Manufacturing an RNA Therapeutic for Duchenne Muscular Dystrophy

A Technical Report
presented to the faculty of the School
of Engineering and Applied Science
University of Virginia

by

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On our honor as University students, we have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Capstone-Related Assignments.

Signed: _____

Approved: _____ Date _____
Eric Anderson, Department of Chemical Engineering

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I. Summary

Our technical project sets out to design a process for the yearly production of 19.31 kg of golodirsen, an RNA therapeutic designed to treat Duchenne Muscular Dystrophy (DMD). The process begins by synthesizing the golodirsen in an *in vitro* bioreactor by feeding a sequence-optimized reaction mixture as well as additional enzymes, cap analogs, cofactors, polyamines, redox reagents, and salts to the reactor. The golodirsen then goes through a series of downstream processes including tangential flow filtrations, affinity chromatography, size exclusion chromatography, and sterile filtration. An overview of the process can be seen in Figure 4.1.1.

An economic analysis was performed based on direct costs, fixed costs, and general expenses, as defined in Turton. Each of these cost components are detailed in Section 6.4. Targeting a yearly revenue of 170% the cost of our raw materials costs would net $159,974,937 per year. After subtracting operational costs, depreciation, and taxes, the annual profit after tax was calculated at $42,998,252. This profit after tax was then used to calculate a return on investment of 980% after a single year of operating the plant post-construction and validation. Additionally, the plant is only set to run for a total of 285 days, leaving open the possibility of repurposing this process for another use during the rest of the year.

II. Introduction

At just three years old, doctors diagnosed Jack Hogan with DMD, a genetic disorder characterized by progressive muscle degeneration and weakness. By age 7, Jack was wheelchair bound and struggled to hold his head up on his own. By age 12, Jack's respiratory and cardiac systems were severely compromised. With the help of corticosteroids and anti-angiotensin enzyme inhibitors to prevent muscle degradation and heart damage, his condition began to stabilize. In late 2018, his doctors predicted that he could live into his mid-20s, around the average life expectancy for DMD patients (Nguyen & Yokota, 2019). Around this time, doctors informed Jack's parents of a newly approved gene therapy for DMD that could address the mutation in Jack's dystrophin gene that caused the disease. The dystrophin gene codes for the production of the dystrophin protein, which is responsible for the structural integrity of muscles during contraction and relaxation cycles. This new gene therapy could trigger the production of a partially functional dystrophin protein in Jack's body, addressing the cause of his medical issues rather than the symptoms. However, with an annual

price tag of $300,000, and Jack's condition relatively stable, his parents decided to delay the use of the therapy until the need was more dire. Unfortunately, on March 31, 2019, Jack's lungs suddenly failed in his sleep. Without any prior indication that his health was worsening again, Jack passed away at just 14 years old.

DMD impacts roughly 1 in 3,500 males born worldwide each year, with an estimated 250,000 active cases in the United States today (Sarepta Therapeutics, 2019). In the majority of those born with DMD, deletions of segments of the gene interrupt the production of dystrophin which leads to the formation of weak, damage-prone muscle cells. DMD causes muscular atrophy, usually starting in the core muscular region, and then impacting the muscles in the limbs. By the age of 12, those affected will experience multiple organ dysfunction, resulting in serious heart and lung conditions (Nguyen & Yokota, 2019). Fortunately, new RNA-based therapies have shown promise in treating the disease at its genetic root rather than treating the associated symptoms, the focus of current therapies. The goal of this technical project is to develop a process to manufacture this oligonucleotide therapeutic and to improve the lives of DMD patients and their families.

III. Previous Work

Currently, there is no cure for DMD, and nearly all treatments for DMD focus on treating the associated symptoms (Malcolm, 2019). In recent years, however, there have been breakthroughs in mutation-targeting oligonucleotide and RNA therapies like the one Jack's doctors recommended to his parents. These new therapies offer an exciting possibility to prolong the lives and improve the standard of living of those afflicted by the disease. Vyondys 53, or golodirsen, is an FDA-approved therapeutic aimed at mitigating DMD-related symptoms and serves as motivation for this project (Sarepta Therapeutics, 2019). The technical aspect of this capstone will center on the design of a process to produce Vyondys 53, including an *in vitro* reactor, tangential flow filtration (TFF), affinity chromatography, and size exclusion chromatography.

These new treatments target the DMD gene mutation itself, tackling the problem at its source. The technology is centered around the *in vitro* synthesis of RNA, genetic material that provides instructions to the body on how to construct proteins. By creating an RNA molecule specific to the mutated DMD gene and introducing it to diseased muscle cells, DMD symptoms can be alleviated and life expectancy can be extended.

Crucial materials in this therapeutic process are antisense oligonucleotides (AOs), short, synthetic, single-stranded DNA or RNA molecules that can alter RNA in a way that can either reduce, restore, or modify protein expression (Rinaldi & Wood, 2017). A study published in 2019 describes how an AO can be designed to modify the RNA sequence associated with the mutated dystrophin gene such that it would produce a truncated and partially functional dystrophin protein rather than no dystrophin protein at all, as can be seen in Figure 3.1 (Nguyen & Yokota, 2019). This enables fully-functional dystrophin to exist in some muscle cells, weakening the symptoms of DMD (The Science and Fundamentals of mRNA Technology, 2020). We aim to apply elements of previous AO designs to create a safe and cost-effective process for manufacturing RNA-based therapeutics that can improve the lives of the millions impacted by DMD.

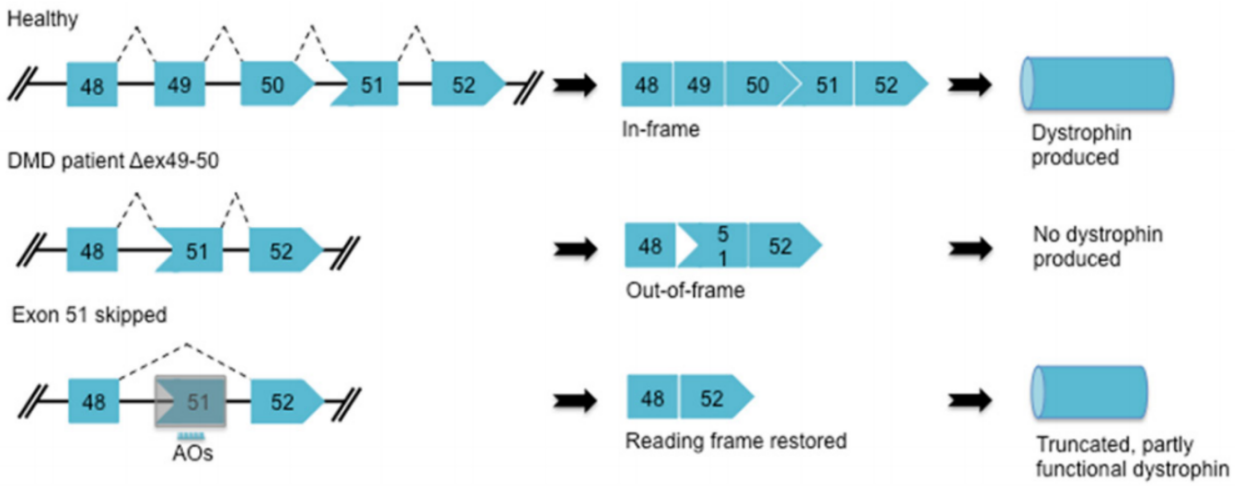

Figure 3.1. Process through which the oligonucleotide treats DMD (Nguyen & Yokota, 2019).

