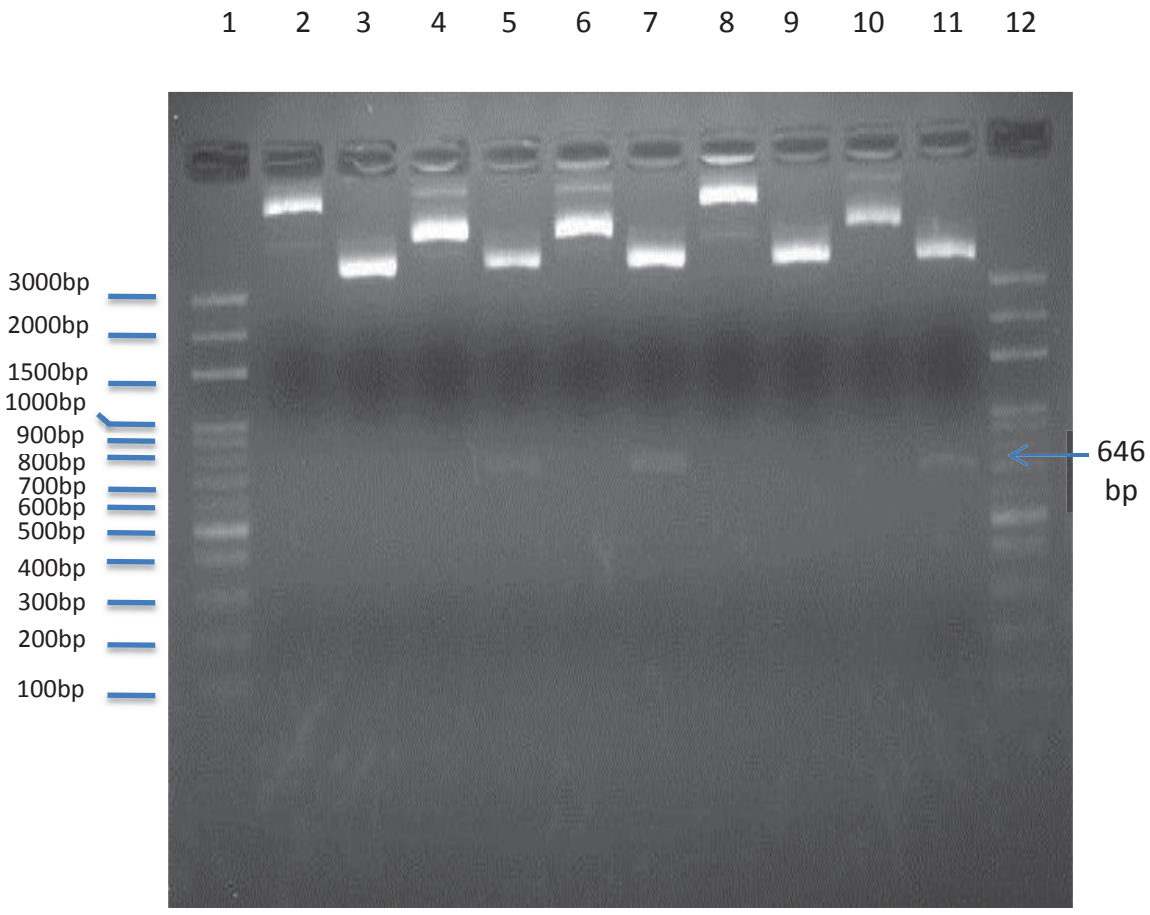

Figure A3.7. Screening Individual clones (40, 41, 42, 43, 44) of Transformation G of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid. Subjecting samples to an *EcoR*1 enzymatic digestion removes the RMMG#6 insert if it is present. Lane 1 Molecular weight marker (100 bp), Lane 2 G40 Control, Lane 3 G40 Digest, Lane 4 G41 Control, Lane 5 G41 Digest, Lane 6 G42 Control, Lane 7 G42 Digest, Lane 8 G43 Control, Lane 9 G43 Digest, Lane 10 G44 Control, Lane 11 G44 Digest, Lane 12 Molecular weight marker (100 bp). Banding at approximately 646 bp in lanes 5, 7, and 11 confirmed these clones contained a vector that incorporated the RMMG#6 insert.

