Creation of αMHC-GCaMP8 Plasmid to Monitor the Differentiation of Human iPSCs into Cardiomyocytes

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Abstract

Due to the poor regenerative potential of adult heart tissue, heart disease is a major cause of death worldwide. With developments in cell biology, researchers have gravitated towards investigating the applications of stem cells to aid in cardiac regeneration through injection of cardiac progenitors into the site of infarction. This method has led to promising results in mammalian studies but to bolster current cardiac research, research tools are required to observe the physiology and quantify the success of differentiation in ES derived cardiac cells. I have attempted to create one such research tool by creating a visual and quantifiable detection method for the differentiation of human iPS cells into beating cardiomyocytes through my creation of an αMHC-GCaMP8 insertion plasmid. Transforming this plasmid into iPSCs will allow GCaMP8 to fluorescently monitor Calcium levels in differentiated cardiomyocytes, taking advantage of the physiological Calcium fluctuations associated with muscle contractions. Though I was unable to confirm creation of this plasmid due to time constraints and unexpected errors, my work can still be finished or expanded upon transforming cells with my plasmid and creating cells with countless applications both in differentiation analysis, disease modeling, and tissue engineering.

Introduction

In an examination of historical art and literature, the heart has been a symbol for love, emotions, and even the soul. As modern science has evolved and humans have learned more about anatomical function, these ideas have been generally abandoned with the heart instead becoming associated with an early death. In the 20th century heart disease surpassed infectious disease as the leading cause of death worldwide and in 2009 1 in 9 death certificates in the United States mentioned heart failure [12]. Developed from the mesoderm, the heart is an organ

that facilitates the pumping of blood throughout the body. Proper heart function is an integral factor in advanced organism life due to its role in delivering nutrients and helping remove waste. In this honors thesis, I attempt to create a research tool for studying heart cells and with future utilization of advances in stem cell studies, gene engineering, and calcium labeling tools; this tool can realize its potential and help study heart disease.

While heart disease stands as an unsolved problem within the medical community, current research has implemented new methods of heart repair particularly through the use of stem cells. Embryonic stem (ES) cells are undifferentiated cells derived from the inner cell mass of a blastocyst and have the potential to give rise to any cell or tissue type within the human body. This pluripotency has led researchers to attempt to create human organs from scratch and inject specific cells into damaged tissue to aid in repair. Due to the extremely low regenerative potential of adult cardiac cells, injection of ES derived cardiac cells into areas of damage is considered a very promising method of heart cell regeneration [11, 14]. This has been confirmed experimentally for mouse models post myocardial infarction using both ES cells and hematopoietic bone marrow cells, and in primate models where human ES derived cardiomyocytes aided in regeneration [10, 23, 24]. These exciting methods of tissue repair hit a speedbump for clinical human therapy due to the ethical and social implications behind the gathering of ES cells from a fertilized human embryo, and the negative immune response to injection of foreign cells into a host.

To circumvent these issues researchers looked for new ways to create endogenous human pluripotent embryonic stem cells without the destruction of embryonic material. Through an understanding of gene expression and frog studies from the 1950s and 60s, researchers found that transfer of a mammalian oocyte nucleus into a terminally differentiated cell caused

reprogramming of the cell into an undifferentiated state suggesting that every differentiated cell has the potential to be reprogrammed based on different gene expression. Further examination of genes with high expression in human ES cells led to the discovery of specific factors (in particular OCT4, SOX2, NANOG, and LIN28) are responsible for the pluripotent phenotype and thus, induced pluripotent stem cells (iPSC) were discovered [36]. These cells are taken from differentiated adult tissue and after activation of specific master transcription factors, are undifferentiated to pluripotency. This exciting new development does have its limitations, though, as the method of iPSC creation and differentiation is not completely perfected. The use of iPSC to aid in regeneration therapies is a dream for modern cell biologists and if perfected will add a personalized and specific new angle to aid in heart repair [11, 26].

The second major biological advance that I utilize in my project is gene editing nucleases that can be engineered to target specific genes and sites. With the advent of iPS cell creation, researchers have become particularly interested in editing and targeting specific genes in attempts to create patient derived cells modeling genetic diseases [4]. These cells can then be used for further testing, exploring novel drugs treatments and the basic biology of genetic disorders [28]. This methodology doesn't come without limitations, though, as transfection of iPSCs is a difficult procedure because they grow poorly as single cells (a practice required for selection of rare targeted clones) and off target DNA insertion may alter differentiation potential or introduce malignant mutations [9, 12]. To avoid the potential confounding off target effects, scientists have developed site specific editing machinery, the most popular being zinc-finger (ZF) domains, CRISPR-Cas9 machinery, and transcription activator-like effector nucleases (TALEN) with TALENs being of particular interest in my project [14]. These editing methods localize to a particular sequence of interest and induce cuts at that sequence with nucleases

thereby causing mutations or insertions through error prone non-homologous end joining (NHEJ), or homology directed repair (HDR) pathways. These methods have allowed scientists to create more effective means of transfecting cells into 'safe harbor' locations where a specific segment such that a gene of interest is inserted through homologous recombination during the HDR pathway [6, 13].

A third biological advance is the utilization of optogenetic markers to monitor expression of specific genes and intracellular ions, particularly calcium. Our current understanding started with the discovery and isolation of green fluorescent protein(GFP) from the jellyfish *Aequorea victoria* [8]. GFP fluoresces green light under specific wavelengths, has no known unwanted phenotypic effects, and allows researchers to test for expression of specific genes when that gene is coupled to GFP. Utilizing GFP, GCaMP, a genetically encoded calcium indicator (GECI), was created through the fusion of GFP, calmodulin and M13 (a sequence from myosin light chain kinase) [21]. With a further research to create mutations in GCaMP, multiple different indicators have been created varying in sensitivity and response [22, 37]. The research potential of these calcium monitoring genes is immense particularly in neurological and cardiac studies due to the major physiological role calcium plays in neuron firing and muscle contractions [3, 17, 31].

Utilizing an understanding of iPS cells, genome editing, and optogenetic markers, I hypothesized that it is possible to create a line of iPSCs that, when differentiated into beating cardiomyocytes, would glow with varying calcium levels associated with the beating. This is the crux of my project and though I only had time to complete some cellular and molecular biology experiments, I have outlined methods to complete this larger gene insertion process. The methodology can be split into distinct parts; creation of insertion plasmid, transfection and transformation of iPSCs, selection for cells with proper gene insertion, differentiation of cells

into cardiomyocytes, and recording fluorescent output associated with beating. To create the plasmid, I needed to have a selection component, insertion machinery, and labeling mechanism. Fortunately, the Kotlikoff lab specializes in optogenetics and I had access to a plasmid with a GCaMP8 coupled to an αMHC resulting in GCaMP8 expression when the cells are differentiated into heart cells due to αMHC being a known marker of cardiac cells providing the labeling mechanism [19]. I then found a study where there was insertion of a plasmid and selection into iPSCs through TALEN transfection and transformation, providing an insertion and selection method [6]. These plasmids were subject to restriction digests creating blunt and sticky ends and then were ligated maintaining the TALEN arms, the neomycin gene, and the αMHC-GCaMP8 cassette (figure 14). The arms will allow specific homologous recombination to occur inserting the neomycin for antibiotic selection and an αMHC-GCaMP8.

With the creation of this plasmid, TALEN transfection and selection protocols similar to Cerbini et al and differentiation protocols used by the Conklin lab, Burridge, and Van Berg [6, 5, 33] should be performed. Utilizing protocols outlined by Cerbini et al, the insertion of this plasmid with be facilitated through electroporation followed by TALEN cutting and insertion of the plasmid into the CLYBL 'safe harbor' locus that is constitively expressed in human cells [6, 16] (figure 2b). The cells will then be subject to antibiotic treatment and differentiated using varying protocols. If all the prior steps are successful, the cells will fluoresce upon beating due to the active αMHC-GCaMP8.

While the methods outlined above were largely incomplete, I successfully isolated an αMHC-GCaMP8 insert, CAG vector, ligated and transformed bacterial cells (with successful plasmid creation still to be determined), created an antibiotic kill curve for my cells, and proved pluripotency and successful cardiac differentiation of my cells through observational and gene

expression analysis. Though the line of engineered cells was not created, the protocols and ideas I use pave the way for new research tools that could change the way we study calcium fluctuations in human cells, particularly cardiac cells.

Methods

The major methods utilized throughout my research is characterized by creation of an insertion plasmid, transfection of iPSCs with said plasmid, selection of cells for proper plasmid insertion, differentiation of cells, and measuring fluorescent output associated with beating. The Kotlikoff lab specializes in creating transgenic mice for different types of cellular imaging so the creation of the insertion plasmid protocol is original created by my colleagues and I through our understanding of molecular biology. The other aspects of the project were based on protocols used by Cerbini et al., particularly when it came to the transfection and colony selection of the engineered iPS cells [6, 7]. Furthermore, many differentiation protocols were attempted and based on protocols from the Conklin lab (through correspondence), Burridge et al, and Van Berg et al [5, 32].