IV. Process Description

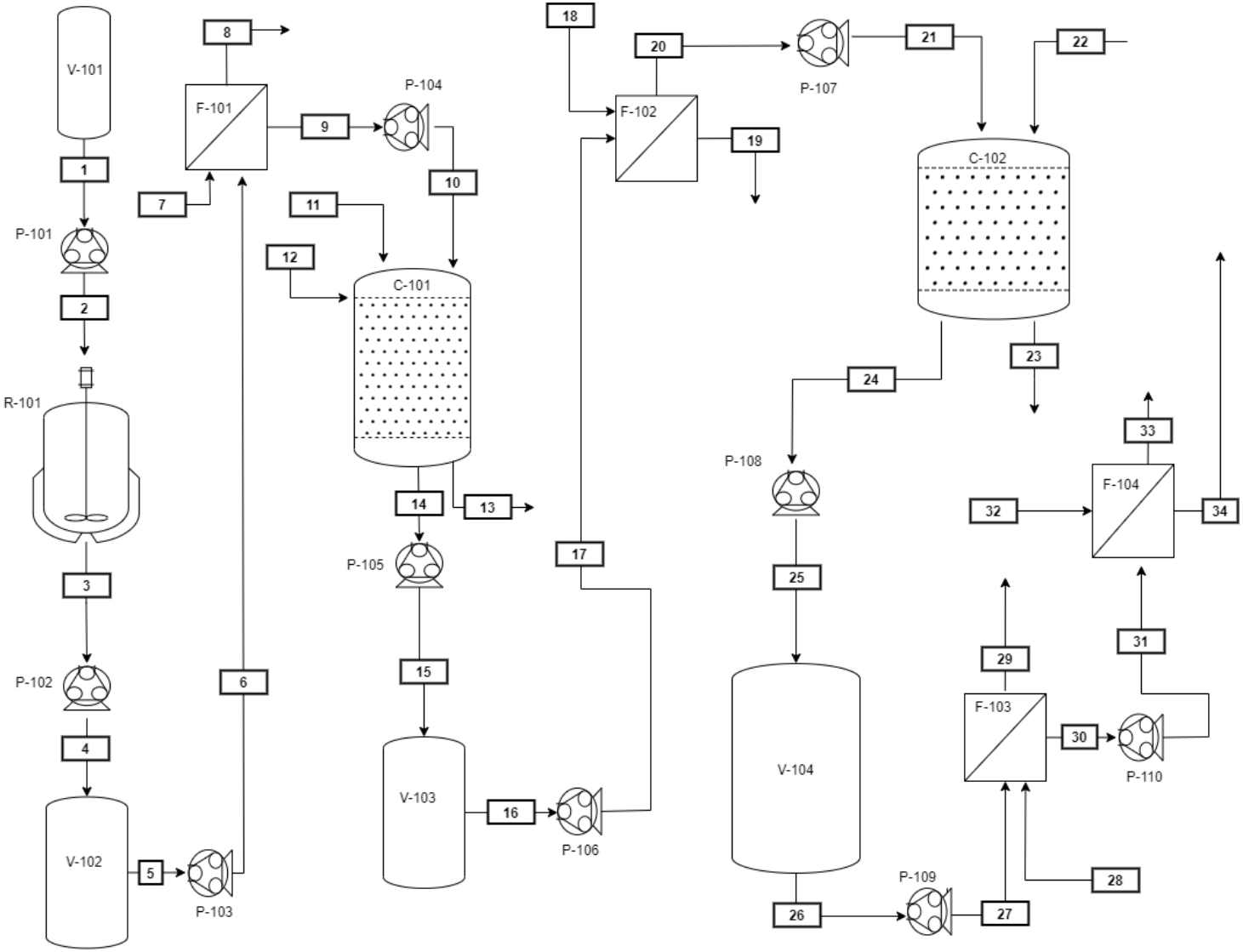

Figure 4.1 Process Flow Diagram for the production of Vyondys 53

4.1 Process Overview

The production of golodirsen begins with 1.25 L of the reaction mixture being loaded into the *in vitro* transcription reactor, R-101. The remaining reaction mixture is fed into R-101 from the reactant storage vessel, V-101, through pump P-101 at 0.625 L/h. The product stream exiting the reactor through pump P-102 is stored in the product mixture storage tank, V-102. This reaction procedure is repeated four times total with the product mixture generated from each reaction stored in V-102. Once the reactions have been completed, the product mixture is pumped through P-103 into the post-reactor tangential flow filtration (TFF) step, F-101, for removal of small molecules and buffer exchange. The material stream exiting F-101 is loaded into the affinity chromatography column, C-101 through pump P-104 at 100 cm/h for further purification. After C-101 is washed and eluted at 888.1 cm/h, the product mixture is pumped through P-105 into a storage vessel, V-103. The material stream is then pumped through P-106 into the post-affinity TFF step, F-102, for concentration and buffer exchange. The concentrated retentate is then loaded into the size exclusion chromatography (SEC) column, C-102, through pump P-107 at 43 cm/h for additional purification. C-102 is eluted at 50 cm/h and the exiting product stream is pumped through P-108 into a storage vessel, V-104. The mixture is then fed into the post-SEC TFF step, F-103, through pump P-109 for final formulation. The retentate is then pumped through P-110 into the sterile filtration step, F-104, for final purification before packaging.

V. Discussion

5.1. Product Specifications

The final product will be a sterile, aqueous, concentrated solution of the active ingredient, golodirsen. The sequence of nucleotides from the 5' end to the 3' end of golodirsen is GUUGCCUCCGGUUCUGAAGGUGUUC. Additionally, there will be a 15-unit poly-A tail attached to the 3' end of the strand. The molecular formula is $C_{305}H_{481}N_{138}O_{112}P_{25}$ with a molecular weight of 8.647 kDa prior to the addition of the poly-A tail. The final formulation of the final product will be:

1. 5.60 g/L golodirsen
2. 0.2 g/L potassium chloride
3. 0.2 g/L potassium phosphate
4. 8 g/L sodium chloride
5. 1.14 g/L sodium phosphate
6. Water for injection (WFI)

The final product will have a pH of 7.5. The output of this process will be a bulk solution of the formulation that can be sent to vial filling, where each vial will contain 2 mL of solution. This solution will be diluted with WFI immediately prior to administration to the patient.

5.2. Project Scale

Vyondys 53 is designed to combat DMD in patients who exhibit exon 53 skipping. This accounts for approximately 10% of all DMD cases in the United States. Thus, the potential patient pool for Vyondys 53 is approximately 25,000 patients. Assuming 25% market penetration, our goal is to produce for 6,250 patients. Treatment with Vyondys 53 requires a dosage of 30 mg per kilogram of body mass once weekly (Sarepta Pharmaceuticals, 2019). We assume that the average mass of the patient pool is 40 kg, as this is the average mass of a 12 year boy in the United States and the median patient age is approximately 12 years old. Thus, the average patient will require 1200 mg of Vyondys 53 each week. This translates to a required production of approximately 390 kg of the final product each year. This requires a production of 19.3 kg of golodirsen each year.

5.3. Social Implications

Because the number of individuals impacted by exon 53 skipping in the United States is fewer than 200,000, Vyondys 53 qualifies for Orphan Drug status. This guarantees market exclusivity for the drug for a minimum of 7 years. We expect demand to be relatively inelastic due to the life-saving nature of the product. Thus, we are able to set the price without risking a loss in the market share. However, other companies have taken advantage of this ability and marked up products by hundreds of percent. Obviously, we must make a profit to make the endeavor worthwhile. However, we set the price according to profit margins common among the general pharmaceutical market rather than the orphan drug market to avoid partaking in this excessive price gouging.

Even still, the anticipated cost of the gene therapy is tens of thousands of dollars per year. Most American families cannot afford out-of-pocket costs this high. Because our product has the ability to save lives, we do not want to limit our customers to wealthy individuals. Thus, we anticipate the implementation of a free-drug access program to families that would otherwise not be able to afford the drug. This program would decrease the overall profitability of the product, but initial analysis indicates a large enough return-on-investment that we are able to sacrifice profits to save more lives. Furthermore, it is clear that a general lack of biosimilars in the pharmaceutical market plays a significant role in the pricing of these drugs. As such, we have planned to make the majority of our profit in the first 7 years of the plant. We do not anticipate an extension of the patent nor additional orphan drug designations, so the product can be produced by generic pharmaceutical manufacturers after the first 7 years. This will increase the long term access to the drug greatly while still ensuring that the process is profitable.

5.4. Design Discussion

5.4.1. In Vitro Transcription Reactor

A sequence-optimized reaction mixture will be prepped and sent from storage vessel V-101 to reactor R-101. This sequence-optimized reaction mixture includes a sequence-optimized nucleotide mixture and several other additives. These additives include immobilized DNA templates, RNA polymerases, soluble magnesium salts, buffer, a reducing agent, RNase inhibitors, and a polyamine. The sequence-optimized nucleotide mixture consists of nucleotides in mole fractions according to the nucleoside composition of the desired RNA molecule. Nucleosides in RNA synthesis include guanosine triphosphate (GTP), uridine triphosphate (UTP), adenosine triphosphate (ATP), and cytidine triphosphate (CTP). Nucleotides will be sourced in the form of nucleotide-Tris (tris(hydroxymethyl) aminomethane) salts. As previously stated, the sequence of our desired RNA product is GUUGCCUCCGGUUCUGAAGGUGUUC. Therefore, the nucleotide mix for this reactor will contain GTP, UTP, ATP, and CTP in mole fractions of 0.20, 0.225, 0.425, and 0.15, respectively. The immobilized DNA templates consist of biotinylated DNA molecules bonded to streptavidin-coupled paramagnetic beads (Figure 5.1.1). The immobilization supports, sold as "Dynabeads," will be sourced from Thermo Fisher Scientific. After each batch, a magnet will be used to retain these paramagnetic beads allowing for the retention of our DNA template.