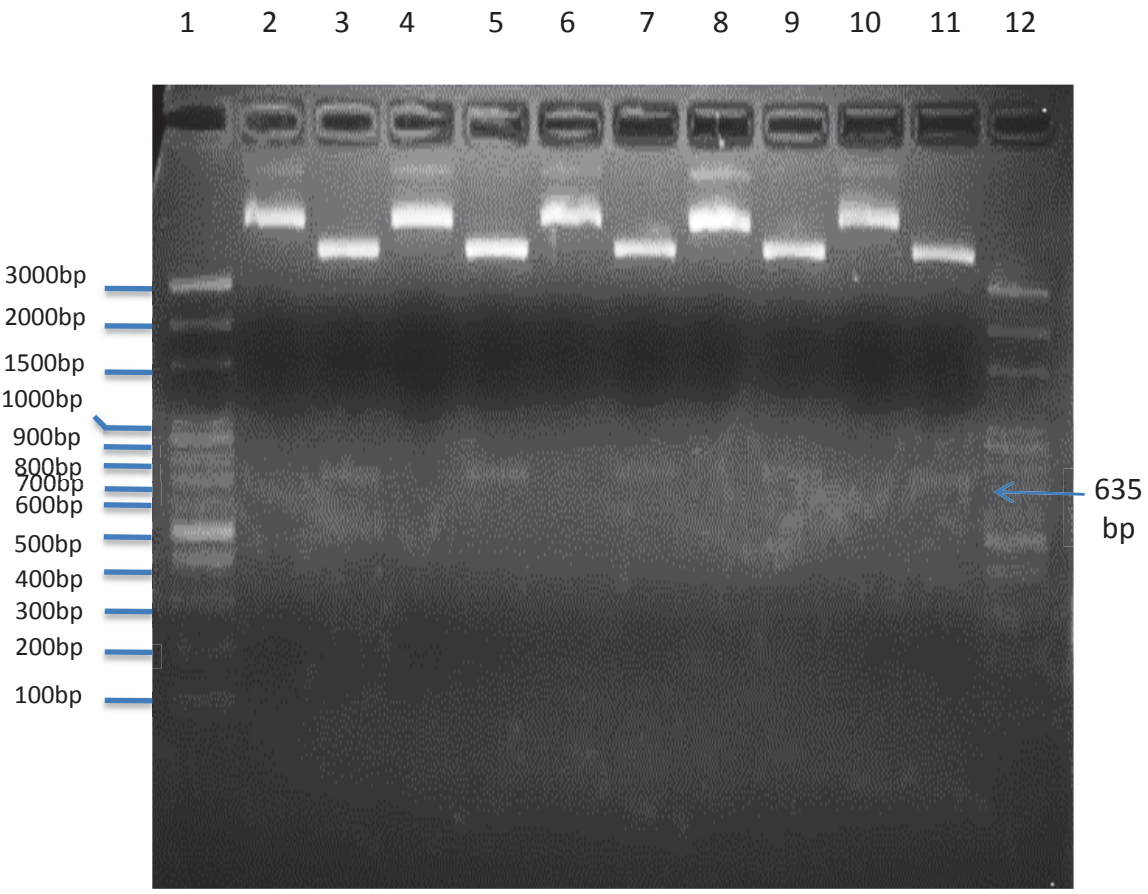

Figure A3.8. Screening Individual clones (34, 35, 36, 38, 39) of Transformation F of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid using a *Hind*III enzymatic digestion. Subjecting samples to a *Hind*III enzymatic digestion removes a fragment that yields a band at approximately 700 bp if the RMMG#6 insert is in the proper orientation. Lane 1 Molecular weight marker (100 bp), Lane 2 F34 Control, Lane 3 F34 Digest, Lane 4 F35 Control, Lane 5 F35 Digest, Lane 6 F36 Control, Lane 7 F36 Digest, Lane 8 F38 Control, Lane 9 F38 Digest, Lane 10 F39 Control, Lane 11 F39 Digest, Lane 12 Molecular weight marker (100 bp). Banding at approximately 635 bp in lanes 3, 5, 7, 9, and 11 confirmed these clones contained a vector that incorporated the RMMG#6 insert and it was in the proper orientation.