Creation of insertion plasmid

1. Preparation of pC13N-iCAG.copGFP (CAG) (Addgene 66578) through removal of CAG-GFP cassette creating a linear vector with a sticky end and a blunt end while retaining homologous TALEN arms and neomycin gene (see Figure 1);

a) Plasmid was acquired in agar stock. Was grown in overnight in kanamycin and isolated using GeneJET Plasmid Miniprep Kit (Thermo Scientific #K0502) according to the manufacturer's protocol.

b) Digest with ASCI (unique cut at 5389):

50 ul plasmid DNA

5 ul NEB cutsmart buffer (NEB)

2 ul ASC1(NEB)

33 ul H2O

total volume = 50ul. Digested at 37°C for 1-2 hour, resulting in a linear fragment of 10973bp with –GCGC 5' overhangs.

c) Fill in 5' overhang with Klenow fragment to create blunt end.

50 ul ASC1 digested plasmid

1 ul Klenow fragment (Fermentas EP0051)

Incubate at 37°C for 10 minutes.

d) Gel purify blunt ended AscI digested plasmid:

1% agarose gel (long) with a wide lane was prepared using SybrSafe as a gel stain in the gel. All 50ul of product was run on the gel, along with a 1kb ladder plus marker.

The gel was imaged, minimizing the exposure to the UV light to avoid DNA damage. On a UV gel box, the band of interest (singular at 10973 base pairs long) was cut out using a

razor blade and put into a 1.5 ml Eppendorf tube (pre-weighed). The mass of my band of DNA was determined and purified from the gel following the instructions for the GeneJet gel purification protocol (Thermo Scientific GeneJET Gel Extraction Kit #K0691). I eluted the DNA in 30 ul of elution buffer and ran ~3 ul of the eluted DNA was then run on a 1% gel to check purity and concentration.

e) I digested the gel purified, blunt ended ASCI fragment with with EcoRI to create a sticky end and excise the CAG-GFP cassette (cuts at 5396 and 7954).

50ul DNA from step (c)

5 ul cutsmart buffer

3 ul EcoRI enzyme

0.5 ul H2O

total volume = 30 ul. Digest at 37°C for 1 hour.

This digest should result in 2 fragments, 2559bp and 8414bp. The 2559 fragment is the CAG-GFP fragment that I wanted to remove. The 8414bp fragment was isolated using the GeneJet gel extraction kit similar to step 1c. This fragment will have one EcoRI end and one blunt end. 3ul was ran to check purity and concentration. The CAG vector is now ready to use.

2. Purification of αMHC-GCaMP8 insert with a sticky end and a blunt end for insertion into the CAG vector (figure 2a).

   a) Digest αMHC -GCaMP8 plasmid (acquired from Kotlikoff lab) with HindIII resulting in a 10-11kb linear fragment, while simultaneously dephosphorylating the 5'ends.

      5 uL DNA

      5 ul 10x fast digest buffer

      5 ul HindIII (NEB)

      5 ul FastAP alkaline phosphatase (NEB)

      30ul water

      total volume = 50 ul. Digest at 37oC for 10 minutes.

   b) Plasmid was gel purified as described in 1c.

   c) A MfeI linker (eurofins MWG Operon) was prepared through;

      i) resuspension in 10mM Tris, pH 7.5-8.0, 50mM NaCl, 1mM EDTA to a 100uM concentration.

      ii) Heat to 94°C for 3-5 minutes

      iii) Remove the heat block from the heater and allow to slowly cool to room temperature (will take 45-60 minutes).

      iv) Store at 4°C until ready to use.

d. Ligate the MfeI linker to the HindIII digested aMHC-GCaMP8 plasmid replacing the HindIII site with an MfeI site.

3 ul of Hind III digested aMHC-GCaMP8

3 ul of MfeI linker

1 ul ligase buffer

1 ul T4 DNA ligase

1 ul H2O

incubated at room temperature overnight. Note: a control ligation was also run where no mfe1 linker was added. Ligation will occur at this site

5' AGCTCTGCAATTGCAG 3'

3' GACGTTAACGTCTCGA 5'

e. Transform DH5α competent cells (Invitrogen #18-265-017) with plasmid from ligation reaction according to manufacturer's protocol. Plated on ampicillin plates and grown overnight.

f. I then picked 10 colonies, grow them overnight in LB-amp stock and performed minipreps to isolate the ligated plasmid.

g. The 10 colonies were tested for proper Mfe1 ligation through trial HindIII and Mfe1 digests along with sequencing analysis for the linker.

h. Digest the MfeI containing plasmid with NotI resulting in a 2 linear fragments of 2,887bp and 7,517bp.

5ul plasmid DNA

2 ul NEBuffer 3.1 (NEB)

1 ul NotI

12 ul H2O

total volume = 20ul. Digest at 37°C for 1-2 hour.

i. Fill in 5' overhang with Klenow fragment creating blunt end at NotI digest site:

20 ul NotI digested plasmid

0.5 ul Klenow fragment (Fermentas EP0051)

incubate at 37°C for 10 minutes. Heat inactivate at 75°C for 10 minutes.

j. Gel purify blunt ended NotI digested plasmid at 7,517 bp as described in 1c.

k. MfeI digest linear purified product creating sticky MfeI site.

25 ul plasmid DNA

4 ul cutsmart

2 ul MfeI

9 ul water

Total volume= 40 ul. Digested at 37oC for 1-2 hour.

1. Gel purify NotI/MfeI digested DNA, isolating the 6,816 bp band.

3. Ligate the Fragments and isolate the TALEN GCaMP8 plasmid.

   a) Ligate 6000bp aMHC-GCAMP8 fragment with CAG fragment at a 1:3 insert to vector ratio overnight. (concentrations determined through nanodropper readings and stoichiometry)

      9 ul aMHC-GCaMP8

      3 ul CAG vector

      2 ul ligase buffer

      1 ul T4 DNA ligase

      5 ul H2O

      Note: A control should be prepared using CAG vector from step 1b without insert

   b) Transform DH5a competent cells and plate on kanamycin selecting for bacteria with correctly ligated plasmids inserted using the same protocol as in 2e. Note: an additional control used should be an undigested plasmid from step 1a or 2a.

   c) Identify clones that are positive, perform miniprep to isolate DNA, and sequence to confirm accuracy of insertion plasmid creation (figure 14).

Transfection of hiPS Cells

The transfection protocol that I would've used is nearly identical to that used by Cerbini

et al. 3*10^6 cells will be harvested using the versene scraping method that I used during regular maintenance and passaging, then resuspended in 100µl P3 Primary Cell 4D-Nucleofector X Solution (Lonza # V4XP-3024) with 5µg each of pZT-C13-R1 and pZT-C13-L1 (addgene) and 10µg insertion plasmid. The cells with then be transfected by putting the mix into a 4D-Nucleofector X Unit (Lonza #AAF-1001X) and running the preset program CB-150. The cells are then re-plated onto 3 wells of a 6 well tissue culture plate in E8 media supplemented with Rock Inhibitor [6].

Colony Selection of Successfully Transfected Cells

To select for the cells with proper insertion, the transfected cells (2-3 days post transfection) were treated with the antibiotic G418(InvivoGen) for presence of an active neomycin gene. A kill curve analysis was performed on the undifferentiated B cells by plating them at different confluencies and exposing them to E8 growth media supplemented with G418 at different concentrations (5µg/ml, 10µg/ml, 25µg/ml, 35µg/ml and 50µg/ml). Additional confirmation of successful transfection through gene expression analysis and sequencing can be performed.

Cell Biology Methods; Maintenance and Differentiation of the iPS cells

Two different lines of WTCII human induced pluripotent stem cells were tested; one older acquired before I joined the lab and one newer, received 2/2016 (henceforth called A and B respectively). Both were acquired from the Coriel Institute and reprogrammed from fibroblast cells using episomal vectors [16]. The thawing, growth, and passaging methods used were all supplied by the Coriel Institute where our cells were acquired. The cells were grown on Matrigel

(Corning cat# 5187009) seeded plates incubated for 30 minutes at 37°C immediately before plating cells. Cells were fed with Essential 8 Medium (Life Technologies #A1517001) daily and split at 70-85% confluency. The A cells were passaged using StemPro Accutase (ThermoFisher A1110501) to single cell suspensions while the B cells were split using Versene (ThermoFisher 15040066) and a scrapping method maintaining the cells as clumps. Cells were plated and allowed to grow for the first day after splitting in E8 media supplemented with ROCK inhibitor (Stemcell tech 72302).

Three differentiation methods were attempted as well—a monolayer method acquired from the Conklin lab, an embroid body differentiation method modeled after the one used by Burridge et al., and another monolayer one modeled after methods used by Van berg et al [5, 33]. The Conklin protocol was received through a correspondence with Po-Lin So, PhD, who works in the lab of Bruce Conklin, MD, at the Gladstone Institutes. I performed the protocol as follows; Cells were plated at different concentrations at Day -2 and on Day 0 were fed with RPMI/B27 medium (Life Technologies) without insulin supplemented with 12μM CHIR (Tocris #4953). Exactly 24 hours after this treatment, the media was changed to just RPMI/B27 without insulin. On Day 3 the cells were again fed with RPMI/B27 but supplemented with 5μM IWP2 (Tocris #3533). On Day 5 the cells were split using a 1:1 ratio and resuspended with RPMI/27 without insulin supplemented with ROCK Inhibitor(Ri). On Day 6 the cells were fed again with RPMI/B27 to remove the Ri, and the medium was replaced every other day until Day 10 when the cells were fed with RPMI/B27 with insulin (Life Technologies #A14666SA). Media should be changed every three to four days and beating will be observed after Day 15.