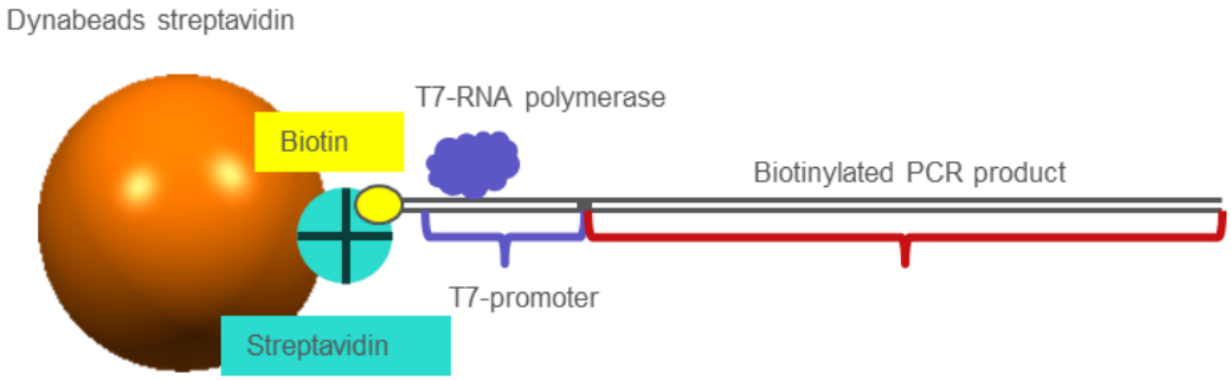

Figure 5.1.1. Biotinylated DNA template, or PCR product, immobilized onto paramagnetic beads seeded with streptavidin proteins ("Dynabeads") (Bosnes, 2018)

Bacteriophage-derived T7 RNA polymerases will be used to synthesize the RNA molecules. T7 RNA polymerases require magnesium ions as cofactors, which will be supplied by magnesium chloride ($MgCl_2$). Suitable buffers for the *in vitro* transcription include 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) or Tris buffers at a pH of 7.5. The reducing agent used will be dithiothreitol (DTT), while the polyamine will be spermidine. The chosen RNase inhibitor is RiboLock by Thermo Fisher Scientific. A cap analog is added to the sequence-optimized reaction mix to promote formation of a Type I cap at the 5' end of each RNA molecule. A Type I cap refers to a dinucleotide cap analog with an OCH_3 substitution on the 2' carbon along the 5-carbon sugar. This cap analog, sold as "CleanCap", will be sourced from TriLink Biotechnologies (Figure 5.1.2).

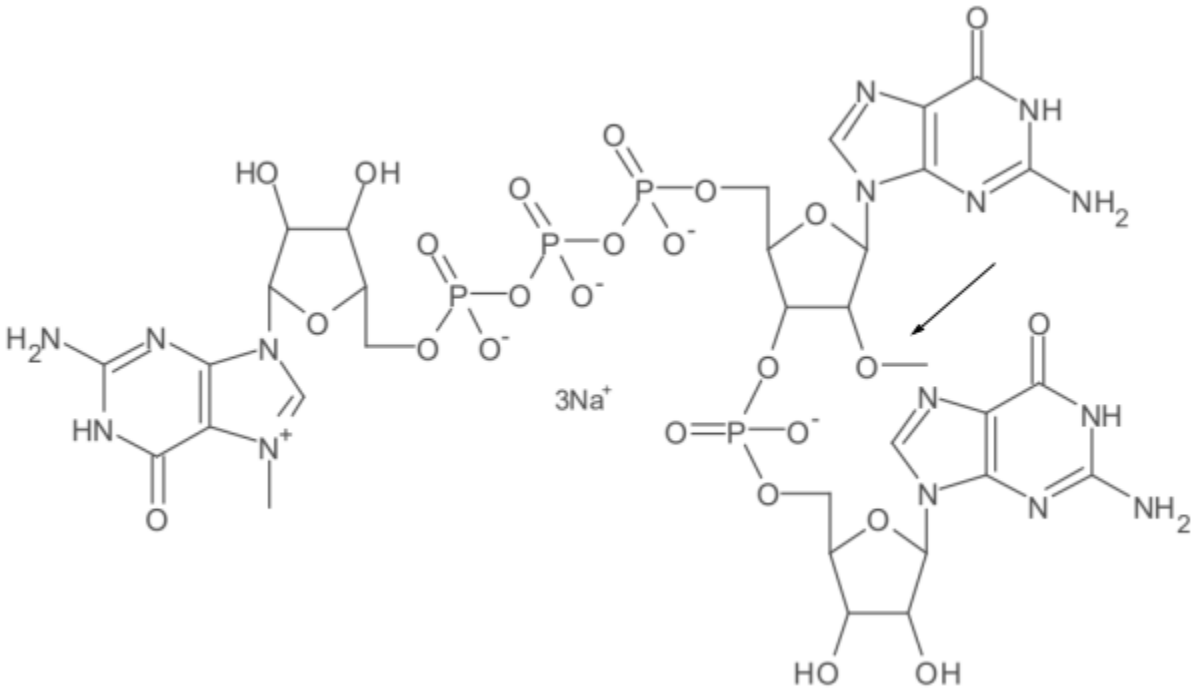

Figure 5.1.2. Proprietary Type I cap analog sold as "CleanCap" by TriLink Biotechnologies, with the arrow indicating the Type I modification (OCH_3) (TriLink Biotechnologies, 2016)

The desired RNA molecule will be enzymatically synthesized using an RNA polymerase in the previously described sequence-optimized reaction mixture. The *in vitro* transcriptions will be carried out in two 2.5 L fed-batch reactors. The sequence-optimized nucleotide

mixture will consist of GTP, UTP, ATP, and CTP concentrations of 4 mM, 4.5 mM, 8.5 mM, and 3 mM, respectively. The nucleotide salts are solubilized in plain ultrapure water. The reaction buffer will consist of T7 RNA polymerase, RNase inhibitor, $MgCl_2$, dithiothreitol, spermidine, and HEPES in concentrations of 40 nM, 200 U/mL, 24 mM, 40 mM, 2 mM, and 80 mM, respectively. The sequence-optimized nucleotide mixture and reaction buffer are components of the reaction mixture. To begin, we will feed 1.25 L of reaction mixture to the reactors. Immobilized DNA template will then be at a concentration of 40 nM to initiate the *in vitro* transcription reaction. As the reaction progresses, additional reaction mixture will be fed at a rate of 0.625 L/hr to maximize product yield per DNA template. Thus, it will take 2 hrs to fill our reactor to its final volume. Once the reactor has been filled, another 4 hrs will be required to allow the formation of our product to take place (Wochner et al., 2015). No turnaround time for this process was listed so a 2 hr turnaround time was assumed. Each batch will therefore take 8 hrs to complete. In order to achieve the required volume for an efficient downstream process, 4 batches must be complete. With our two reactors running simultaneously, the overall upstream process time is 16 hrs. In addition to reducing costs associated with excess of starting materials, the sequence-optimized reaction mixture allows for real-time tracking of the extent of reaction. A decrease in the total nucleotide concentration will directly correlate with the quantity of RNA produced.

Co-transcriptional capping will be utilized for this RNA synthesis. Capping protects the RNA molecules from degradation and reduces *in vivo* immunogenicity (Van Hoecke & Roose, 2019). GTP is the preferred cap structure for *in vivo* transcriptions because it may then be post-transcriptionally modified by enzymes to produce Type I cap structures. However, Type I cap analogs are preferred for *in vitro* transcriptions due to the cost associated with scaling enzymatic post-transcriptional modifications of RNA molecules. The use of a cap analog in the sequence-optimized reaction mixture will ensure that the desired cap structure is placed at the 5’ end of the RNA molecule. However, the cap analog will need to be fed in excess of GTP to ensure preferential capping over GTP present in solution. Therefore, the cap analog and GTP will need to be fed in at least a 4:1 molar ratio in order to ensure proper capping. As a result, GTP will be the limiting reagent for the RNA production. This method of co-transcriptional capping achieves >99% yield of RNA molecules with the desired Type I cap structure (*TriLink Biotechnologies*, 2016).

As the reaction progresses, a sample of the reaction mixture may be passed through an ultrafiltration membrane with a molecular weight cut-off in order to retain any large molecules. The filtered material may then be analyzed via spectrophotometry to determine the total concentration of nucleotides remaining in the reaction mixture. A process control module will maintain a constant total nucleotide concentration in the reactor by regulating the inflow of additional sequence-optimized feed. Additional process control modules include pH and magnesium probes. Potassium hydroxide may be added as necessary to maintain the pH of the reaction mixture at a constant 7.5. As RNA molecules are synthesized, free phosphate ions may bind with free magnesium ions to form insoluble magnesium phosphate. Intermittent injections of magnesium chloride will maintain sufficient concentrations of magnesium ions to prevent cofactor-limiting transcription.