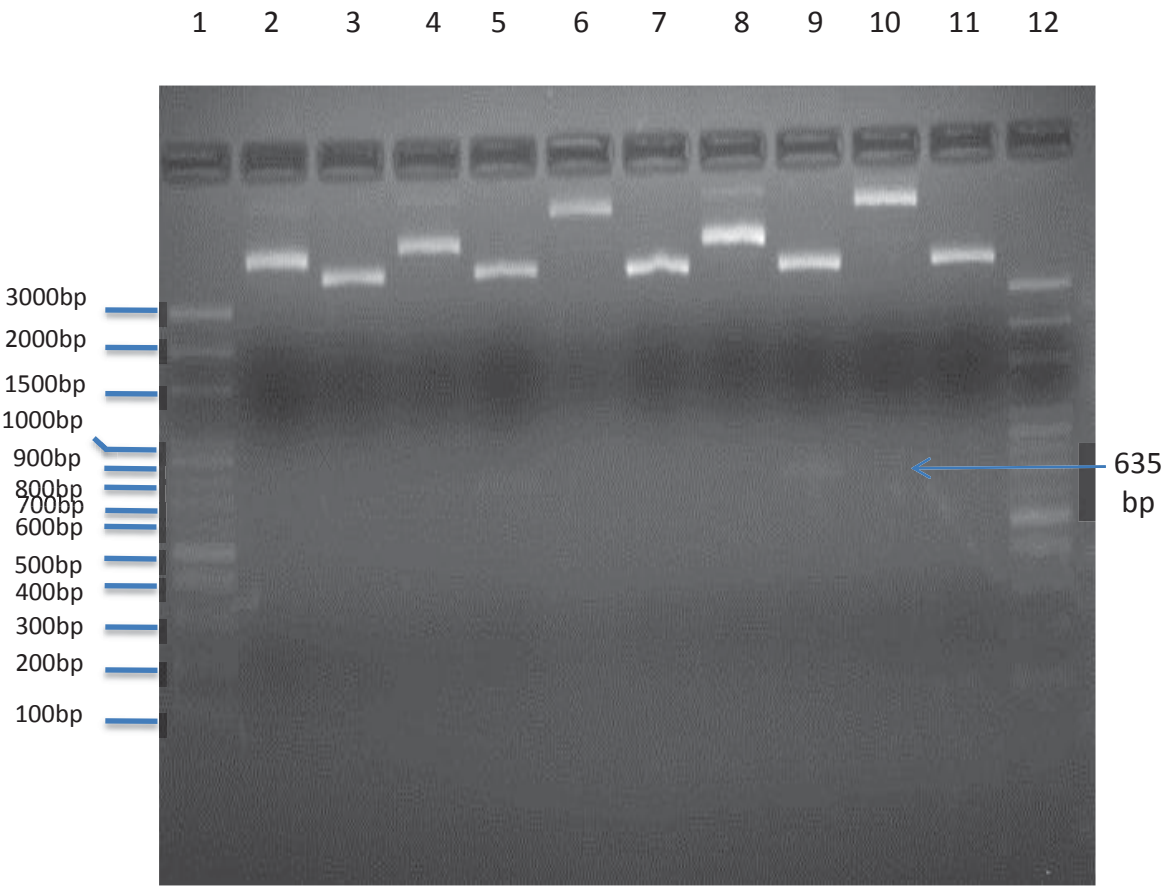

Figure A3.9. Screening Individual clones (40, 41, 42, 44, 46) of Transformation F of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid using a *Hind*III enzymatic digestion. Subjecting samples to a *Hind*III enzymatic digestion removes a fragment that yields a band at approximately 635 bp if the RMMG#6 insert is in the proper orientation. Lane 1 Molecular weight marker (100 bp), Lane 2 F40 Control, Lane 3 F40 Digest, Lane 4 F41 Control, Lane 5 F41 Digest, Lane 6 F42 Control, Lane 7 F42 Digest, Lane 8 F44 Control, Lane 9 F44 Digest, Lane 10 F46 Control, Lane 11 F46 Digest, Lane 12 Molecular weight marker (100 bp). Banding at approximately 635 bp in lanes 5 and 9 confirmed these clones contained a vector that incorporated the RMMG#6 insert and it was in the proper orientation.