For further analysis, the two lines were tested for expression of different pluripotency and cardiac makers through RNA analysis. The cells were first removed from their plate using the

respective splitting methods, then spun down and flash frozen with liquid nitrogen and stored at -80°C. The RNA of these cells were then isolated using a RNEasy Mini Kit according to the manufacturer's specifications. The concentration of RNA was determined using a nanodrop machine, then 1μg of RNA was converted to cDNA using a high capacity RNA to cDNA kit (Applied Biosystems 4387406). PCR testing on this cDNA was performed with primers for αMHC, NANOG, and OCT4 to test for expression within the cells. Specific primer sequences are outlined in table 1(Eurofins).

Fluorescent Analysis of Beating Cells

Though I was unable to analyze successfully differentiated and beating transgenic cardiomyocytes, in the coming months, upon successful gene insertion and differentiation, the cells will be recorded beating and glowing green with the calcium expression associated with beating.

Results and Discussion

Creation of Insertion Plasmid

The digest of the CAG plasmid was rife with error and utterly unsuccessful throughout the majority of my research. After starting again from square 1—the agar stab received from addgene—I achieved more positive results and eventually got to step 3c in the plasmid construct. I will outline my research and thought process through my negative followed by my more promising.

Upon initial digest two bands were observed suggesting incomplete digest (Figure 3a). In attempts to fix this issue, the volume of the digest was changed and additional minipreps were performed in case my initial prep was compromised. These results also showed incomplete digest and little difference between the undigested control and the digested samples (Figure 3b). Though the data is not included, this procedure was repeated multiple times with varying preps, digestion times, restriction enzyme concentrations, and DNA concentrations. Additionally, a new ASCI restriction enzyme was ordered in case the sample I was using had gone bad but all results were identical—no digest at the ASCI site. All samples prepared had a characteristic 7kb band and <12kb band. The 7kb band is hypothesized to be a supercoiled plasmid which will travel more quickly due to its small size, while The 12kb band is considered an open circle of plasmid DNA that will travel more slowly [38]. I expected to see and isolate a clean ~11Kb linear fragment but this wasn't observed in any of my digests. The top line on the gel in figure 2b is an unknown very long fragment that may be contamination. Through further investigation of the ASCI, I found that this enzyme is blocked by CpG Methylation, and methylation of DNA can result in unsuccessful digests [18]. Despite this potential confounding factor, I found that CpG methylation is mainly a concern in eukaryotic DNA and isn't retained when the DNA is transferred to a bacterial host [2]. Since the cells were acquired in DH5α bacterial cells, there is no CpG methylation present.

Due to the repeated failure of the ASCI digest, I performed multiple other digests using EcoRI, SalI, and XhoI (Figure 4). The SalI digest was unsuccessful, showing bands identical to the undigested plasmid and the ASCI while the EcoRI and XhoI digests showed linear fragments. Despite the EcoRI having 2 restriction sites on the plasmid, there is only one strong linear band suggesting digestion at only one site. On the other hand, XhoI digest was successful

and reveals that the plasmid has the potential to be digested. With this data I concluded that there is something wrong with the bases around the ASCI site (~5400 bp figure 1) due to the unsuccessful digest of SalI and the partial digest of EcoRI, both of which are within 50bps of the ASCI site (genotype and restriction enzyme locations acquired from addgene supplied data). Assuming either deletion or some other base pair alteration of this site, plasmid 1 and 2 from figure 3 were sequenced (figure 5). Despite the unsuccessful digests of ASCI and EcoRI, both sites were clearly observed in the sequence. The SalI site is present on plasmid 2 but on plasmid 1 slight modifications were observed confirming my gel data. This data concluded my suspicion that there are slight modifications to this segment of DNA that may be negatively affecting my digests but the true reason for the digest failures remains largely unknown.

Three of my superiors have examined the results as well, performing their own separate minipreps and digests yet the results remain consistent. These perplexing and contradictory results have thrown a wrench in my plasmid protocol and effectively halted my whole project. A potential way to circumvent this issue is to find another digestion site within the 4000-5500 region in attempts to excise the CAG GFP site while maintaining the neomycin gene to allow for antibiotic selection post transfection. Upon genomic analysis of the plasmid (though I am unsure I can still trust the addgene reference), I have been unsuccessful in finding such a site. An additional option is to create a blunt end at the XhoI site excising the neomycin gene (Figure 1a, XhoI at 3014). This has been proven successful through gel analysis though we would be unable to select the cells immediately post transfection. The only way to select for proper transfection would be to grow the cells in small wells from single colonies, genotype the colonies after growth and select the cells with the αMHC GCaMP8 insert. This process would be tedious and extremely inefficient as iPS cells have trouble growing as single cells. Due to time and financial

constraints I was unable to order an analyze a new CAG stock and I will continue to analyze this plasmid with the molecular biologists in this lab in attempts to elucidate the mechanism of my failure. If necessary, I will contact the manufacturer and Cerbini for further consultation.

After fishing the original bacterial stab of the CAG plasmid out of the freezer, I was able to retry this procedure starting completely over. This new trial was met with much more success and clear linear bands after CAG digest. After isolation the EcoRI digest was also successful showing presence of expected 8414bp fragment. The lack of success above can be attributed to contamination of my glycerol CAG stock or of my initial minipreps because there was unsuccessful ASCI digests from the very beginning.

Despite the errors in the CAG digest, the isolation of the αMHC-CaMP8 insert (figure 2a) was fairly successful. Upon initial digest, similar failures were observed with undigested plasmid present in my first HindIII digest. Upon using a larger volume with more DNA and removing an unnecessary heat inactivation step, a clear linear fragment was observed post HindIII digest (Figure 6). The band is widened and seemingly long potentially due to overloading the gel and after gel isolation a much cleaner band was observed. Like the digests, initial ligation attempts were met with failure but after tweaking the amounts of linker and total volume, successful ligation was achieved with bacterial growth on ampicillin plates (Figure 7b). The plates show growth of satellite colonies as well which is common for ampicillin. My control plate showed no bacterial growth confirming that there was no self-ligation of the digested plasmid, and that the HindIII digest and gel isolation was successful (Figure 7a). Additionally, a dephosphorylation step was performed in step 2a in attempts to minimize self-ligation and this was proven successful by the lack of growth on the control plate [32]. Ten colonies were picked, being careful not to pick satellites, and six were found to be strong positives through a MfeI digest

confirming that the MfeI linker has replaced the HindIII site (Figure 8a). Further confirmation of MfeI presence was confirmed in all six positive colonies through sequencing analysis (Figure 8b). NotI digestion was also successful showing 2 clear bands after digest (Figure 9a). The 3kb band is expected while the longer band is expected to be ~7kb. MfeI digest of the longer band could not be confirmed as the band was extremely faint and there was no observable 700bp fragment (Figure 9b). This could be due to the fact that the band was run in an extremely wide lane distributing the DNA and potentially the 700bp band was too faint to be seen. MfeI digest was assumed to be successful due to previous data proving its presence and ability to be digested and isolation of the top band was successful though a very small amount of final insert was present (Figure 9c). Further testing, particularly of steps 2j-l, will be performed to examine the slight inconsistencies in data and the presence of a longer than expected final product. Also for successful insertion and ligation, there must be a large amount of insert fragment present so more DNA will have to be isolated than the tiny amount seen in figure 9c.

The ligation of the two plasmid fragments was performed and the product was transformed in DH5α bacteria plated on kanomycin. After allowing incubation, I saw the presence of three colonies on my many plates. This was a very low ligation success rate and is potentially due to the extremely low concentration of fragment vector and insert, about .0014 pmol/μL of each. This is much lower than the concentrations recommended by the manufacturer, but nonetheless there was growth suggesting a circular plasmid with kanamycin present in the colonies. The reason for this growth can be attributed to three things; either successful ligation and plasmid transformation, self-ligation of CAG fragment and insertion, or possible contamination. To confirm that there was proper insertion I performed an EcoRI digest where the TALEN αMHC-GCaMP8 plasmid would linearize due to the presence of an EcoRI site on the

insert and a ligated CAG fragment or contamination would remain circular. This diagnostic test was inconclusive and due to time constraints I was unable to perform another. To continue my work one should perform more digests and potentially sequence to verify proper plasmid creation. If my plasmid isn't present in this bacteria, I would suggest performing my procedure again but this time with much more DNA and elute into lower volumes causing higher concentrations to avoid the potential difficulties associated with ligating at such a low concentration of fragment DNA.

The utilization of a blunt and sticky end ligation was outlined because it is efficient and causes proper orientation of the insertion fragment. My sticky ends, despite being different digests, are expected to join because EcoRI and MfeI have complementary overhangs (AATT). The blunt end reactions didn't require dNTPs in addition to the Klenow despite having 5' overhangs because they were performed directly after digests where the digestion buffer would supply the needed material.

Differentiation of iPSCs to Cardiomyocytes

The differentiation of the B cells was successful using the Conklin differentiation protocol. This was confirmed through observation of beating after day 15 and expression of αMHC (Figure 10 and 11a). The largest number of beating cells were observed from cells plated at 2.5E4 and 5E4 cells per well at day -2. The differentiation of the A cells was unsuccessful when subject to the Conklin, Burridge, and Van Berg protocols. Though there is expression of αMHC in these cells, there was never observed beating suggesting incomplete differentiation (Figure 11b). NANOG expression was seen in both populations of differentiated cells (Figure 11). This can be attributed to presence of not completely undifferentiated colonies suggesting that the protocol is not completely efficient. Despite this expectation, there is no Oct4 RNA in

the differentiated cell lines though implying that NANOG expression is turned off later in the differentiation process. Research confirms this data and while Oct4 plays a large role in maintaining pluripotency, NANOG is considered to only inhibit ectoderm formation and not affect other lineages [35]. Both cell lines were confirmed to be initially pluripotent before testing through presence of Oct4 and NANOG RNA (figure 11). In comparing RNA expression between differentiated cell lines, there is much higher expression of αMHC in B and more NANOG expression in A. This reinforces my conclusion that the B cells were differentiated much more efficiently.

