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The Preparation of Synthetic MyoD mRNA for Cellular Differentiation and Innate Immune Response Downstream Application

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The Preparation of Synthetic MyoD mRNA for Cellular Differentiation and Innate Immune Response Downstream Application

by

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Abstract

The development of therapeutic immune responses from the manipulation of embryonic stem cells (ESCs) via induction of synthetic RNA with 5' capped and 3' poly(A)tailed ends would lead to development of stem cell therapy. A necessary step in attaining such a goal is to first produce an mRNA transcript from a plasmid containing the open reading frame (ORF) for a transcription factor for cellular activation.

In this research, the DNA plasmid pMD4 encoding MyoD was transcribed into synthetic mRNA. The plasmid was first amplified using Polymerase Chain Reaction (PCR), and analyzed using gel electrophoresis. The amplified template was purified via ethanol precipitation. The template was then transcribed using T7 RNA polymerase and an RNase inhibitor along with RNase free buffers and solutions. Gel filtration Spin Column Chromatography was then used to separate and purify the RNA transcript so that it could be properly quantified using UV-Vis Spectroscopy. A concentration yield of 4.2μg/μL of the RNA transcript was obtained (Figure 9) and stored for further downstream cellular differentiation and innate immune response application.

Key Words: mRNA synthesis, MyoD, stem cell differentiation, innate immune response

Dedication

To Mom, Dad, and John Caleb: Every success and accomplishment of mine will follow a common thread back to you before finally reaching our Creator. Your love and encouragement have been my backbone through everything.

And to the brothers and sisters who suffer from any invisible Autoimmune disease: Keep fighting and working hard through every pain. It is worth it. It is always worth it.

Abbreviations

DNA: Deoxyribonucleic acid

dNTPs: Deoxynucleotide triphosphate

ESCs: Embryonic Stem Cells

EtBr: Ethidium Bromide

iPSCs: Patient Specific Induced Pluripotent Stem Cells

kb: Kilobase

mRNA: Messenger Ribonucleic acid

ORF: Open reading frame

PCR: Polymerase Chain Reaction

RNA: Ribonucleic Acid

UV-Vis Spectroscopy: Ultraviolet-Visible Spectroscopy

Table of Contents

Chapter 1: Introduction

The purpose of this study is to efficiently direct patient specific induced pluripotent stem cells (iPSCs) towards a clinically competent cell type via synthetic messenger RNA (mRNA). So far, derivations of such an idea have produced toxic results and modified cellular reprogramming (Warren, et al., 2010). Therefore, methods of properly synthesizing RNA that is not seen as viral by the innate immune system are key to this project. The first step of such an application is to synthesize mRNA.

Messenger RNA is a conveyor of genetic information from DNA to the ribosome. DNA is transcribed into RNA, which translates into proteins. This central dogma of molecular biology is key to how proteins, and therefore, our cells are made and function.

The innate immune system is a subsystem of the overall immune system that is comprised of cells and mechanisms that defend our body against foreign pathogens in a generic way by providing immediate response to foreign invaders. Cells that mature in the innate immune system are first stem cells. Stem cells can simply be thought of as cells that reproduce through mitosis without changing cell type. If stem cells can be made for our innate immune system in a specific manner, our body can quickly defend itself from foreign invaders that might be detrimental to the human body.

For the purpose of this research, synthetic mRNA was transcribed from a MyoD DNA template in order to inoculate stem cells to observe change in their potential function in immune responses.

MyoD is a transcription factor that prepares myoblasts for efficient cellular differentiation (Ishibashi, Perry, Asakura, & Rudnicki, 2005). Muscle development begins to be conveyed through MyoD in myoblasts when mesodermal cells begin

developing in the third week of embryonic development. Differentiation is kept dormant by various mechanisms that inhibit potent myogenic factors during early development. During transcription, to efficiently differentiate cells, a 5' cap (Figure 1) must be added to each precursor mRNA molecule, creating a stable and mature mRNA molecule allowing translation to occur.