Each reactor will be temperature-controlled to maintain a constant temperature of 37 °C. An impeller will provide sufficient mixing and agitation to aid the reaction progression. After the completion of the RNA transcription, a magnetic platform under the reactor will attract the paramagnetic beads to preserve the DNA template. The remainder of the reaction mixture will be passed to storage vessel V-102 and then to TFF unit F-101 for purification and concentration. The yield of desired RNA molecules is approximately 6.78 grams per liter (Wochner et al., 2015).

5.4.2. Post-Reactor Tangential Flow Filtration

The purpose of TFF unit F-101 will be to remove small particles including salts, spermidine, dithiothreitol (DTT), and spare nucleotides prior to entering the affinity chromatography. Additionally, the replacement buffer will contain a 0.5 M NaCl concentration that will be used in the chromatography step that follows.

The filter chosen for this process is a Reusable Hydrosart Ultrafilter. In order to determine the molecular weight cutoff, we calculated the size of our RNA strand using an online oligonucleotides properties calculator (Kibbe et al., 2015). We found that our RNA strand had a molecular weight of roughly 13 kDa, so a filter with a 10 kDa cutoff was chosen. The filter comes in a variety of cross-sectional areas, but the one chosen for this filtration was 0.6 m^2. Additionally, the filter comes with a manual discussing recommended average flux as well as inlet, outlet and permeate pressures for its usage with water. To see if these numbers would be a good estimate for our RNA solution, we first needed to check that our RNA

solution's viscosity was approximately that of water. To do this, the intrinsic viscosity of the solution was first found using Boedtker's Mandelkern-Flory equation by relating the intrinsic viscosity to the molecular weight of our RNA (Freerksen et al., 1990). It was determined that our RNA solution's viscosity was 1.14 cP, approximately that of water. Thus, the values for the average flux and pressures for the Reusable Hydrosart Ultrafilter with a cross-sectional area of 0.6 m^2 were used. These values are J_{avg} = 50.0 L/h*m^2 , P_{inlet} = 2 bar, P_{outlet} = 0.5 bar, and P = open valve. Therefore, we will have a transmembrane pressure of about (2+0.5)/2 - 1.01 bar = 0.24 bar. Additionally, our filter will need to be cleaned once a week with 1M NaOH for 30 minutes.

In order to perform all the calculations for this TFF step, certain assumptions had to be made. First, we assumed constant flux through our TFF unit due to the concentration change of the product having minimal effect on the viscosity of the solution. Second, 0% of the impurities would be retained in the filter. Lastly, we targeted 99% retention of our golodirsen.

As discussed in Section 5.3, the chromatography steps will only be performed once every four batches, so it was decided that the first TFF step would also be run once for every four batches to create a more efficient process. Thus, this TFF step will have an initial volume of about 10 L. Using the following equation, we managed to calculate the volume of wash needed to remove 99% of impurities with an initial volume of 10 L.

$$\frac{V_W}{V_0} = ln(\frac{C}{C_0})_{impurities}/(1 - \sigma_{impurities}) = ln(0.01)/1 = 4.61 \quad (5.4.2\text{-}1)$$

$$V_W = 46.1\ L \quad (5.4.2\text{-}2)$$

Next, the final concentration of the product was calculated for an initial concentration of 6.78 g/L coming out of the bioreactor.

$$(\frac{C}{C_0})_{product} = 0.99 \quad (5.4.2\text{-}3)$$

$$C_{product} = 6.71\text{g/L} \quad (5.4.2\text{-}4)$$

Then, the retention rate for our RNA was calculated in order to calculate the concentration of RNA permeate.

$$\sigma_p = 1 + \frac{ln(\frac{C}{C_0})_p}{(\frac{V_0}{V_W})} = 0.998 \quad (5.4.2\text{-}5)$$

$$C_{permeate} = (1 - \sigma_p)C_{product} = 0.015\ g/L \quad (5.4.2\text{-}6)$$

Lastly, the process time was calculated using the following equation.

$$t = \frac{V_W}{AJ_0} = \text{ 1 hr and 32 minutes} \quad (5.4.2\text{-}7)$$

5.4.3. Affinity Chromatography

After passing through unit F-101, the solution will be run through an affinity chromatography step in unit C-101. Initially, the chromatography steps were designed to process one batch of reactor effluent at a time. However, the resin used in affinity chromatography must be replaced after a set number of batches (*POROS Oligo dT(25) Affinity Resin,* 2020). Thus, it is more efficient to process larger volumes of solution at a time. The columns were redesigned to process 4 batches, or about 10 L, of reactor effluent at a time. Furthermore, the original design of the column did not specify a pressure drop. Thus, the pressure drop in the initial design would not have been compatible with a glass material of construction. The column was redesigned with a design specification of a 2.5 bar pressure drop.

Thus, the input to the affinity chromatography step will be 10 L of HEPES Buffer with 0.5 M NaCL and 67.1 g of mRNA, 60.48 g of which will be the desired golodirsen strand. Thermo-Fisher POROS Oligo dT(25) Affinity Resin, a rigid, 50 μm polymeric resin, will be used in the column. In high salt concentrations, the resin binds extremely efficiently to the poly-A tail of the completed mRNA strands. In low salt concentrations, the poly-A tail will separate from the resin, allowing for efficient elution. The optimal binding conditions for this resin were used in the design of this column.

One mL resin captures 2 mg mRNA. To capture 67.1 g of mRNA (ibid.), then, 33.6 L of resin is required. The ideal compression factor for the resin is 1.06, which translates to an extra particle void fraction of ε = 0.372. The viscosity of the solution leaving F-101 will be η=1.14 cP. The pressure drop across the column should be kept under 3 bar, ideally less than

2.5 bar. Thus, 2.5 bar was chosen as the column's pressure drop. Pressure drop impacts the retention rate of the mRNA strand, so experimentation would be done to determine the optimal pressure drop if time and resources allowed. The Carman-Kozeny Equation was then used to calculate the product of column length, L, and superficial velocity, u.

$$\Delta P = 150\frac{(1-\varepsilon)^2}{\varepsilon^2}\eta\frac{Lu}{d_p^2} \quad (5.4.3\text{-}1)$$

Plugging in, we find

$$250,000Pa = 150\frac{(1-0.372)^2}{0.372^2}(0.0014Pa \cdot s)\frac{Lu}{(0.005cm)^2} \quad (5.4.3\text{-}2)$$

$$Lu = 12.78\ \frac{cm^2}{s} \quad (5.4.3\text{-}3)$$

The retention time of the solution must be greater than 3 minutes (ThermoFisher Scientific, 2020). Ideally, experimentation would be done with the resin and golodirsen to determine the peak of productivity of the column with respect to retention time. No data was publicly available regarding the relationship between the productivity and retention time of golodirsen, so an estimate was made. 3.5 minutes was chosen to ensure adequate retention time without a significant drop in productivity. Thus, we have

$$\frac{L}{u} = 3.5\,min = 210\,s \quad (5.4.3\text{-}4)$$

Combining equations 5.4.3-3 and 5.4.3-4, we find that $L = 51.805\,cm$ and $u = 0.247\frac{cm}{s} = 888.1\ \frac{cm}{h}$. In order to calculate the column diameter, the total column volume (CV) was calculated using the required resin volume (RV) and the extra particle porosity (ε).

$$\varepsilon = \frac{CV-RV}{CV} \quad (5.4.3\text{-}5)$$

$$\varepsilon = \frac{CV - 33.6L}{CV} \quad (5.4.3\text{-}6)$$

It was found that $CV = 53.5\,L$ The column diameter was then found using the volume of a cylinder:

$$CV = \pi r^2 L \quad (5.4.3\text{-}7)$$

$$53L = 53500cm^3 = \pi r^2 \cdot 51.81cm \quad (5.4.3\text{-}8)$$

$$r = 18.13cm;\ d = 36.26cm \quad (5.4.3\text{-}9)$$

Thus, the column will have an inner diameter of 36.26cm and a length of 51.81 cm. The volumetric flow rate (Q) can then be calculated from the superficial velocity (u) and cross sectional area of the column (S).

$$u = \frac{Q}{S} \quad (5.4.3\text{-}10)$$

$$0.247\ cm/s = \frac{Q}{\pi(18.13cm)^2} \quad (5.4.3\text{-}11)$$

Thus, the volumetric flow rate must be 255.06 cm^3/s or 15.30L/min. This flow rate and interstitial velocity will be used in each step of the affinity chromatography process other than loading of the mRNA solution. This solution must be loaded at 100 cm/h, or 1.296 L/min in order to ensure the sample is evenly distributed across the diameter of the column. Initially, the column was designed such that each step of the chromatography process was fed at this flow rate. However, with a residence time of 3.5 minutes, the column would have been 500 cm long. Most industrial columns are much shorter and wider to avoid a large pressure drop, so the column was redesigned to ensure a pressure drop of only 2.5 bar.