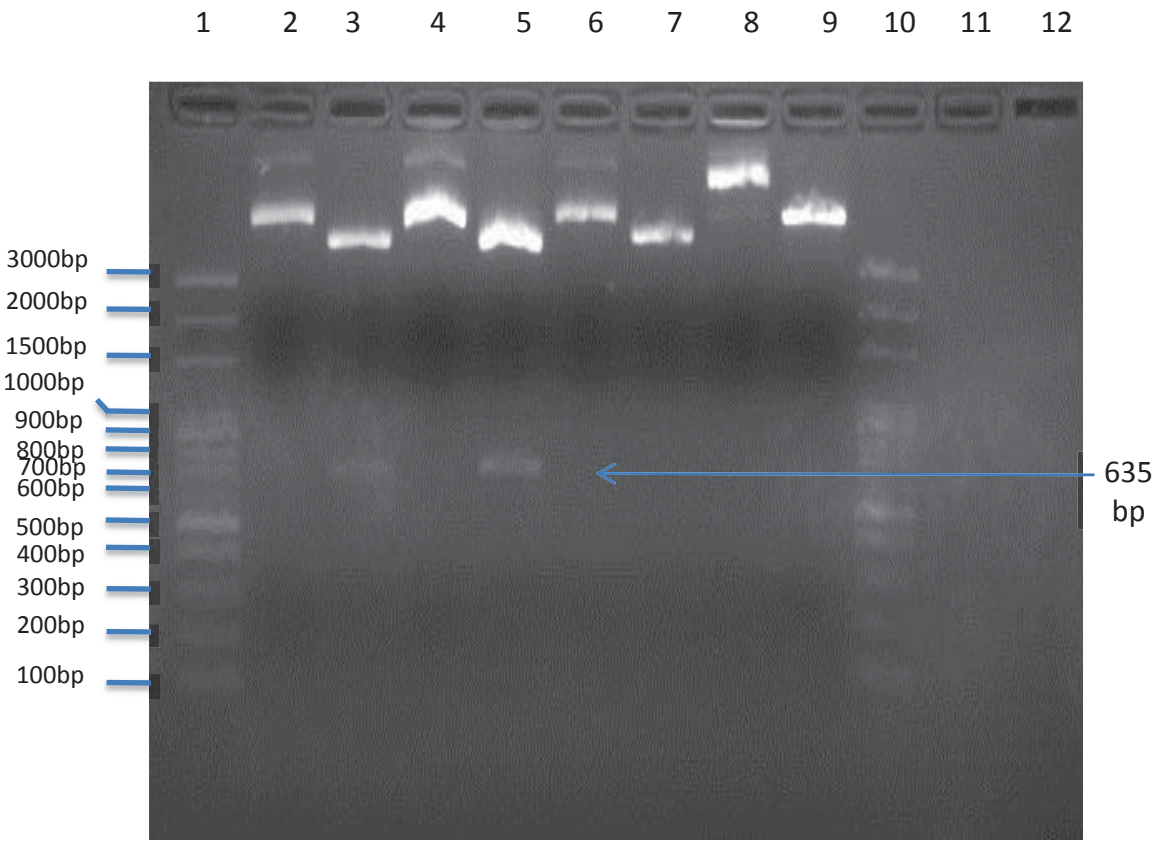

Figure A3.10. Screening Individual clones (47, 52, 53, 55,) of Transformation F of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid using a *Hind*III enzymatic digestion. Subjecting samples to a *Hind*III enzymatic digestion removes a fragment that yields a band at approximately 635 bp if the RMMG#6 insert is in the proper orientation. Lane 1 Molecular weight marker (100 bp), Lane 2 F47 C, Lane 3 F47 Digest, Lane 4 F52 Control, Lane 5 F52 Digest, Lane 6 F53 Control, Lane 7 F53 Digest, Lane 8 F55 Control, Lane 9 F55 Digest, Lane 10 Molecular weight marker (100 bp). Banding at approximately 635 bp in lanes 3 and 5 confirmed these clones contained a vector that incorporated the RMMG#6 insert and it was in the proper orientation.