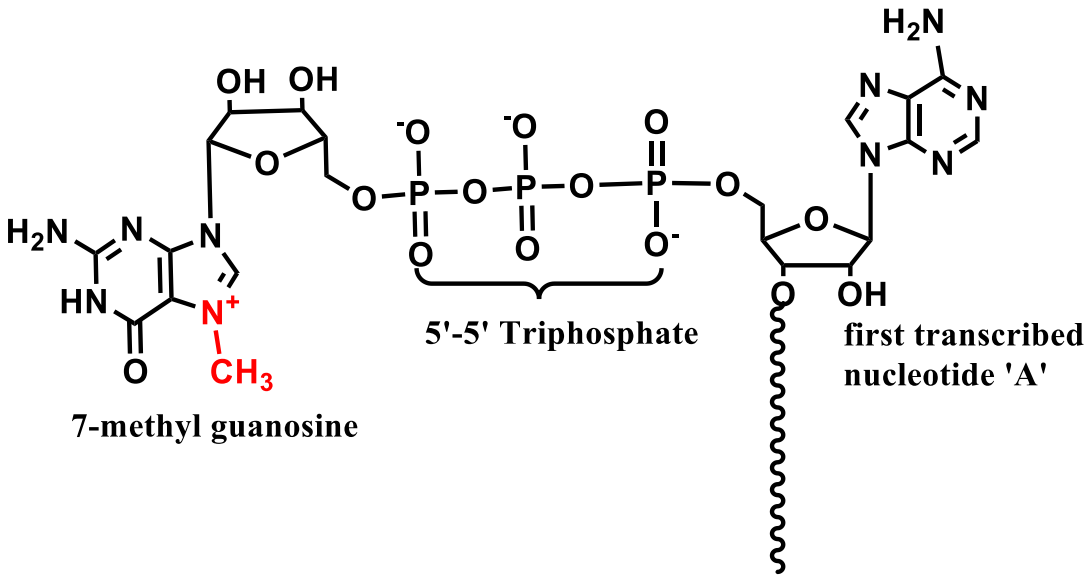

Figure 1: 7-methyl-guanosine caps precursor mRNA on the 5' end to protect it from degradation from nucleases all the while producing mature mRNA able to undergo translation (Lewis & Izaurralde, 1997).

The steps taken for such a project were to use a plasmid as a template to amplify the MyoD gene with a primer. Using Polymerase Chain Reaction, the template was amplified and then observed using gel electrophoresis. The DNA template was then purified using ethanol precipitation. After purification, the DNA template was transcribed *in vitro* into mRNA using RNA polymerase and an RNase inhibitor. The mRNA was then purified using spin column chromatography and quantified using UV-Vis Spectroscopy.

Chapter 2: Literature Review

As explained in chapter 1, synthesizing mRNA with a 5' cap for stem cells to differentiate can be used to manipulate our innate immune system in a positive way is key to this research project. Similar research has been conducted that has led to this research question, and key variables must be considered.

Modifying mRNA to direct cell production:

Modifying mRNA using a synthesized 5' cap to form a unique form of mRNA is key to the manipulation of generating cells that can perform specific tasks utilizing our innate immune system to defend our bodies. Warren, Manos, and their team found that synthesized mRNA can be used as a non-integrating strategy to inoculate stem cells, reprogramming cell fate and directing the differentiation of pluripotent stem cells into differentiated myogenic cells (2010). The application of this idea guides the future of regenerative medicine through the manipulation of such stem cells.

Generating stem cells via modified mRNA:

A stem cell is an undifferentiated cell that can be replicated an indefinite amount of time; yet it can also give rise to specialized cell types. Controlling these cells by manipulating their original stages could lead to an endlessly complex form of therapy, due to the wide range of potential tissues and organs to which the differentiation of these stem cells could be directed.

To differentiate these stem cells, mRNA must first be modified in an appropriate manner to lead translation into an effective protein that could be transiently expressed,

directing the specifically desired cell-type . In the same study mentioned before, Warren, Manos, and their team found that factors induced into this system were "robustly expressed and correctly localized to the nucleus" (2010). This study alone is a promising characterization of the theory for stem cells to be adequately manipulated and directed.