The only true differences between the cells was method of passaging and passage number where the A cells were older by 5 passages. This suggests that passaging iPSCs with accutase into single colonies can affect the differentiation potential of the cells and that with more passages the cells lose differentiation potential as well. To avoid unwanted results, the RNA expression analysis utilizes primers on either side of long stretching introns such that DNA from the cells that may have been mistakenly isolated will not be replicated during the PCR step. This ensures only cDNA replication and that data is acquired only from RNA present in the cells.

Kill Curve Analysis

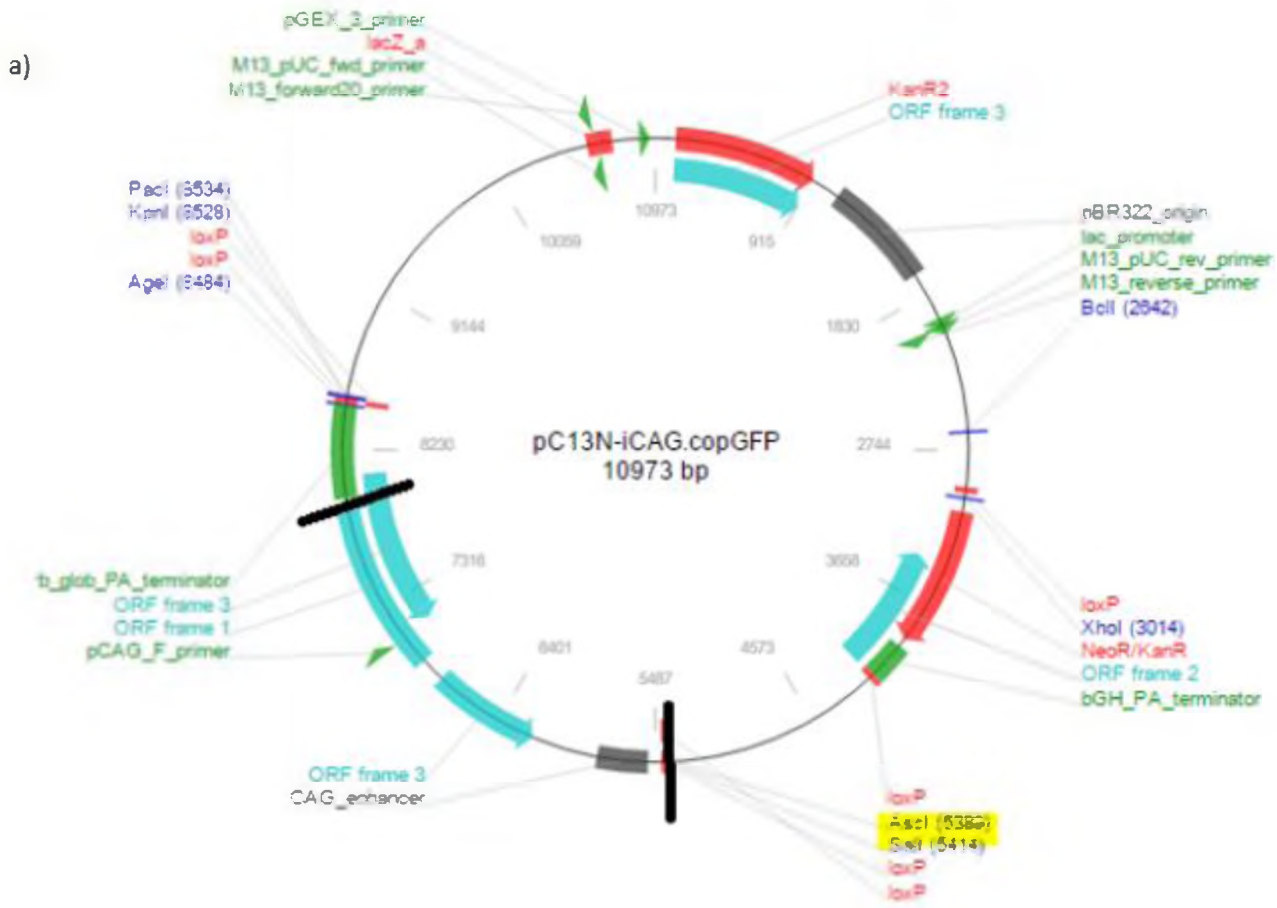
Though colony selection was unable to be performed, my kill curve analysis showed that that with a higher confluency, a longer amount of time and a higher concentration of antibiotics are needed to effectively kill cells. With a 20-40% initial plating confluence, three days at 10ug/ml are required to effectively kill cells after 4 days, while at 15% confluency 5ug/ml is required to kill cells after 2 days. In the future I will repeat this analysis at 15% confluency with lower concentrations of G418 because 2 days is an extremely quick death and this concentration may kill my transfected cells if they are lightly expressing neomycin. Additionally, I would've

preferred to have numerical data to quantify the cells present rather than just observational analysis.

With current research in tissue engineering progressing, researchers require tools to confirm not only the creation of proper cells and tissues but also the functionality of these tissues. The cells I intended to create do both of these things and effectively adds an additional quantifiable measurement of successful differentiation, association, and beating of cardiomyocytes. While I was unable to complete this task due to failures in the molecular biology component and time constrants, I am poised to transfect and differentiate upon confirmation of plasmid creation. With the protocols and data I have presented, one could continue my work and effectively create a line of αMHC-GCaMP8 iPS cells. Though there has been research transfecting iPS cells with monitors for calcium expression, there is nothing currently (as of April 2016) like the cells I have intended to make and my research is unique in my usage of αMHC-GCaMP8, and TALEN transfection [1, 28]. This research would not only provide innovation in heart research tools but also in human iPS cell editing. With more positive results showing viable methods of human iPSC editing, clinical trials are more likely to be approved further progressing the field of medicine. Additionally, the logic and research I have used can be applied to other calcium promoting cells and theoretically I could create multiple insertion fragments with varying promoters specific to different cell lines. A library of iPSCs transfected with different promoters for calcium expression coupled to GCaMP8 could provide limitless research potential particularly in studies of heart, muscle, or nerve cells. With the completion of this project, these tools can become a reality and human iPSC research can be revolutionized.

This project has taught me how to research, create, and implement new technologies and ideas both alone and as part of a larger collective. Most importantly I have learned the true fluidity of biological research. My project and protocols involved dozens of changes, slowly chipping away and sculpting until results are finally beheld. Though I was unable to marvel at success, I can still see the art in the marble and know that my work is far from finished.

Figures and Tables:



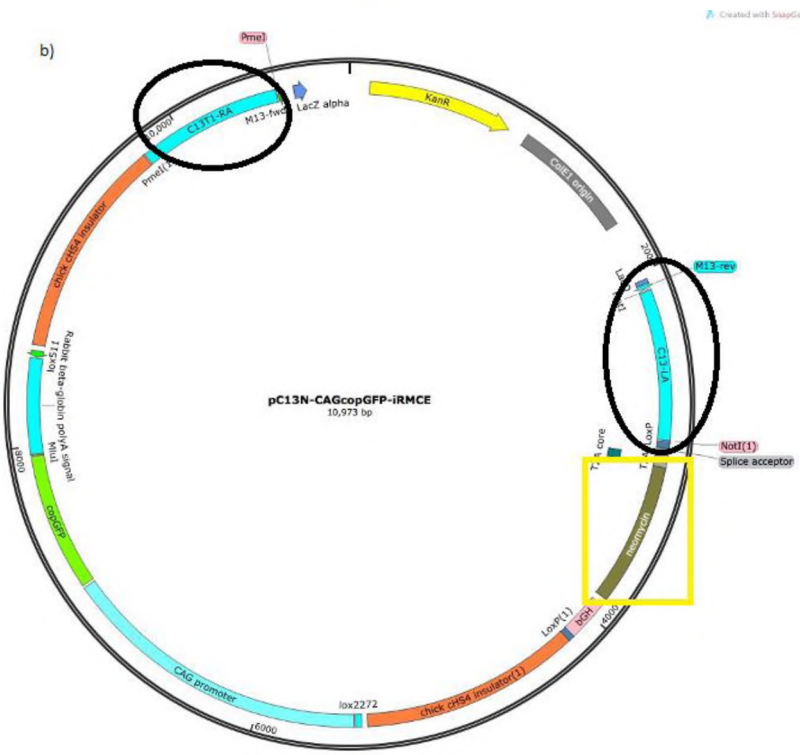
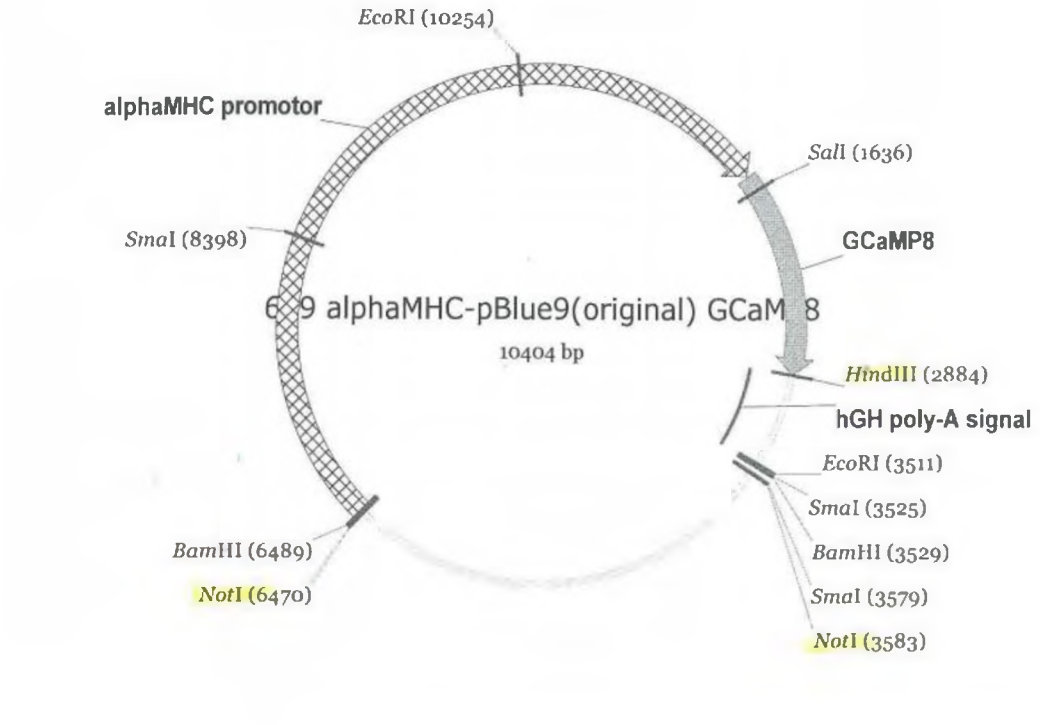