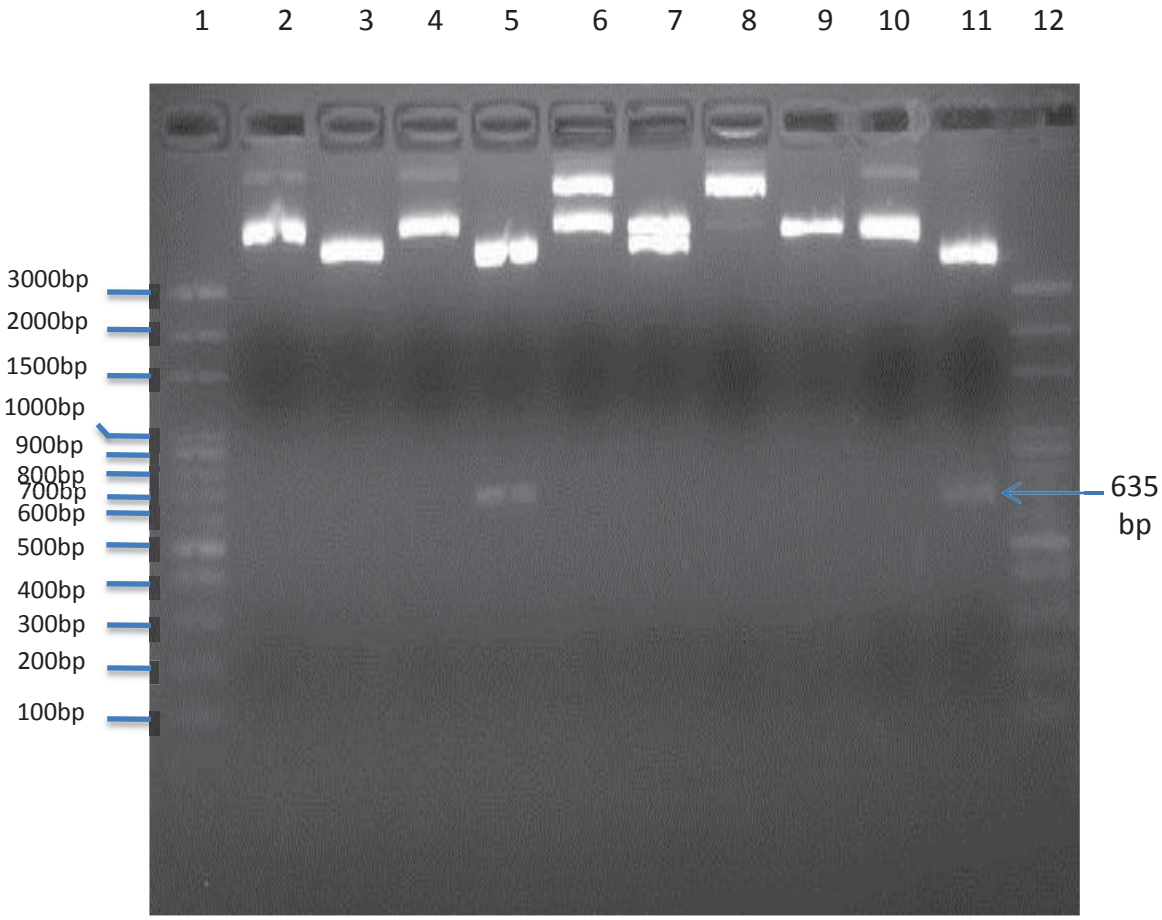

Figure A3.11. Screening Individual clones (13, 14, 16, 17, 18) of Transformation G of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid using a *Hind*III enzymatic digestion. Subjecting samples to a *Hind*III enzymatic digestion removes a fragment that yields a band at approximately 635 bp if the RMMG#6 insert is in the proper orientation. Lane 1 Molecular weight marker (100 bp), Lane 2 G13 Control, Lane 3 G13 Digest, Lane 4 G14 Control, Lane 5 G14 Digest, Lane 6 G16 Control, Lane 7 G16 Digest, Lane 8 G17 Control, Lane 9 G17 Digest, Lane 10 G18 Control, Lane 11 G18 Digest, Lane 12 Molecular weight marker (100 bp). Banding at approximately 635 bp in lanes 5 and 11 confirmed these clones contained a vector that incorporated the RMMG#6 insert and it was in the proper orientation.