Synthetic mRNA inoculated into cells without a proper 5' cap and poly(A) tail has shown to be toxic by the immune system, thus causing a problem when used for stem cells. Some stem cells in mice, however, have been shown to lack the properties that fight against viruses and foreign bodies. Wang, Teng, and their team including my advisor Dr. Faqing Huang have studied the effects of synthetic mRNA on stem cells in mice and have concluded that, "…[there is] evidence to a general conclusion that mESCs [(mice stem cells)] have underdeveloped antiviral mechanisms" (2014). This could be beneficial for further research in differentiating stem cells into healthy cells that contain various genes to encode proteins that help defend areas affected by foreign bodies.

Response of Innate Immune System from such cells:

Because of the lack of antiviral responses in mESCs, proteins can be efficiently translated from synthetic mRNA because the stem cells can have nucleic acids repeatedly introduced to them without any immune responses arising. Therefore, it is believed that the innate immune system can be manipulated by gene expression from synthetic mRNA that avoids the safety concerns of viral derivatives in regenerative medicine.

It was shown that some single-stranded strands and protein-encoding synthetic mRNA produced amounts of cytotoxicity to the stem cells that would cause unfavorable conditions for the immune system, causing adverse effects in the organism. Therefore, different transcription factors to facilitate synthesizing appropriate mRNA for the activation of gene expression in stem cells is key to future projects (Wang et al., 2014).

Chapter 3: Research Methods

The means taken to undergo research amplifying a DNA template and transcribing the template into mRNA were completed with several different instruments in the lab. The following instruments are regularly used to amplify DNA: Polymerase Chain Reaction and Gel electrophoresis. After amplification, the DNA template was purified using ethanol precipitation, and it was then transcribed into mRNA using RNA T7 polymerase followed by gel filtration spin column chromatography. The mRNA was then quantified using UV-Vis Spectroscopy.

Amplification and Purification of MyoD DNA Template

Polymerase Chain Reaction (PCR)

A known efficient MyoD DNA template pMD4 was amplified using the primers M13f/Ma34. A 50 μL mixture was made using 37.75 μL RNase free water, 5 μL for a 1X Buffer, 5 μL Mg^{2+}, 1.25 μL M13f/Ma34 primers, 0.5 μL dNTPs, 1.0 μL pMD4 template, and 0.5 μL Taq Polymerase.

The process of PCR is outlined in Figure two, where the template must first be denatured, then annealed, and finally elongated. Depending on the template, the temperature, time, and number of cycles must be properly controlled. This mixture was denatured at 98°C for 50 seconds, annealed at 55°C for 50 seconds, and extended at 72°C for 1 minute and 15 seconds. From previous experimentation and statistically based understanding, 20 total cycles of the above procedure were induced for complete and thorough amplification.