Thus, the overall affinity chromatography process will be as follows (*POROS Oligo dt(25) Affinity Resin,* 2020):

1. The column will be equilibrated with 3 CVs of the binding buffer, HEPES with 0.5 M NaCl.

2. The mRNA solution will be loaded on to the column at a velocity of 100 cm/h.
3. The column will be washed with 3 CVs of the binding buffer at a velocity of 881.1 cm/h. All enzymes, remaining small molecules, and unbound proteins will be washed through the column in this step.
4. The column will be eluted with 5 CVs of HEPES with 0 M NaCl at a velocity of 888.1 cm/h. The bound mRNA will unbind and wash out of the column with this buffer.
5. The column will be regenerated with 5 CVs water at a velocity of 888.1 cm/h to ensure nothing remains in the column.
6. The column will be sanitized with 5 CVs 0.1 M NaOH at a velocity of 888.1 cm/h, followed by 3 CVs of HEPES with 0.5 M NaCl at 888.1 cm/h.
7. The column will be loaded with 16.7 L HEPES with 0.5 M NaCl and stored in a cold room at 4˚C.

The overall process will take 1 hour and 40 minutes. Once the stream has exited the column, it will be stored in storage vessel V-103.

5.4.4. Post-Affinity Tangential Flow Filtration

Because the affinity column is designed to capture all RNA strands with poly-A tails, only golodirsen and unwanted RNA strands remain in solution. To separate the desired from undesired RNA strands, a size-exclusion chromatography (SEC) must be performed. The material stream exiting the affinity column is very dilute (0.22 g/L) due to the large volume of buffer required for the elution step. To minimize the volume of solution entering the SEC column, TFF unit F-102 was inserted into the process design between the two chromatography steps to concentrate the mRNA solution as well as exchange the buffer for 0.05 M Na_3PO_4. Based on calculations done for the SEC, we needed to reduce our initial volume of 267.5 L to 11.76 L.

The filter chosen for this process is once again a Reusable Hydrosart Ultrafilter with a 10 kDa cutoff. This time, the area of the filter was increased from 0.6 m^2 to 3 m^2 to process the larger volume. The operating conditions remain the same as diafiltration, so J_{avg} = 50.0$\frac{L}{h \cdot m^2}$, P_{inlet} = 2 bar, P_{outlet} = 0.5 bar, and P = open valve. In order to perform all the calculations for this ultrafiltration and diafiltration step, we made two of the same assumptions as the

post-reactor diafiltration: constant flux and 99% retention of our golodirsen. Next, the concentration factor was calculated.

Ultrafiltration Calculations.

$$CF = \frac{V_0}{V} = \frac{267.5}{11.76} = 22.75 \quad (5.4.4\text{-}1)$$

Using the concentration factor, we were able to calculate the final concentration coming out the the ultrafiltration step using the following two equations:

$$\left(\frac{C}{C_0}\right)_p = (CF)^{\sigma_p} = (22.75)^{0.998} = 22.6 \quad (5.4.4\text{-}2)$$

$$C = \left(\frac{C}{C_0}\right)_p * C_0 = 22.6 * 0.22 g/L = 4.97\ g/L \quad (5.4.4\text{-}3)$$

The retention rate for the RNA was assumed to be the same for this TFF step as the last, so we find that:

$$\sigma_p = 1 + \frac{ln\left(\frac{C}{C_0}\right)_p}{\left(\frac{V_0}{V_w}\right)} = 0.998 \quad (5.4.4\text{-}4)$$

$$C_{permeate} = (1 - \sigma_p)C = 0.01\ g/L \quad (5.4.4\text{-}5)$$

Then, the product yield was calculated for our ultrafiltration:

$$\frac{CV}{C_0 V_0} = (CF)^{\sigma_p - 1} = 0.99 \quad (5.4.4\text{-}6)$$

Lastly, the process time for our ultrafiltration was calculated using the following equation:

$$t = \frac{V_0 - V}{A * J_{avg}} = 1 \text{ hr and } 42 \text{ minutes} \quad (5.4.4\text{-}7)$$

Diafiltration Calculations.

Using the following equation, we again calculated the volume of wash needed to remove 99% of impurities with an initial volume of 11.76 L.

$$\frac{V_W}{V_0} = ln(\frac{C}{C_0})_{impurities}/(1 - \sigma_{impurities}) = ln(0.01)/1 = 4.61 \quad (5.4.4\text{-}8)$$

$$V_W = 54.2\ L \quad (5.4.4\text{-}9)$$

Next, the final concentration of the product was calculated for the concentration following ultrafiltration.

$$(\frac{C}{C_0})_{product} = 0.99 \quad (5.4.4\text{-}10)$$

$$C_{product} = 4.92\ \text{g/L} \quad (5.4.4\text{-}11)$$

Then, the retention rate for our RNA was calculated in order to calculate the concentration of RNA permeate.

$$\sigma_p = 1 + \frac{ln(\frac{C}{C_0})_p}{(\frac{V_0}{V_W})} = 0.998 \quad (5.4.4\text{-}12)$$

$$C_{permeate} = (1 - \sigma_p)C_{product} = 0.011\ g/L \quad (5.4.4\text{-}13)$$

Lastly, the process time was calculated using the following equation.

$$t = \frac{V_W}{AJ_0} = 22\ \text{minutes} \quad (5.4.4\text{-}14)$$

After filter F-102, the process fluid will be pumped to SEC Column C-102.

5.4.5. Size Exclusion Chromatography

Transcribed RNA strands that are longer than the target mRNA sequence will be removed through size-exclusion chromatography (SEC) in unit C-102. The SEC column designed for this process was based on the Cytiva HiLoad Superdex 75 prep-grade column (*HiLoad Superdex 75 Pg Preparative Size Exclusion Chromatography Columns*, n.d.). This column was selected because it has been used previously to purify transcribed RNA of similar lengths to golodirsen (Kim et al., 2007; McKenna et al., 2007). The Superdex 75 prep-grade resin will be used in this process due to its high selectivity for proteins and oligonucleotides ranging from 3 to 70 kDa (golodirsen is ~13 kDa). This is a gel filtration resin with a dextran and cross-linked agarose matrix.

Scale-up.

The scale-up of the Superdex 75 column was guided by instructions provided by Cytiva that advised maintaining sample-to-column volume ratio, flow velocity, and bed height (*Fundamentals of size exclusion chromatography*, n.d.). To adhere to this guidance, the column volume was increased by increasing the column diameter. The maximum sample-to-column volume ratio of the Superdex 75 is 0.041. Based on the 11.76 L load volume coming out of F-102, maintaining this ratio requires a column volume of 287 L. The column diameter (D) was determined by this volume using the equation for the volume of a cylinder with the bed height (L) of the Superdex 75 (60 cm):

$$CV = \pi L\left(\frac{D}{2}\right)^2 \tag{5.4.5-1}$$

$$D = \sqrt{\frac{CV}{\pi L}} = \sqrt{\frac{2.87{\cdot}10^5\ cm^3}{\pi * 60\ cm}} = 78.0\ cm \tag{5.4.5-2}$$

Based on the column diameter (D) and flow velocity (u), the volumetric flow rate through the column can be calculated. For the Superdex 75 column, the recommended flow velocity range is 10-50 cm/h. To maximize throughput, 50 cm/h was selected for this design and used to determine the volumetric flow rate of the elution buffer.

From the column diameter, the cross sectional area (S) can be calculated based on the area of a circle:

$$S = \pi\left(\frac{D}{2}\right)^2 = \pi\left(\frac{78\ cm}{2}\right)^2 = 4780\ cm^2 \quad (5.4.5\text{-}3)$$

This can then be used to calculate the volumetric flow rate from the flow velocity:

$$Q = uS = (50\ cm/h)(4780\ cm^2) = 2.39 \cdot 10^5\ cm^3/h = 3.98\ L/min \quad (5.4.5\text{-}4)$$

To estimate the concentration of golodirsen exiting the SEC column a mathematical model was used to generate a chromatogram assuming a linear isotherm, linear driving force (LDF) model and periodic injections (Carta, 1988). This model required estimations of the effective pore diffusivities of both species which were assumed to be 10^{-7} cm^2/s. The partition coefficients used in this model were obtained from Superdex 75 resin selectivity curves based on the assumption that elongated mRNA strands were roughly the size of golodirsen dimers, 26 kDa. Based on the peak areas obtained from the chromatogram, the volumes of buffer exiting with the waste and product streams were determined to be 40.2 L and 258.6 L respectively, as shown in Figure 5.4.5.1. The selectivity curves, modeled chromatogram, input parameters, and calculation of golodirsen concentration are displayed in Appendix A.