Figure 1: CAG Plasmid Images. Both images show maps of the same plasmid while the top map (A) shows restriction sites with important ones used highlighted. Though EcoRI sites aren't labeled, they are marked by the black lines. B) Depicts the genes present note the two TALEN homologous arms circled in black and the neomycin gene boxed in yellow. (both images acquired from addgene site)

a)



b)

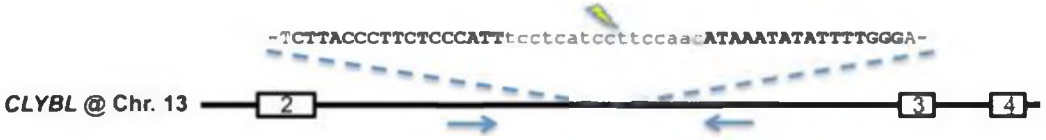


Figure 2: Map of insertion fragment and site of plasmid insertion into genome. A) Map of the αMHC-GCaMP8 insert where restriction sites of interest are highlighted. Though excised genes aren't shown, there is an active ampicillin gene (image acquired from Bo Shui). B) Image of the site of plasmid insertion where the TALEN arms are bold and the site of DNA cutting is marked by lightning (figure 1D from [1]).

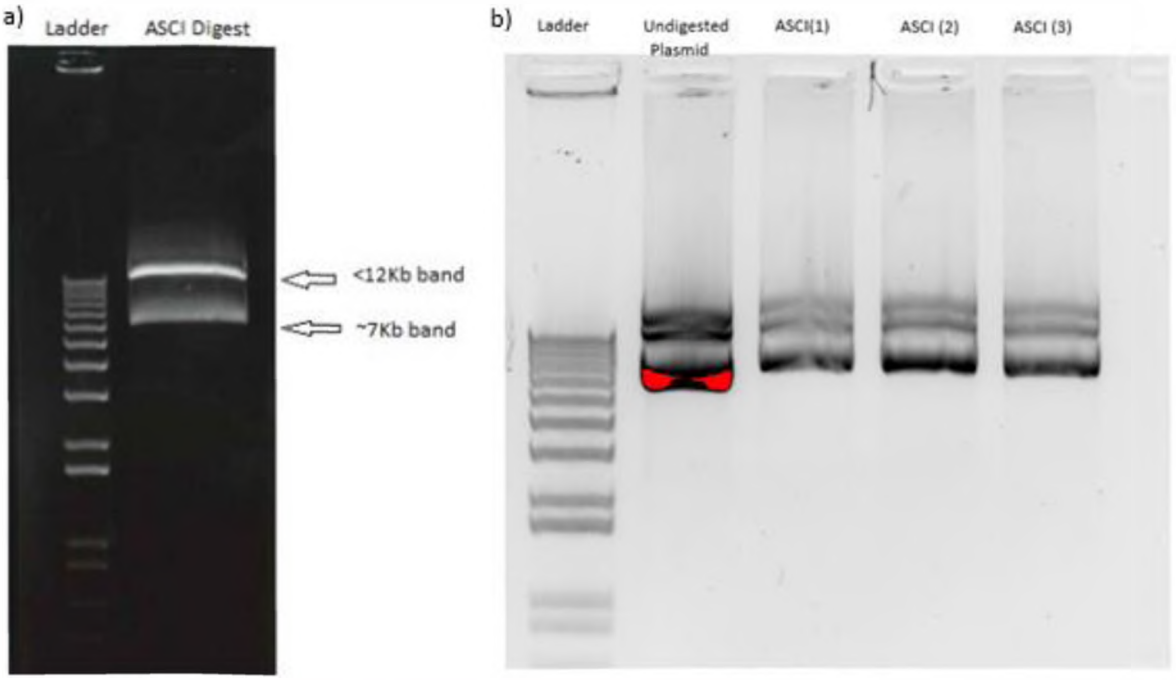


Figure 3: ASCI Digests. A) First ASCI Digest with two bands suggesting incomplete digestion. B) The same digest run with different minipreps and an undigested control.

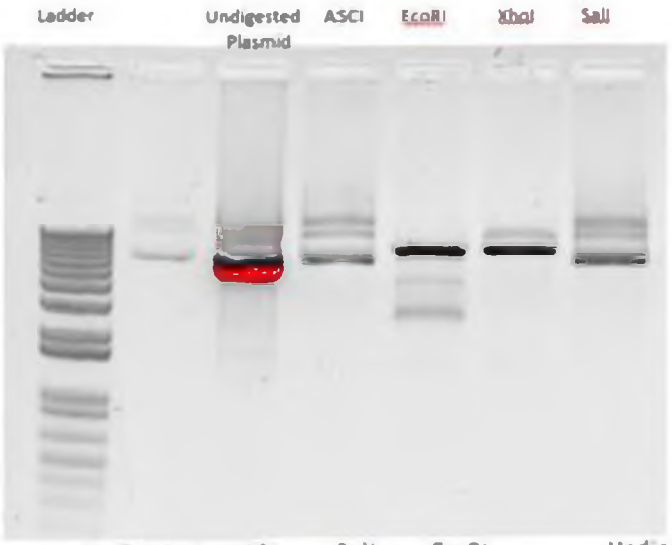


Figure 4: CAG Plasmid Test Using 3 Different Preps. Note that all the digestions in our area of interest were unsuccessful, though there might be slight EcoRI as observed by the faint band in the top gel.