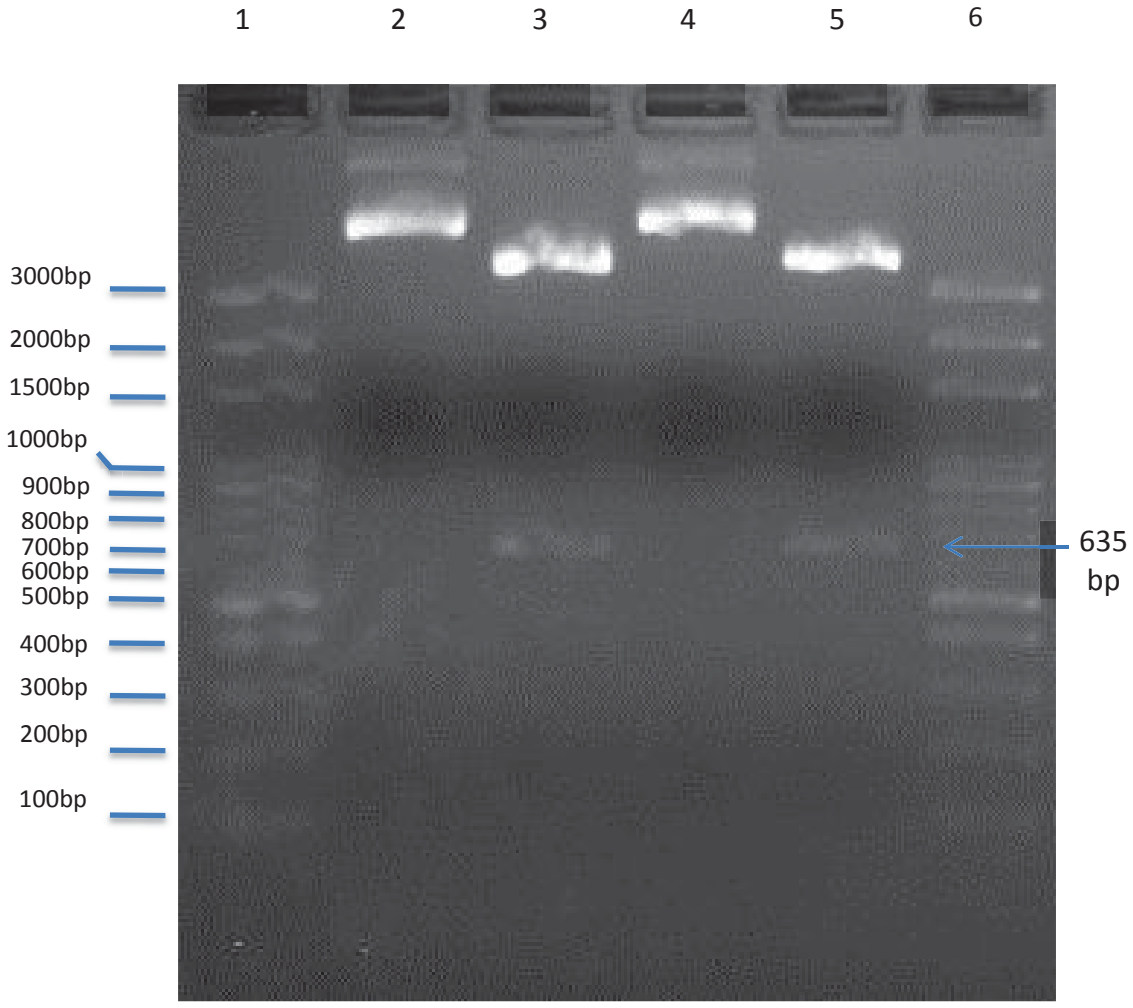

Figure A3.12. Screening Individual clones (42, 44)of Transformation F of One Shot TOP 10 E. coli with RMMG#6/pAcGFP1-C1 plasmid using a *Hind*III enzymatic digestion. Subjecting samples to a *Hind*III enzymatic digestion removes a fragment that yields a band at approximately 635 bp if the RMMG#6 insert is in the proper orientation. Lane 1 Molecular weight marker (100 bp), Lane 2 G42 Control, Lane 3 G42 Digest, Lane 4 G44 Control, Lane 5 G44 Digest, Lane 6 Molecular weight marker (100 bp). Banding at approximately 635 bp in lanes 3 and 5 confirmed these clones contained a vector that incorporated the RMMG#6 insert and it was in the proper orientation.