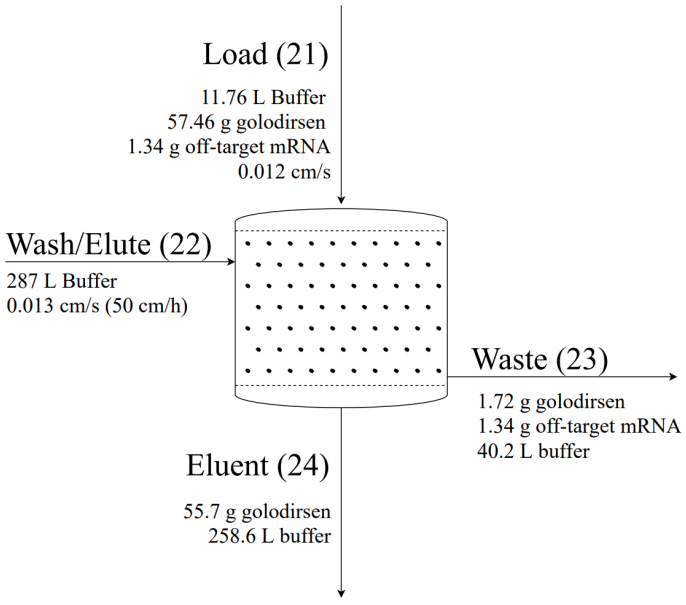

Figure 5.4.5-1 Diagram of SEC material flow

To verify that the pressure drop over the packed bed (ΔP) during operation of this column does not exceed the maximum allowable pressure, 300,000 Pa, the Carman-Kozeny Equation was used to calculate ΔP. The viscosity of column fluid (η) used in this equation was approximated as that of water at room temperature, 0.00105 Pa, because the column will be operated at room temperature. The extraparticle porosity (ε) used in this equation was assumed to be 0.3. The porosity of the resin was not provided by Cytiva but column porosity generally falls between 0.3 and 0.4 so 0.3 was chosen as a conservative estimate. The resin particle diameter (d_p) is specified by Cytiva as 34 μm.

$$\Delta P = \frac{150(1-0.3)^2}{(34 \cdot 10^{-6} m)^2 * 0.3^3} * (0.00105\ Pa \cdot s)(0.6\ m)(0.00014\ m/s) = 208,000\ Pa \quad (5.4.5\text{-}5.)$$

Since the pressure drop calculated above is less than the maximum recommended value, the Superdex 75 resin can be used for this scaled up separation.

Cycle time estimate.

To calculate the total time required for the SEC step, filtering time, load time, and elution time was calculated. Prior to being loaded into the column, the 11.76 L sample solution must be filtered through a 0.22 μm filter to remove particulate matter. This is estimated to take 0.25 h. The sample load time (t_{load}) was calculated based on the column's dynamic binding capacity at 10% of the breakthrough curve ($DBC_{10\%}$) which is dependent on the load volume (V_{load}), feed concentration (C_F), and column volume (CV).

$$DBC_{10\%} = \frac{V_{load} C_F}{CV} = \frac{(11.76\ L)(5\ g/L)}{287\ L} = 0.20\ g/L \quad (5.4.5\text{-}6)$$

Load time can then be calculated from this value, load velocity (u_{load}), bed height (L), and feed concentration (C_F). Load velocity was selected to be 43 cm/h because this value provides better resolution during injection based on chromatographic modeling. Load time (t_{load}) was then calculated using the equation below:

$$t_{load} = \frac{L \cdot DBC_{10\%}}{C_F u_{load}} = \frac{(60\ cm)(0.20\ g/L)}{(5\ g/L)(43\ cm/h)} = 0.06\ h \quad (5.4.5\text{-}7)$$

Elution time is calculated based on the volume of the elution buffer required, expressed in terms of column volume (CV_{elute}), flow velocity (u), and bed height (L). This process requires one column volume of buffer for elution and the flow velocity is 50 cm/h as specified previously.

$$t_{elute} = CV_{elute} \cdot \frac{L}{u_{elute}} = 2.87 \times 10^5\ cm^3 \cdot \frac{60\ cm}{50\ cm/h} = 1.2\ h \quad (5.4.5\text{-}7)$$

Summing the filter time, load time, and elution time gives the total cycle time:

$$t_{cycle} = t_{filter} + t_{load} + t_{elute} = 0.25\ h\ + 0.06\ h\ + 1.2\ h\ = 1.51\ h \quad (5.4.5\text{-}8)$$

The overall SEC process will be as follows:

1. Filter sample through 0.22 μm filter
2. Load sample into column at 43 cm/h
3. Wash/elute column with 1 CV of buffer (0.05 M Na_3PO_4 and 0.15 M NaCl) at 50 cm/h
4. Collect purified sample (target product elutes last)

The separation will be performed at ambient pressure and room temperature.

Cleaning and equilibration procedure.

The column also requires cleaning and equilibration every 10-20 uses. This is estimated to take 9.6 h for cleaning and 2.4 h for equilibration, 12 h total. The cleaning and equilibration processes are as follows:

Cleaning:

1. 1 CV of 0.5 M NaOH solution at 25 cm/h
2. 1 CV of distilled water at 25 cm/h
3. 2 CV of buffer (0.05 M Na_3PO_4 and 0.15 M NaCl) at 25 cm/h

Equilibration:

1. 2 CV of buffer (0.05 M Na_3PO_4 and 0.15 M NaCl) at 50 cm/h

SEC Yield.

This SEC process is assumed to yield a 97% recovery of golodirsen. SEC column scale-up is typically designed based on small-scale experimentation that enables optimization of column design and operating conditions. If optimized, SEC processes can yield total separation of species in the sample given that there is a significant difference in molecular weight as there is in this sample. Because we are unable to perform these experiments and do not have access to any experimental data, we are assuming a 3% loss of product during this separation. 97% recovery gives a process yield of 55.7 g of golodirsen.

Alternative to SEC.

An alternative to SEC is anion exchange chromatography (AEX) which is a method of purification that separates based on ionic interaction between positively charged sorbents and negatively charged molecules. AEX sorbents typically include a positively charged functional group cross-linked to solid phase media. AEX is an effective alternative to SEC the components of the solution exiting the affinity column, golodirsen and elongated mRNA strands, are negatively charged due to the basicity of the RNA backbone and have charge differences large enough to be separated using this method due to their difference in size. Easton et al. (2010) used AEX to purify RNA oligonucleotides between 30 and 500 nt in length and is a good resource for further investigation. We ultimately chose to use SEC instead of AEX because it was a faster method of purification, about 1.5 h compared to 3-4 h reported by Easton et al. AEX also requires a salt gradient in the elution buffer in order to separate each component which requires additional time and effort to optimize and control compared to the simple elution of the SEC column.

5.4.6. Post-SEC Tangential Flow Filtration

After our SEC, we are ready for final formulation. This formulation involves both an ultrafiltration and a diafiltration step. First, the solution exiting C-102, will be concentrated from 258.6 L to 10 L. Then, we will replace our HEPES buffer with phosphate buffered saline in a diafiltration step.

The filter used for this final step is another Reusable Hydrosart Ultrafilter with a 10 kDa cutoff and area equal to 3 m^2. The values for pressure and flux will be the same as the previous two steps. The assumptions made for the previous diafiltration and ultrafiltration processes will be used again.

Ultrafiltration Calculations.

To start, the concentration factor factor was calculated

$$CF = \frac{V_0}{V} = \frac{258.6}{10} = 25.86 \quad (5.4.6\text{-}1)$$

Using the concentration factor, we were able to calculate the final concentration coming out the the ultrafiltration step using the following two equations:

$$(\frac{C}{C_0})_p = (CF)^{\sigma_p} = (25.86)^{0.998} = 25.86 \quad (5.4.6\text{-}2)$$

$$C = (\frac{C}{C_0})_p * C_0 = 25.86 * 0.22 g/L = 5.69\ g/L \quad (5.4.6\text{-}3)$$

The retention rate for the RNA was again assumed to be the same as the previous two TFF steps so:

$$\sigma_p = 1 + \frac{ln(\frac{C}{C_0})_p}{(\frac{V_0}{V_W})} = 0.998 \quad (5.4.6\text{-}4)$$

$$C_{permeate} = (1 - \sigma_p)C = 0.0124\ g/L \quad (5.4.6\text{-}5)$$

Then, the product yield was calculated for our ultrafiltration

$$\frac{CV}{C_0 V_0} = (CF)^{\sigma_p - 1} = 0.999 \quad (5.4.6\text{-}6)$$

Lastly, the process time was calculated using the following equation:

$$t = \frac{V_0 - V}{A * J_{avg}} = 1 \text{ hr } 40 \text{ minutes} \quad (5.4.6\text{-}7)$$

Diafiltration Calculations.