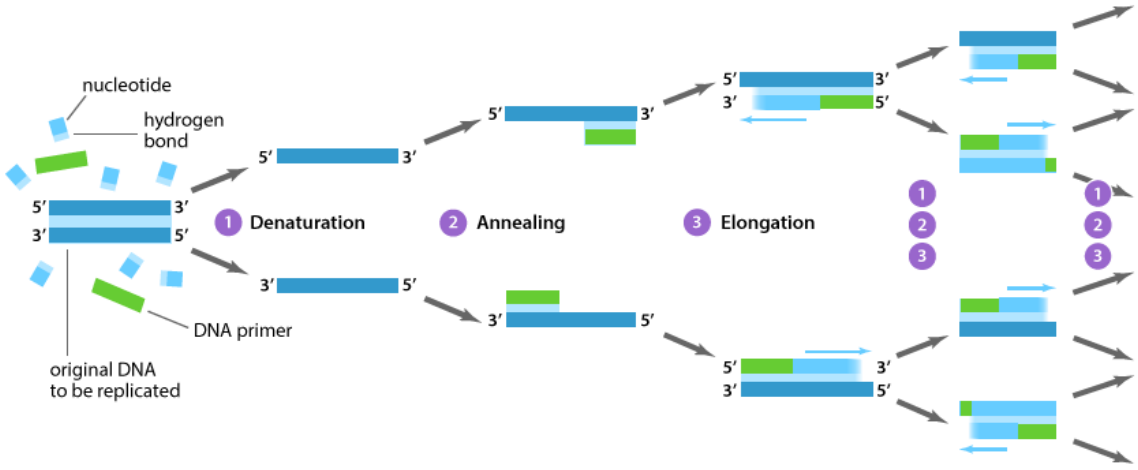

Figure 2: How Polymerase Chain Reaction works: 1) Denaturation: the reaction is heated to 98 °C for 50 seconds to break the hydrogen bonds between the strands. 2) Annealing: the reaction temperature is lowered to 55 °C for 50 seconds to allow primers to anneal to the template strands. 3) Extension: the temperature is increased to 72 °C to allow for the addition of dNTPs. The amount of target sequence doubles with each thermal cycle which leads to an exponential amplification represented by $2^{20)}$.
https://www.abmgood.com/marketing/knowledge_base/polymerase_chain_reaction_introduction.php

Gel Electrophoresis

After amplification of the DNA template, the mixture was observed using gel electrophoresis with a DNA ladder standard. Gel Electrophoresis is used to separate and analyze fragments of macromolecules based on size and charge using an electrical field. Figure 3 shows the apparatus and electrical input and output receivers, indicating the flow of the macromolecules. The macromolecules travel through the pores of the gel at a speed that is inversely proportional to their sizes (*Nature*, scitable).

A 30 mL 1X gel was made by putting 0.45 g agarose into an Erlenmeyer flask with 10X TBE buffer solution. It was heated for 30 seconds in a microwave, and then 0.5 µL Ethidium bromide (EtBr) was put as a stain in the solution. At ~60°C, the solution was put in the mold with wells for 30 minutes to form the gel. Then, 5 µL of

the DNA ladder and the amplified DNA stock were run for 30 minutes in gel electrophoresis.

Figure 3: Gel Electrophoresis Apparatus: Tested Molecules are run through a gel and electrical field where the smaller molecule will travel a greater distance through the gel than the smaller.

Ethanol Precipitation

After the MyoD DNA template was amplified and observed, it was purified using ethanol precipitation. Ethanol functions as an anti-solvent in the presence of a monovalent cation, and efficiently precipitates nucleic acids out of a mixture. The 50 μL PCR stock solution was added to a new tube with 5 μL 3M Sodium Acetate, 150 μL Pure ethanol, and was then vortexed for several seconds. The solution was stored at -20°C for one hour and then centrifuged at 14,000 RPM for 8 minutes. The DNA pellet was obtained by collecting the supernatant into a new tube. Finally, 5 μL Pure H_2O was added to the DNA pellet.

RNA Transcription

Following DNA purification, *in vitro* transcription was performed. A 10 μL sample was made by adding 3.25 μL of RNase free water, 1X buffer (1 μL), 1mM dithiothreitol (DTT)

(1 μL), 7.5 mM NTPs (3 μL), 0.5X (0.5 μL) purified DNA template, 1 μL T7 promoter, and 0.25 μL RNase Inhibitor. The solution was incubated at 37°C for 1 hour.

Gel Filtration Spin Column Chromatography

After incubation, the mRNA transcript was purified by adding 100 μL of RNase free water and transferring it to a 100k DA molecular cut-off spin column. It was centrifuged for 2 minutes at 14,000 RPM, and 50 more μL was added to the column. It was again centrifuged for 6 more minutes, and the supernatant was then discarded. For two minutes at only 2,000 RPM, the RNA transcript was centrifuged into a new tub in order to collect the sample without contamination.

Using UV-Vis spectroscopy, the sample was quantified between 220 and 320 nm in a 500-fold dilution in a 500 μL glass cuvette. Figure 4 shows a schematic, where a beam of light is reflected into the sample, and an absorbance value due to the wavelength of light is recorded. The μM concentration due to the absorbance value and dilution factor was then calculated.