A

```
1         10         20         30         40         50
|         |          |          |          |          |
NCTCAAGNCNNCATNNNNNNNNNNNNNNNNCGGACKCTNNNTCGAGCTCAA
GCTTNNNNTTCCCTAGCTCACAACCTAACCAATGAGTCCTGCAAACTGAC
ATGGTTTTCTCCAGAAGATGATGGAGGCTCTCCAATCACCAATTATGTCA
TTGAAAAGCGTGAATCTGACCGCAGAGCATGGACCCCAGTGACATATACA
GTTACCCGACAAAATGCTACTGTCCAGGGTCTCATTCAAGGAAAAGCCTA
CTTTTTCCGAATTGCGGCTGAAAATAGTATTGGCATGGGTCCATTTGTTG
AGACATCAGAGGCACTTGTTATCAGAGAGCCAATAACTGTACCAGAGCGT
CCTGAAGACCTGGAAGTCAAAGAAGTTACTAAAAATACTGTAACTTTGAC
TTGGAATCCTCCTAAGTATGATGGTGGGTCAGAAATTATTAACTATGTCC
TAGAAAGTCGGCTCATTGGNGACTGAGAAGTTCCACAAAGTTACAAATGA
CAACTTGCTTAGCAGAAAATACACTGTTAAAGGCTTAAAAGAAGGTGATA
CCTATGAGTACCGTGTCAGTGCTGTCAACATTGTTGGACAAGGCAAACCA
TCATTTTGCACCAAACCAATTACTTGCAAGGATGAGCTGGCACCCCCAAC
GCTTCACCTAGACTTCAGAGATAAGCTCACGATTCGAGTTGGTGAAGCTT
TTGCCCTCACTGGCCGTCGTTTTACAAGGGCGAATTCTGCAGTCGACGGT
ACCGCGGGNCCCGGGATCCACCGGATCTARANGAACTGATCATAWTCAGC
CATACCACNNNNNTWTTGTNNNNNNNAGCGGTTWTACNNGTATTATTY
```

B

Length	848		
Frequencies:	#	%	%
A	236	27.8	29.5
C	187	22.1	23.4
G	173	20.4	21.7
T	203	23.9	25.4
K	1	0.1	
N	43	5.1	
W	3	0.4	
R	1	0.1	
Y	1	0.1	
GC	360	45.1	

Figure A3.13. Consensus sequence of clone G14 constructed using Genious software. A. The nucleotide sequence. **B.** The statistical output of the consensus sequence.

A

```
1         10         20         30         40         50
|         |          |          |          |          |
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNCCCTAGCTCACAACCTAACCAATGAGTCCTGCAAACTG
ACATGGTTTTCTCCAGAAGATGATGGAGGCTCTCCAATCACCAATTATGT
CATTGAAAAGCGTGAATCTGACCGCAGAGCATGGACCCCAGTGACATATA
CAGTTACCCGACAAAATGCTACTGTCCAGGGTCTCATTCAAGGAAAAGCC
TACTTTTTCCGAATTGCGGCTGAAAATAGTATTGGCATGGGTCCATTTGT
TGAGACATCAGAGGCACTTGTTATCAGAGAGCCAATAACTGTACCAGAGC
GTCCTGAAGACCTGGAAGTCAAAGAAGTTACTAAAAATACTGTAACTTTG
ACTTGGAATCCTCCTAAGTATGATGGTGGGTCAGAAATTATTAACTATGT
CCTAGAAAGTCGGNMTCATTGGGACTGAGAAGTTCCACAAAGTTACAAAT
GACAACTTGCTTAGCAGNAAAATACACTGTTAAANGGCTTNNAAAAGAAR
GTGATACCTATGRGTACCGTGTCAGTGCTGTCAACAWTGTTGGACAARGC
AAACCATCATTTTGCACCAAACCAATTACTTGNNCAAGGATGANGCTNGG
CNNNMCCCCCAACNNGCTTCACCTANGACTTCAGAGNATAAGCTCANNNN
TCGARTTNNTSAAGSTTTKGSSYKCAMWGGNNNNCGTTTTNNNWGGGCSN
NNNNNNCMSTCSACNNNNCYKSGGGCCCGNNNTYCNNNNGWTYTNNNNNN
NNNNNNNNTTAANNNNNNNNNNNNACAWATGGNNNNGGNNNNNNNNNGRY
```

B

Length	849		
Frequencies:	#	%	%
A	206	24.3	30.7
C	150	17.7	22.3
G	146	17.2	21.7
T	170	20.0	25.3
K	3	0.4	
M	4	0.5	
N	147	17.3	
R	5	0.6	
S	8	0.8	
W	5	0.6	
Y	5	0.6	
GC	296	44.0	

Figure A3.14. Consensus sequence of clone G41 constructed using Genious software. A. The nucleotide sequence. **B.** The statistical output of the consensus sequence.