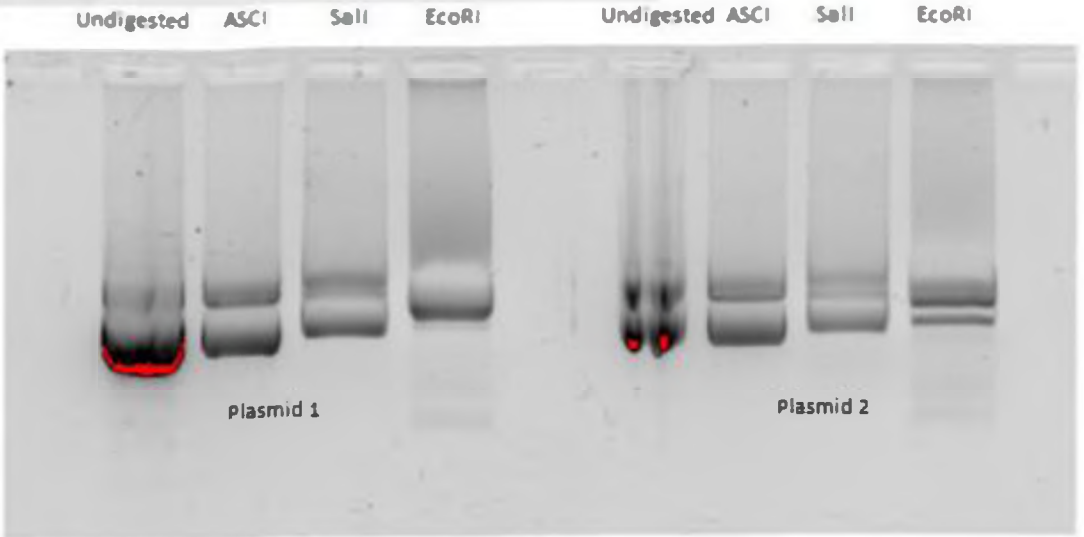
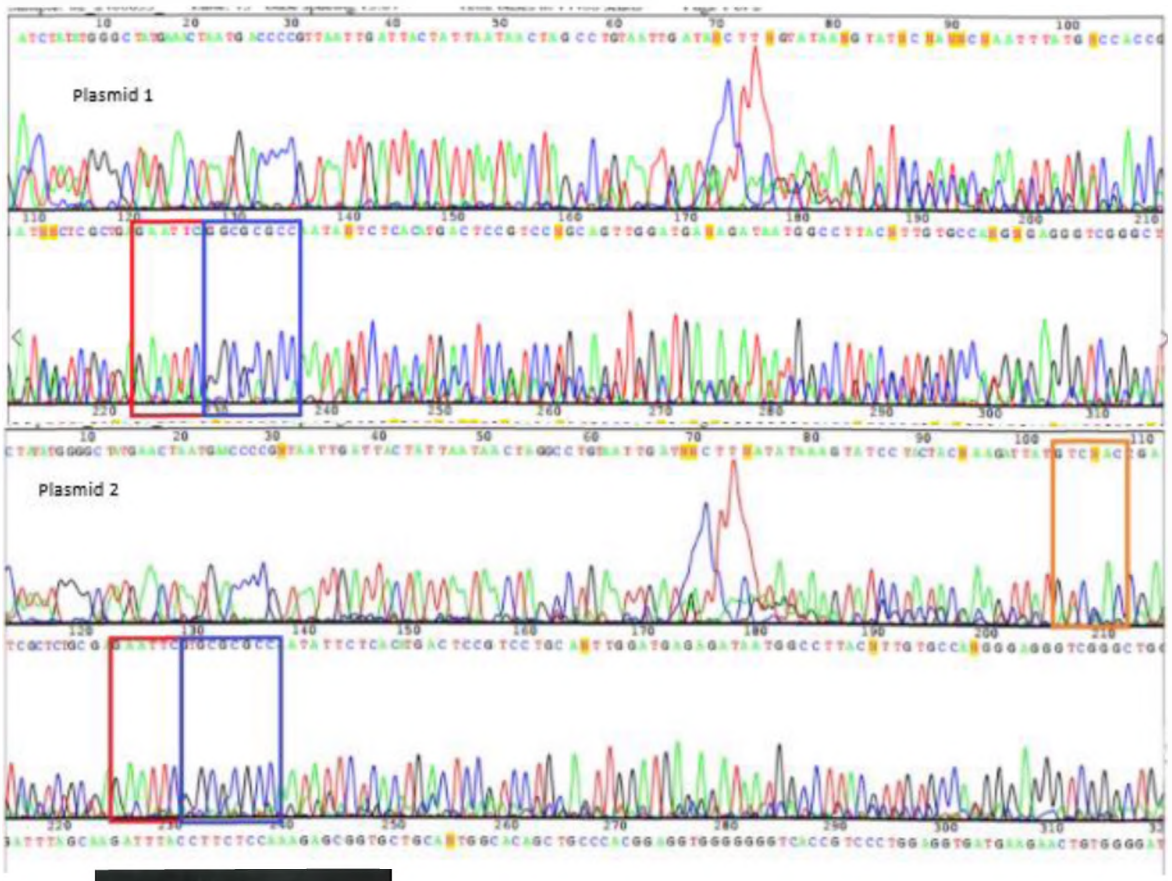


Figure 5: Sequencing data from iCAG plasmid 1 and 2. These are the same plasmids used in figure 3. The restriction sites are marked by a rectangular box with SalI being orange, EcoRI red, and ASCI blue. Based findings on peak readings more so than given sequences.



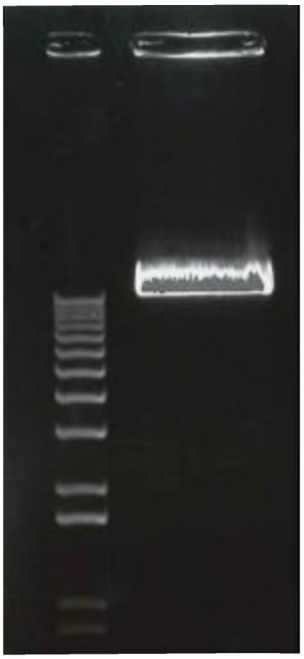
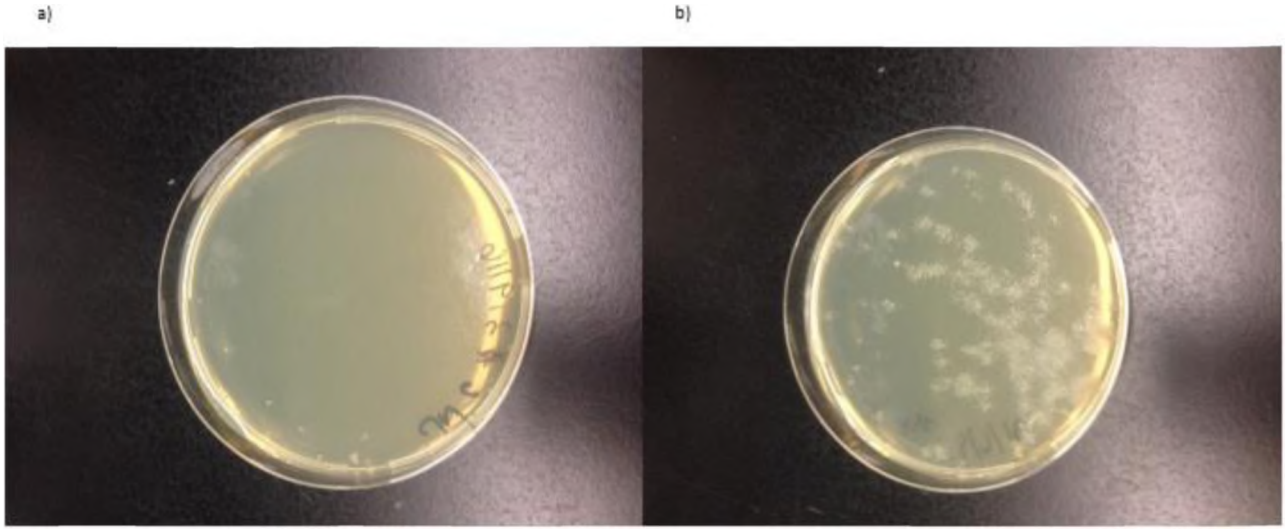


Figure 6: Initial GCaMP8 Plasmid Digest

Figure 7: Bacterial growth on Ampicillin plates after MfeI ligation. A) negative control with no MfeI added B) treatment with MfeI. Many satellite colonies observed around growth as image was taken a week after plating.



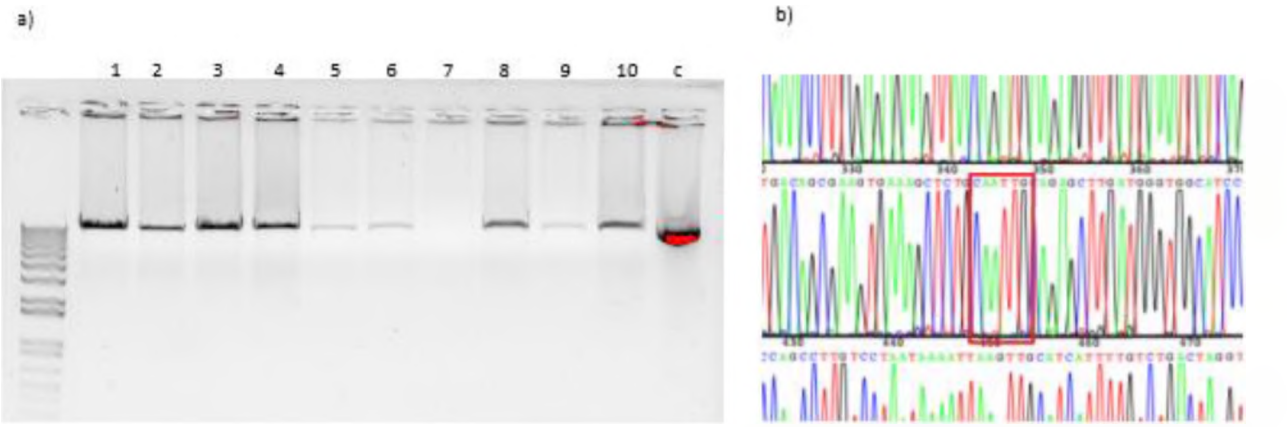
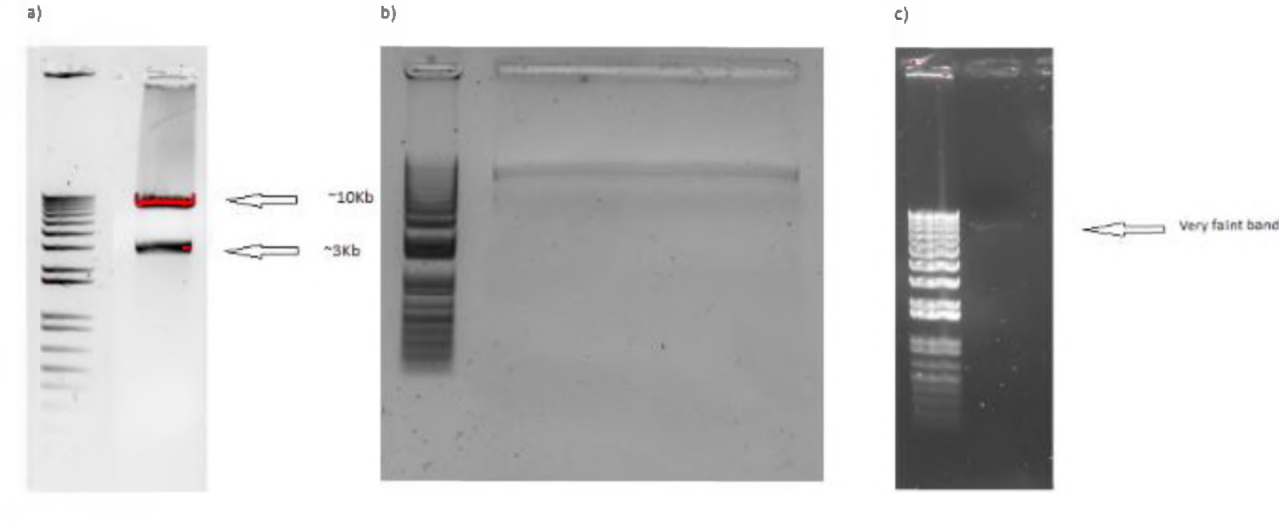


Figure 8: Confirmation of successful replacement of HindIII site with MfeI. A) Depicts MfeI digest of 10 picked colonies with samples 1, 2, 3, 4, 8, and 10 being positive. C is an undigested GCaMP8 plasmid control. B) Sequencing data confirming the presence of an MfeI site in sample 1. MfeI CAATTG in red box. Similar date was observed in all positive samples.