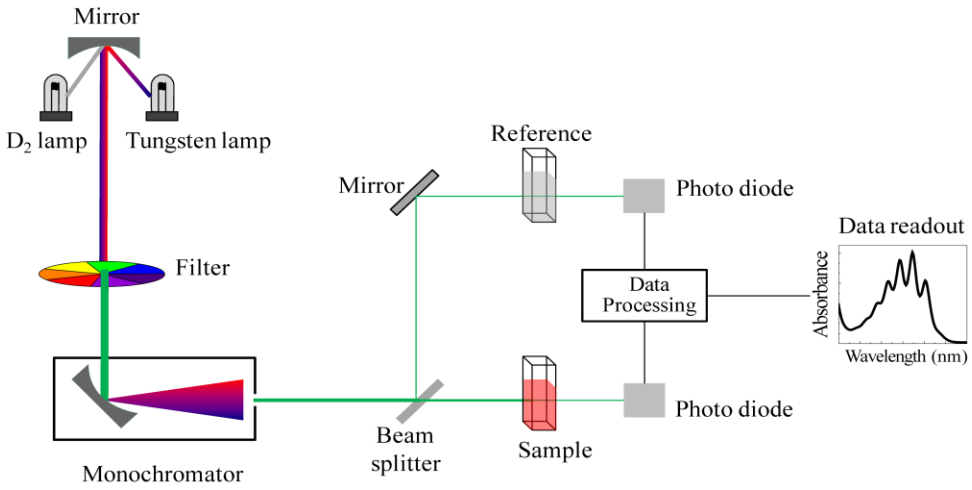

Figure 4: UV-Vis Spectroscopy schematic - Public Domain

Chapter 4: Results

Amplification and Purification of MyoD DNA

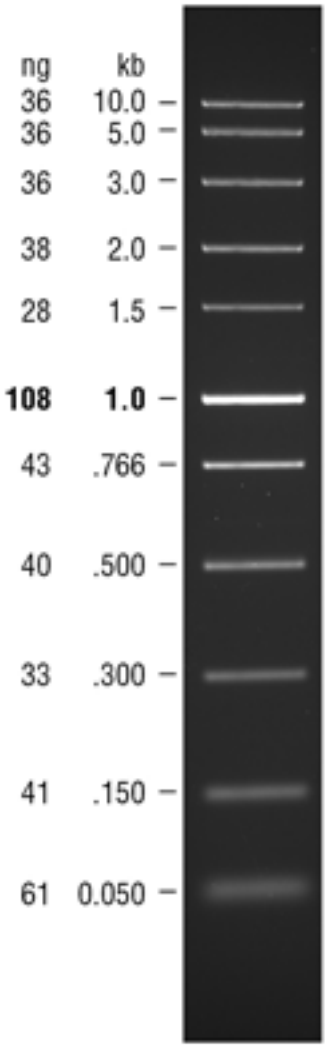

Figure 5: Fast Ladder DNA - a Standard to appropriately quantify the size and molecular weight of a sample being tested

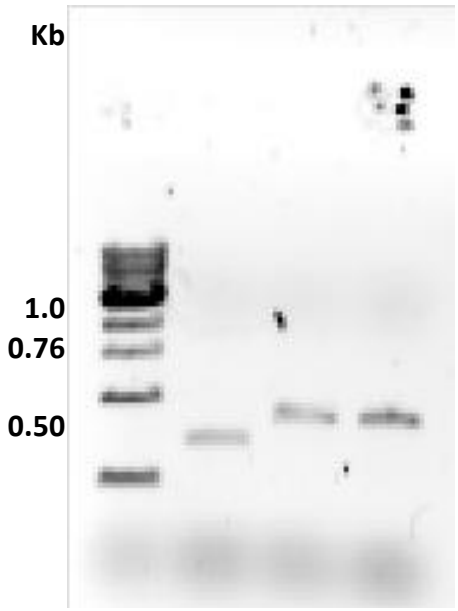

Figure 6: Initial EtBr 1% gel run from pMD4 PCR was induced with incorrect primers. Faint bands at ~0.55-0.50 kb yielded with the DNA ladder.

Before M13f/Ma34 primers were obtained, incorrect primers for a similar MyoD template were used to attempt amplification. Though incorrect, the validity of the correct primers was backed even more efficiently once obtained, used for amplification, and observed via gel electrophoresis.