Using the following equation, we calculated the volume of wash needed to remove 99.999% of impurities with an initial volume of 10 L to meet sterilization requirements.

$$\frac{V_W}{V_0} = ln(\frac{C}{C_0})_{impurities}/(1 - \sigma_{impurities}) = ln(0.00001)/1 = 11.5 \quad (5.4.6\text{-}8)$$

$$V_W = 115\ L \quad (5.4.6\text{-}9)$$

Next, the final concentration of the product was calculated for an initial concentration of 5.69 g/L coming out of the reactor.

$$(\frac{C}{C_0})_{product} = 0.99 \quad (5.4.6\text{-}10)$$

$$C_{product} = 5.63\ \text{g/L} \quad (5.4.6\text{-}11)$$

Then, the retention rate for our RNA was calculated in order to calculate the concentration of RNA permeate.

$$\sigma_p = 1 + \frac{ln(\frac{C}{C_0})_p}{(\frac{V_0}{V_W})} = 0.998 \quad (5.4.6\text{-}12)$$

$$C_{permeate} = (1 - \sigma_p)C_{product} = 0.005\ g/L \quad (5.4.6\text{-}13)$$

Lastly, the process time was calculated using the following equation.

$$t = \frac{V_W}{AJ_0} = \text{2 hrs and 25 minutes} \quad (5.4.6\text{-}14)$$

5.4.7. Final Sterile Filtration

A sterile filtration will then be used to ensure our product meets FDA requirements for purity in order to ensure patient safety. This sterile filtration will use a Sartopore 2 HF cartridge that has a pore size of 0.2 μm. The diameter of the filter is 70 mm and the height will be 568 mm. The filter area is 1.4 m^2. The maximum diffusion through the filter is 42 mL/min, or 2.52 L/hr. Thus, processing all 10 L of our solution will take approximately 3 hrs and 58 minutes. These cartridges have a max allowable differential pressure of 5 bar, which is lower than the 2.5 bar that will be used to achieve our diffusion rate. Because the desired product is smaller than our filter, we will assume a 100% retention rate.

5.4.8. Pump Design

A total of 10 pumps will be required to run the process, feeding into each major unit operation. Table 5.4.8.1 provides the flow rate, pressure differential, and power requirement for each pump. Originally, the process designed accounted for the use of centrifugal pumps. However, because the power requirement of each pump is relatively low, peristaltic pumps will be used.

The tangential flow filtration systems that will be used will contain pumps. Thus, pumps P-103, P-106, P-109 will not be separate units, but will be contained within the TFF systems itself.

Table 5.4.8.1. Pump Design Specifications

Pump	Total Pressure Differential (kPa)	Flow Rate (m^3/s)	Power Requirement (watts)
P-101	49.34	1.74E-07	0.01
P-102	48.34	1.74E-07	0.01
P-103	149.34	8.33E-06	1.24
P-104	350.34	2.26E-04	79.29
P-105	49.34	2.26E-04	11.17
P-106	148.34	4.17E-05	6.18
P-107	308.34	6.63E-05	20.45
P-108	49.34	6.63E-05	3.27
P-109	148.34	4.17E-05	6.18
P-110	244.34	3.00E-07	0.073

5.5. Plant Schedule

As mentioned previously, our process is designed to produce 19.3 kg of golodirsen each year. The process times for each unit operation can be found in Table 6.1. Since our upstream process takes a total of 24 hrs to complete compared to only 10 hrs 28 minutes for the downstream process, we will begin producing the next 4 batches while the downstream process is being run. Therefore, we will be producing approximately 4 batches worth of product every 24 hrs.

Table 5.5.1. Process time by unit operation

Unit Operation	Time
In Vitro Reactor	16 hr
Post-Reactor Diafiltration	1 hr 32 min
Affinity Chromatography	1 hr 40 min
Post-Affinity UF/DF	2 hr 8 min
Size-Exclusion Chromatography	1 hr 41 min
Post-SEC UF/DF	4 hr 5 min
Sterile Filtration	3 hr 58 min

Our reactor has a volume of 2.5 L and will produce 6.78 g of golodirsen per liter. Therefore, every full cycle of upstream and downstream processes will produce approximately 67.8 g. In order to meet the desired 19.3 kg/year, 285 cycles must be processed.

5.6. Environmental Concern and Waste Management

This process produces waste streams at each downstream separation step. The contents of each waste output and their associated environmental concerns are detailed in Table 7.1. Due to the small scale of this manufacturing process, waste collection and treatment will be outsourced to a professional chemical waste management company. Based on federal, state, and local regulations for Boston, Massachusetts, the location of the manufacturing facility, all waste produced in this process will be labeled as hazardous *(310 CMR 30.000: Massachusetts Hazardous Waste*, 2019; *Chemical Waste*, n.d.). Waste from each step in the process will be collected and sealed in a waste container that will then be labeled for collection.

Due to the large volume of liquid waste produced at C-101 and C-102, waste containers with a capacity of at least 300 L will be required at these steps to store the aqueous waste output at these steps. While all process waste is liquid, when chromatography resin from C-101 and C-102 requires replacement, used resin will be collected in solid waste containers and picked up by a chemical waste service. Used filters will also be disposed of as solid waste as they are replaced. Large volumes of corrosive waste are generated during the cleaning procedures of C-101 and C-102 which require washing with 0.1 M and 0.5 M solutions of sodium hydroxide (NaOH) respectively. A smaller volume of 1 M NaOH is also required in the TFF cleaning procedure. Because NaOH is a strong base, waste solutions must be stored in plastic containers resistant to corrosion and labeled as corrosive for pickup (*Waste*, n.d.). NaOH also has associated environmental concerns and should not be disposed of through drainage systems at risk of harming aquatic life.

A main environmental concern of the process as a whole is the consumption of water. The total volume of water required per batch was determined to be 1716 L which translates to 31,000 kg of water per kg of product. For reference, this water consumption ratio is about six times higher than that of the production of monoclonal antibodies (Idris et al., 2016). The vast majority of the total water consumption occurs during the downstream processes, specifically the two chromatography steps, C-101 and C-102. To decrease the water consumption of this process, the column volumes of each chromatography step could be decreased. Potential consequences of this change would be decreased column throughput and longer cycle time. Another option to decrease water consumption is to recycle aqueous solutions. This would require additional purification steps but could have a profound impact on this process's water use. A cost-benefit analysis would have to be performed to determine if this change is worthwhile. This process's demand for water for injection (WFI) also increases power consumption due to extensive use of the WFI system which requires power for vaporization and compression of water (Idris et al., 2016). A great deal of power is also devoted to the heating, ventilation, and cooling (HVAC) system required to maintain a controlled environment for therapeutic manufacturing (Ho et al., 2010). Investing in more efficient design and operation of the controlled environment would create a more energy efficient process overall by decreasing HVAC power requirements.

Table 5.6.1. Waste produced and environmental concerns of each unit operation

Unit Operation	Waste Produced	Environmental Concerns
F-101	*Permeate Solution* - Nucleotides - Dithiothreitol (DTT) - Spermidine - Salts - Golodirsen	- DTT impacts air quality and can migrate in air, soil, and water, affecting animal, aquatic, and plant life. - Spermidine and nucleotides are mobile in water and pose risk to aquatic ecosystems if released. - The small amount of salts and golodirsen exiting the process at this step do not pose an environmental risk.
C-101	*Waste Solution* - HEPES buffer - NaCl - golodirsen - Proteins - Enzymes	- HEPES buffer is mobile in water and poses risk to aquatic ecosystems if released through drains. - Salt water can be damaging to plant and animal life in aquatic ecosystems but does not pose a significant threat at the low concentration used in this step. - Proteins, enzymes, and golodirsen do not present environmental concerns because the small amount exiting the process will be collected in liquid waste vessels for disposal.
F-102	*Permeate Solution* - HEPES buffer - golodirsen	- HEPES buffer is mobile in water and poses risk to aquatic ecosystems if released through drains. - The small amount of golodirsen exiting the process at this step does not pose an environmental risk.
C-102	*Waste Solution* - $NaCl/Na_3PO_4$ buffer - golodirsen - Elongated mRNA strands	- The salt buffer solution containing low concentrations of mRNA material will be treated prior to release into the environment and does not present any environmental risks.
F-103	*Permeate Solution* - HEPES buffer - golodirsen	***See F-102 environmental concerns***
F-104	*Permeate Solution* - HEPES buffer - golodirsen	***See F-102 environmental concerns***

5.7. Health and Safety

To maintain compliance with current FDA regulations, this production facility will align with current Good Manufacturing Practices (cGMP). Production according to cGMP assures consistent production and product control. Utilization of cGMP minimizes risks associated with pharmaceutical production. Every part of the process will be controlled by cGMP, including, starting materials, processes, equipment, and employee training. Procedures dictated by cGMP greatly reduce the risk of both product contamination and employee injury. All individuals working in the facility will be trained and follow all cGMP regulations, including operators, engineers, supervisors, and maintenance personnel.