Figure 9: Isolation of alpha MHC GCaMP8 insert. A) Gel post Not1 digest where the top band was isolated. B) Gel post MfeI digest where again the top band was isolated. C) 5ul from gel isolation of long band in b to test for concentration.



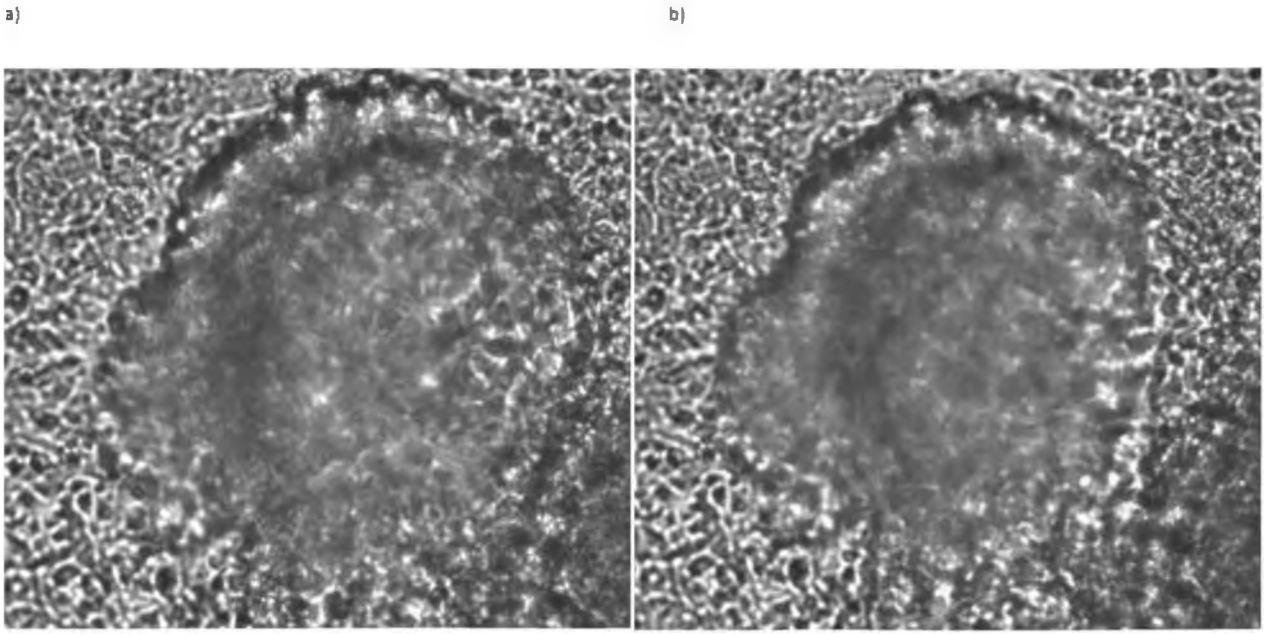
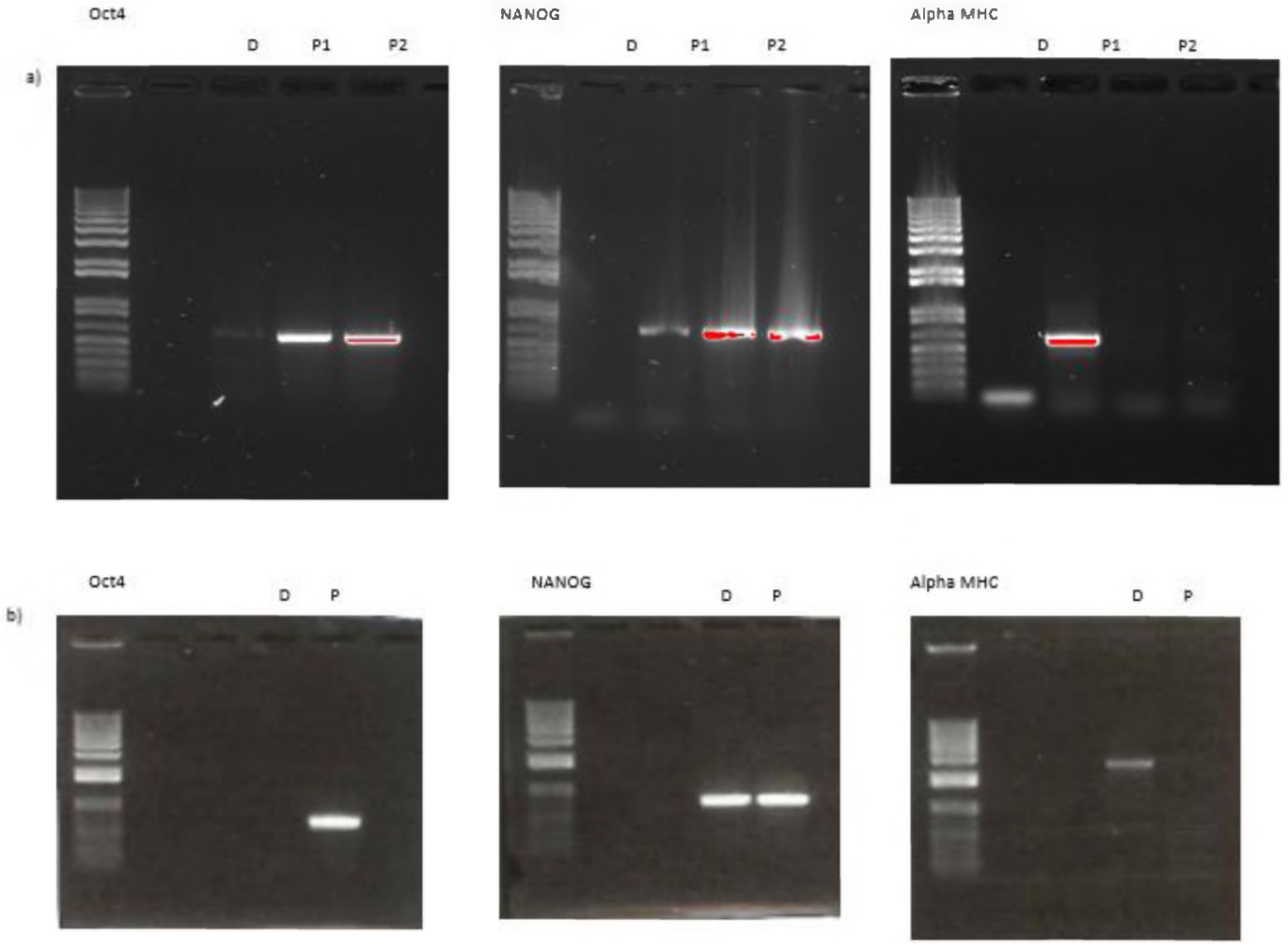


Figure 10: Screenshots from video of differentiated and functionally beating cardiomyoctes. A) Depicts the cells at their resting state. B) Shows contraction of the cardiac cells. Note the change particularly in the middle and on the right end of the cells. Images taken under 20X magnification.

Figure 11: Analysis of RNA expression in cultured cells. A) represents B cells with the 3rd lane marked D and the 4th and 5th marked P while B) represents analysis of the older A cells with the 4th lane marked D and the 5th lane marked P. The D lane is derived from cells that have been subject to a differentiation protocol while the P lanes are harvested pluripotent cells. The cells were tested for expression of Oct4, NANOG, and Alpha MHC. Note the differences in alpha MHC and NANOG expression.



	Alpha MHC	NANOG	Oct4
Forward Sequence	CGGCCCAGATTCTT CAGGATT	GACTGAGCTGGTTGC CTCAT	GTAGTCCCTTCGC AAGCCCT
Reverse Sequence	TCCCGTGATGAGGA TGGACT	AGGGCCTTCTGCGTC ACAC	ACCTTCCCAAATA GAACCCCCA

Table 1: 5' to 3' sequences of primers used for RNA analysis

Figure 12: Successful CAG results: a) Shows post ASCI digest with the ~11kb linear band shown. For better understanding compare to unsuccessfull digests in figure 3 and 4. b) shows post EcoRI digest where the longer 8,414bp band is the vector plasmid, note the two bands confirming successful digest.