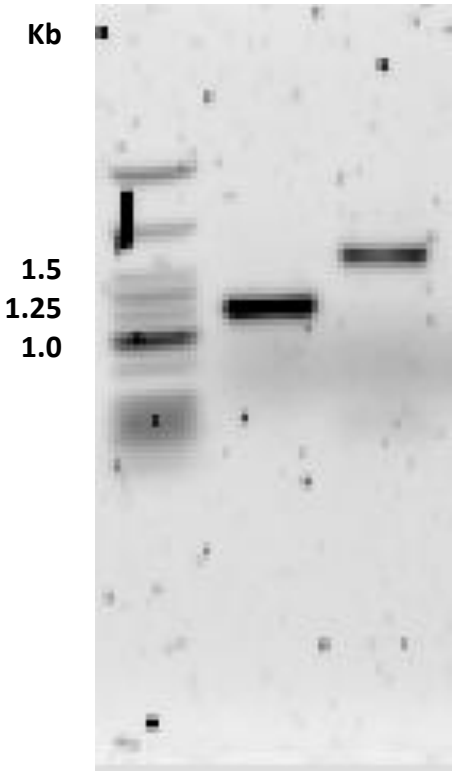

Figure 7: EtBr stained 1% gel with DNA ladder poor resolution but pMD4 dark stain at ~1.25kb

A second gel gave a dark band downstream in an area that would be considered appropriate but could not be relatively observed due to a poor DNA ladder.

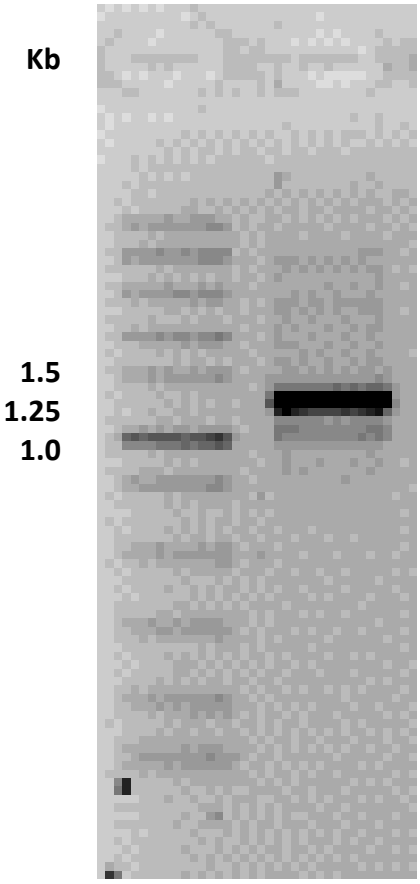

Figure 8: A final EtBr stained 1% gel shows pMD4 after a 20 cycle PCR with lane 1 being the DNA ladder and lane 2 being the sample at 1.259kb.

Following a 20 cycle PCR of pMD4 with primers M13f/Ma34, an EtBr stained 1% gel electrophoresis helped quantify its size to be 1.259kb. This ensured that the reaction was successful and that the template could then be purified and transcribed.