Figure A3.15. Alignment of G14 Consensus sequence and titin immunogenic domain (EU 428784). Sequences were aligned using Genious software. Statistical analysis of the alignment gave 76.2% pairwise % similarity with 646/646 identity.

```
                1        10        20        30        40        50        60
                |        |         |         |         |         |         |
G41 Consensus   NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
     EU428784   ------------------------------------------------------------

G41 Consensus   NNCCCTAGCTCACAACCTAACCAATGAGTCCTGCAAACTGACATGGTTTTCTCCAGAAGA
     EU428784   -TCCCTAGCTCACAACCTAACCAATGAGTCCTGCAAACTGACATGGTTTTCTCCAGAAGA

G41 Consensus   TGATGGAGGCTCTCCAATCACCAATTATGTCATTGAAAAGCGTGAATCTGACCGCAGAGC
     EU428784   TGATGGAGGCTCTCCAATCACCAATTATGTCATTGAAAAGCGTGAATCTGACCGCAGAGC

G41 Consensus   ATGGACCCCAGTGACATATACAGTTACCCGACAAAATGCTACTGTCCAGGGTCTCATTCA
     EU428784   ATGGACCCCAGTGACATATACAGTTACCCGACAAAATGCTACTGTCCAGGGTCTCATTCA

G41 Consensus   AGGAAAAGCCTACTTTTTCCGAATTGCGGCTGAAAATAGTATTGGCATGGGTCCATTTGT
     EU428784   AGGAAAAGCCTACTTTTTCCGAATTGCGGCTGAAAATAGTATTGGCATGGGTCCATTTGT

G41 Consensus   TGAGACATCAGAGGCACTTGTTATCAGAGAGCCAATAACTGTACCAGAGCGTCCTGAAGA
     EU428784   TGAGACATCAGAGGCACTTGTTATCAGAGAGCCAATAACTGTACCAGAGCGTCCTGAAGA

G41 Consensus   CCTGGAAGTCAAAGAAGTTACTAAAAATACTGTAACTTTGACTTGGAATCCTCCTAAGTA
     EU428784   CCTGGAAGTCAAAGAAGTTACTAAAAATACTGTAACTTTGACTTGGAATCCTCCTAAGTA

G41 Consensus   TGATGGTGGGTCAGAAATTATTAACTATGTCCTAGAAAGTCGGNMTCATTGGGACTGAGA
     EU428784   TGATGGTGGGTCAGAAATTATTAACTATGTCCTAGAAAGTCGGC-TCATTGGGACTGAGA

G41 Consensus   AGTTCCACAAAGTTACAAATGACAACTTGCTTAGCAGNAAAATACACTGTTAAANGGCTT
     EU428784   AGTTCCACAAAGTTACAAATGACAACTTGCTTAGCAG-AAAATACACTGTTAAA-GGCTT

G41 Consensus   NNAAAAGAARGTGATACCTATGRGTACCGTGTCAGTGCTGTCAACAWTGTTGGACAARGC
     EU428784   --AAAAGAAGGTGATACCTATGAGTACCGTGTCAGTGCTGTCAACATTGTTGGACAAGGC

G41 Consensus   AAACCATCATTTTGCACCAAACCAATTACTTGNNCAAGGATGANGCTNGGCNNNMCCCCC
     EU428784   AAACCATCATTTTGCACCAAACCAATTACTTG--CAAGGATGA-GCT-GGC---ACCCCC

G41 Consensus   AACNNGCTTCACCTANGACTTCAGAGNATAAGCTCANNNNTCGARTTNNTSAAGSTTTKG
     EU428784   AAC--GCTTCACCTA-GACTTCAGAG-ATAAGCTCACGATTCGAGTTGGTGAAGCTTTT-

G41 Consensus   SSYKCAMWGGNNNNCGTTTTNNNWGGGCSNNNNNNNCMSTCSACNNNNCYKSGGGCCCGN
     EU428784   ------------------------------------------------------GCCC--

G41 Consensus   NNTYCNNNNGWTYTNNNNNNNNNNNNNNTTAANNNNNNNNNNNNACAWATGGNNNNGGNNN
     EU428784   ------------------------------------------------------------

G41 Consensus   NNNNNNGRY
     EU428784   ---------
```

Figure A3.16. Alignment of G41 Consensus sequence and titin immunogenic domain (EU 428784). Sequences were aligned using Genious software. Statistical analysis of the alignment gave 74.1% pairwise % similarity with 629/646 identity.