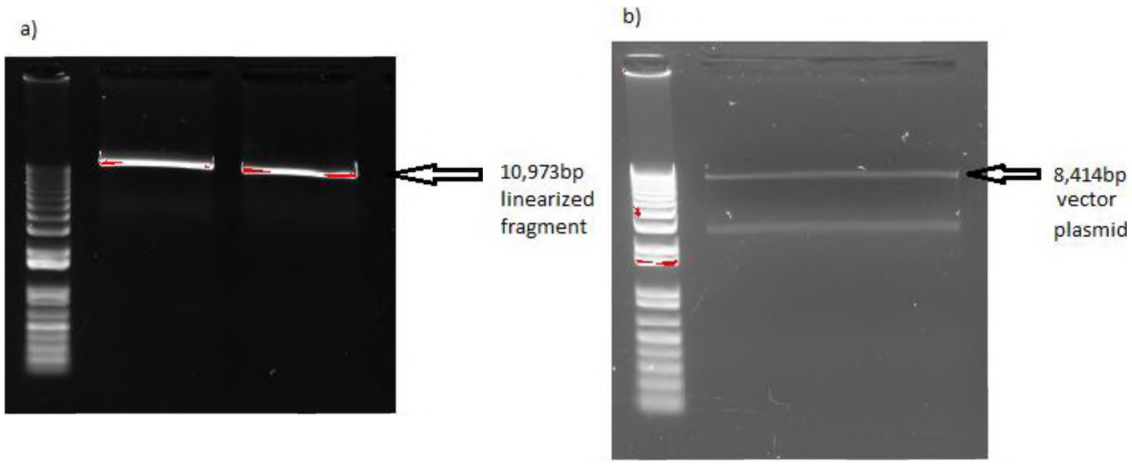
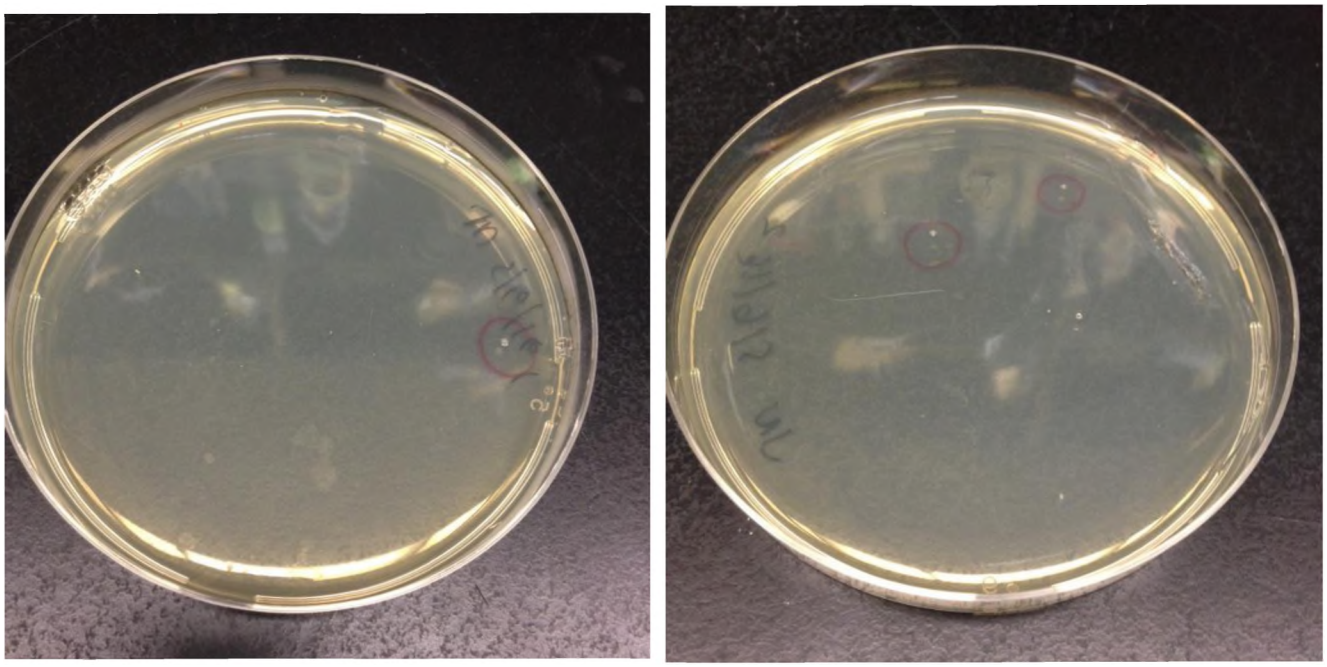


Figure 13: Bacterial Growth on Kanomycin Plates Post Ligation: Shows the three bacterial colonies on the two plates suggesting the presence of a kan gene on a circular ligated plasmid. The colonies are circled in red. Low incidence of growth can be attributed to low fragment concentrations in the ligation.



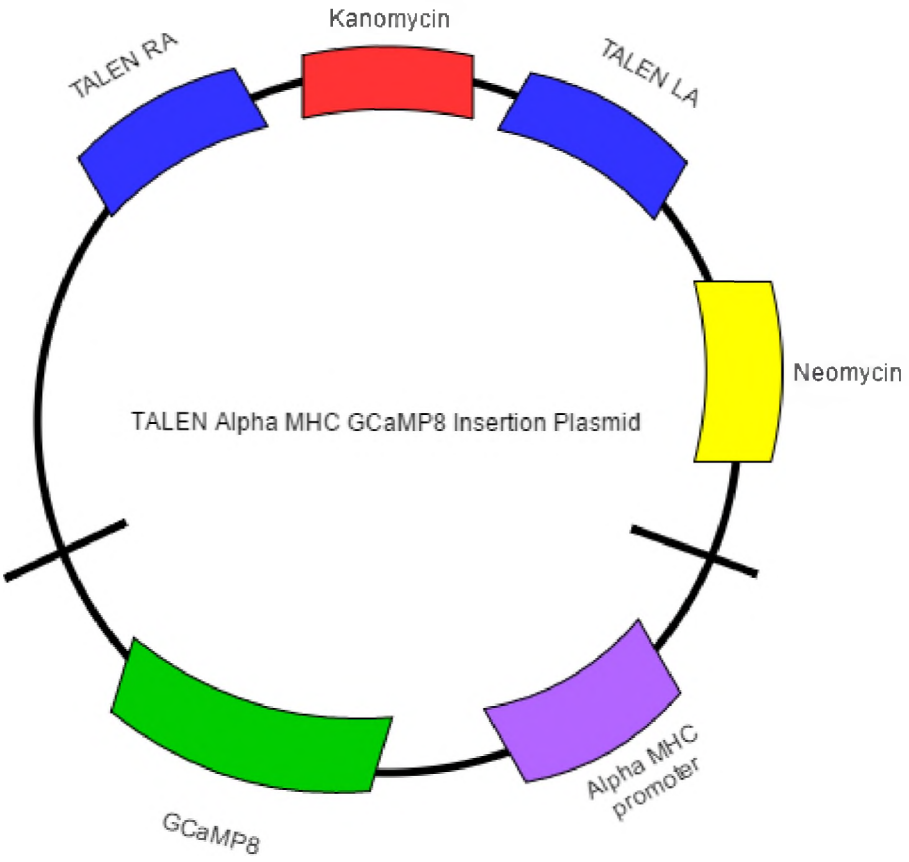


Figure 14: Diagram of insertion plasmid: Note that the image is not to scale. Also the black dashes mark the site where the ligation occurred.

Table 2: Kill Curve analysis. Three different kill curves were performed but it appears that with a higher confluency, a longer amount of time and a higher concentration of antibiotics are needed to effectively kill cells. With a 20-40% initial plating confluence, three days at 10ug/ml are required to effectively kill cells, while at 15% confluency 5ug/ml is required to kill cells after 2 days. Different trials separated by black row.

G418 concentration	Control	5ug/ml	10ug/ml	25ug/ml	35ug/ml	50ug/ml
Day 0	All plates have some cell death but show consistent levels of growth and					

	are around 15% confluency					
Day 1	Some floating cells observed but mainly good growth	Large number of dead, floating cells observed - still ~10% confluent with viable cells	Similar to 5ug... but instead with ~5% confluency	Majority of cells dead >5% confluent	Tons of death (maybe plated at higher concentration, ~5-10% confluency)	All cells floating/ dead, no pure iPS colonies remain
Day 2	Floating cells again observed but definite cell growth is taking place, ~ 20-30% confluent	No observable cellular growth				
Day 0	All cells appear to be in large well defined colonies with well at ~20-40% confluency					
Day 1	Floating dead cells observed with many embryonic colonies. Many have 'skirt' and appear to be growing upwards but still viable and well defined cells.	Many floating cells and seemingly dying colonies, though there are still some well defined colonies.	Many dead/dying cells-- characteristic defined edges appear lost	Almost complete death- very few viable colonies still growing	Extreme cell death, most cells appear as smudged cluster that doesn't appear to be growing	
Day 2	Many cells appear to be dying	Most cells are dissociating-	Living cells observed but not well	Most cells appear to be dead or dying.		

	(smudged) but there are still viable cells on the outsides of the plate	but a few viable colonies still remain on the top.	defined in the characteristic iPS nature.	No growth observed		
Day 3	Still plenty of living cells, skirt appears to be growing g and some of the larger colonies appear to be growing up suggesting slight differentiation	Floating cells suggest death but still colonies present >10%	No more death observed, but cells present appear non iPS and not growing	No additional floating cells but cells still plated appear to be dead/dying	-more death observed	
Day 4	Lot of cell death with floating cells but seemingly viable iPS colonies remain	No cell growth/ no living cells				

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