RNA Transcription

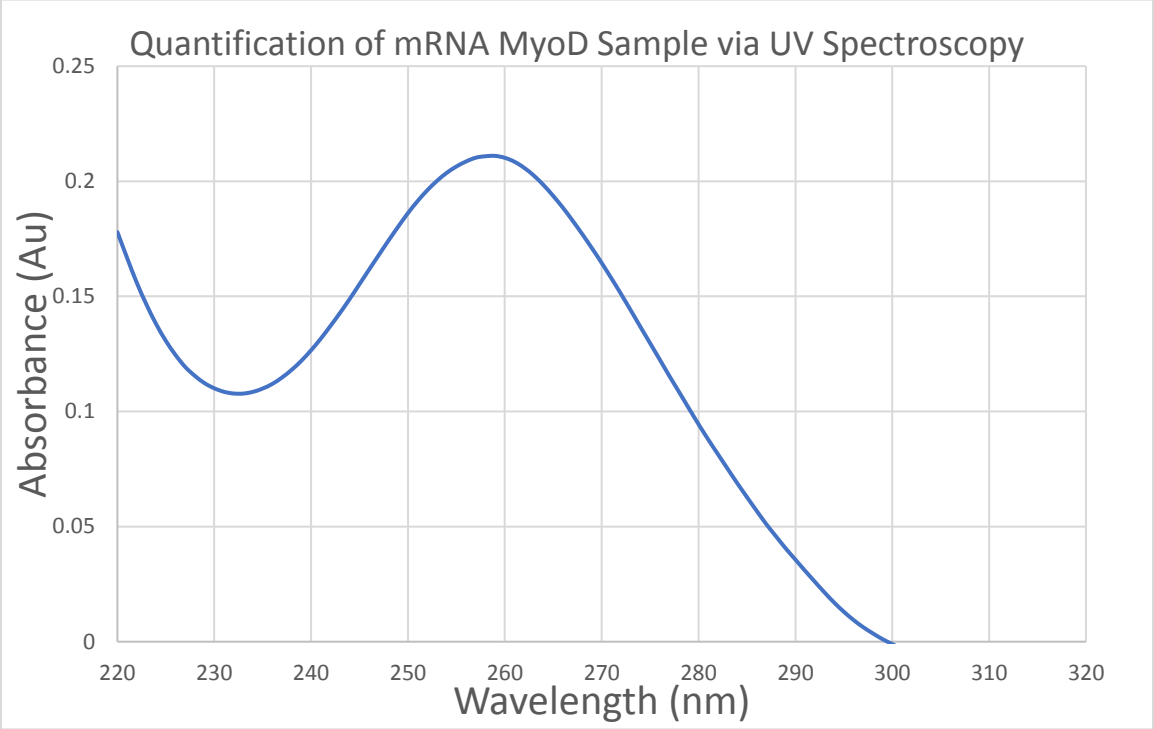

Figure 9: Transcribed MyoD sample was quantified using UV-Vis Spectroscopy between 220 and 320 nm with absorbance peak at 0.211 AU.

Following DNA purification, the template was transcribed using the protocol described in Chapter 3. After incubation and purification of the mRNA, it was quantified using UV-Vis Spectroscopy between 220 and 320 nm. The peak absorbance was 0.211 AU. Using the absorbance value 0.211 OD/μL in a 500-fold dilution, it was calculated that a concentration of 4.2 μg/μL of mRNA was transcribed from the pMD4 template with a mass yield of 42μg.

Chapter 5: Discussion and Conclusion

The pMD4 plasmid prepared by Dr. Huang proved to be an efficient template to amplify, purify, and transcribe under proper conditions and mechanisms. Original primers used showed that the amplification of pMD4 needed a new set of primers in order to be successful. After thorough research, the M13f/Ma34 primers proved to be most successful in fully amplifying the MyoD template.

After observing the amplification of the pMD4 template via gel electrophoresis, it cannot help but be recognized that a small band under the broad, main band exists after several trials (Figure 8), thus showing a small impurity in the plasmid or separate binding site of the M13f/Ma34 primers. This could have deterred transcription from offering an even more significant yield of mRNA. Also, replication of the amplifying step to gauge the most precise number of cycles of amplification could have also resulted in a larger yield of the amplified template, which would yield more mRNA following transcription. Nevertheless, mRNA was produced that could be used for downstream application in cellular differentiation.

Further progress in the experiment could be to introduce a 5' cap with a ϕ2.5 T7 promoter and a 3' poly(A) tail essential for transfection into stem cells during the transcription process. Purifying the properly capped mRNA from the non-capped would also be necessary so less toxic mRNA molecules would not transfect the stem cells and cause an undesired immune response.

Molecular data work up of efficient caps and tails were beyond the scope of this particular project, but would guide the necessary steps of making components necessary to transfect stem cells that could potentially be used for positive innate immune response.

One end goal of this entire project is to develop new forms of regenerative medicine in order to prevent problematic and unnecessary innate immune responses, where stem cells could differentiate into mature and desirable, healthy muscle cells. Being able to initiate a response that targets the source before maturation ever begins would lead to a more efficient, less toxic, and more desirable outcome of differentiation of healthy cells. Such a development would lead to biochemical derivatives the would span across more than just differentiation of stem cells into muscle cells, allowing this same principle to be targeted to any type of tissue or organ in an organism.

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