The process described above yields very few serious health concerns as the majority of chemicals used in the process are not toxic to humans. However, caustic cleaning chemicals and high concentrations of some buffer solutions may pose a threat to human health. The safety concerns of all chemicals utilized throughout the process are summarized in Table 5.7.1.

Table 5.7.1. Safety Concerns of Each Compound

Component	OSHA PEL (mg/m^3)	Safety Concerns
HEPES Buffer	N/A	Mild skin irritation possible
RNA Polymerase	N/A	Not hazardous material according to GHS
Magnesium Chloride	10	Skin and eye irritant; dust may be explosive
RNAse Inhibitor	N/A	Mild skin and eye irritation possible
Dithiothreitol	N/A	Harmful if swallowed; produces highly toxic gases when burned; reacts strongly with oxidizers
Spermidine	N/A	Severe skin and eye irritant; avoid inhalation; combustible
Nucleotides	N/A	No known significant hazards
Golodirsen	N/A	No known significant hazards
Sodium Chloride	N/A	Mild skin, eye, and respiratory tract irritant; can absorb moisture from air
Sodium Phosphate	N/A	Not considered hazardous by OSHA, but may decompose upon heating
Potassium Chloride	N/A	Mild skin, eye, and respiratory tract irritation; dust may cause irritation
Potassium Phosphate	N/A	Mild skin, eye, and respiratory tract irritation
Sodium Hydroxide	2	Serious eye and skin corrosion, corrosive to metals
Water for Injection	N/A	Potentially dangerous upon ingestion

The table shows that most hazards can be effectively combated with proper personal protective equipment (PPE). All individuals who enter the workspace will be required to wear chemical-resistant gloves, tightly fitting safety glasses, and a face shield. The workspace will be well-ventilated to ensure no toxic or dangerous vapors accumulate. As in any lab, no individual will bring any food or drink into the workspace to avoid accidental ingestion.

To design an inherently safer process, we intend to keep the amount of chemicals in stock as low as possible. By reducing the amount of each chemical that we store, the risk of a release, unwanted reaction, combustion, and contamination. Thus, we plan to store no more than a month’s supply of any of the chemicals used in the process. Furthermore, the storage of these chemicals will be carefully considered. All chemicals will be stored in tightly sealed

containers in cool, well-ventilated areas. All potentially reactive chemicals will be stored separately to avoid accidental mixing. Sodium hydroxide is not needed in concentrations higher than 1 M, so pure sodium hydroxide will be diluted immediately upon purchase to reduce the odds of a toxic release or reaction.

VI. Economic Analysis

6.1. Fixed Capital Investment

Due to the small-scale and relatively new pharmaceutical technology in our process, Turton was not able to provide accurate correlations. The fixed capital investment for this project was instead calculated via the Peter-Timmerhaus-West method of scaling total equipment cost. The total equipment cost consists of the sum of ancillary and major equipment costs. Ancillary equipment for this process consisted of stainless steel storage vessels, peristaltic pumps, and a water for injection system. Major equipment included a bioreactor system, tangential flow filtration units, chromatography columns, and sterile filtration housing. Prices for each piece of equipment were sourced from the sites of each manufacturer or through requested price quotes. The equipment costs and source are summarized in Table 6.1.1.

Table 6.1.1. Ancillary and Major Equipment Costs

Equipment	Manufacturer	Price/Unit	No. of Units	Cost
15 L Stainless Steel Jacketed Storage Vessel	USA Lab	$5,000	5	$25,000
L/S Standard Digital Pump System	Masterflex	$3,070	9	$27,630
I/P Digital Network-Compatible Pump System	Masterflex	$8,025	1	$8,025
Vapor Compression Distillation Module	MECO	$350,000	1	$350,000
SciVario Twin Dual Reactor System	Eppendorf	$178,465	1	$178,465
KrosFlo KMPi	Repligen	$40,000	3	$120,000
ACE Large Chromatography Column	Sigma-Aldrich	$10,000	2	$20,000
Single Round Housing T-Type	Sartorius	$2,000	1	$2,000
Total Equipment Cost (Ancillary + Major)				**$731,120**

Based on general industry estimates and other capstone projects, it was estimated that total equipment cost would account for 20% of the total fixed capital investment. In turn, this would equate to a total fixed capital investment of $3,655,600. Based on Peter, Timmerhaus, and West, this fixed capital investment may be broken down into direct and indirect costs

associated with setting up the process plant. Direct costs include purchased equipment, installation, instrumentation, piping, electrical, building, service facilities, and land expenses. Indirect costs include engineering and supervision, legal, contractor's fees, and contingency expenses. Allocation for each component of the direct and indirect costs were made based on the suggested ranges given by Peter, Timmerhaus, and West. The breakdown of fixed capital investment is summarized in Table 6.1.2.

Table 6.1.2. Breakdown of Fixed Capital Investment by Component

Type of Cost	**Component**	**% of Fixed Capital Investment**	**Cost**
Direct Costs	Purchased equipment	20%	$731,120
	Installation	9%	$329,004
	Instrumentation	7%	$255,892
	Piping	6%	$219,336
	Electrical	4%	$146,224
	Buildings	12%	$438,672
	Service Facilities	8%	$292,448
	Land	1%	$36,556
Indirect Costs	Engineering and Supervision	12%	$438,672
	Construction Expenses	8%	$292,448
	Legal Expenses	1%	$36,556
	Contractor's Fees	2%	$73,112
	Contingency	10%	$365,560
		Total Fixed Capital Investment	**$3,655,600**

6.2. Utilities, Labor, and Waste Treatment

The power requirements for each component of the process was determined and is summarized in Table 6.2.1. The values for each of the listed pieces of equipment was determined based on their respective data sheets. The process times presented in Table 5.5.1 were used to determine the annual uptime for reactors and the tangential flow filtration. For the reactor, the downtime required for cleaning the reactors was excluded from the annual uptime. For the pumps, the chromatography and sterile filtration process times were used. The pumps associated with the reactors and tangential flow filtration units were ignored as they are included in the power requirement for those units. The required water for injection per year was used alongside the maximum flow rate of the unit to determine its annual uptime. For the storage tanks, an average daily uptime of 8 hours was assumed since only one storage tank of the three needs to be on during any 24 hour period. The average commercial electricity rate in Boston is $0.14/kWh. To determine the annual power consumption of each piece of equipment, the following equation was used:

$$\text{Power Consumption} = \text{Power Requirement} * \text{Annual Uptime} \quad (6.2.1)$$

The total electrical cost associated with operating this process for a year would therefore be around $6,000. The cost of process equipment and plug loads in pharmaceutical plants is typically around 39% of total electrical costs for pharmaceutical plants (Capparella, 2013). Using the process equipment electrical cost estimate to approximate the total annual electrical cost results in $15,773 per year. Approximately 489,000 liters of water would be needed for this process per year. The price of water in Boston is $81.05 per 1000 cubic feet. The annual water cost would therefore be $1,400. The total annual utilities cost would be equal to the sum of the electrical and water costs, or $17,173.

Table 6.2.1. Electrical Costs associated with Power Consumption of Process Equipment

Equipment	Power Requirement (kW)	Annual Uptime (hr)	Annual Cost
Reactors	3.4	3420	$1,627.92
Pumps	2.53	2085.25	$738.60
Water for Injection System	9.25	322.17	$417.22
Storage Tanks	9.0	2280	$2,872.80
Tangential Flow Filtration	1.6	2208.75	$494.76
		Total Process Electrical Cost	**$6,151.29**

The costs of labor for this process are summarized in Table 6.2.2. The process will require twelve operators split across the upstream and downstream unit operations. Each operator will work 40-hour work weeks for 49 weeks of the year, leaving 3 weeks for vacation, sick days, and holidays. Six engineers will act as technical support for the process. Each engineer will work 40-hour work weeks for 48 weeks of the year, leaving 4 weeks for vacation, sick days, and holidays. Two plant supervisors will manage day-to-day operations of the entire plant. Each supervisor will work 40-hour work weeks for 47 weeks of the year, leaving 5 weeks for vacation, sick days, and holidays. The annual salary for an operator will be $60,000. Estimating the gap in pay between each position to be 40%, the annual salaries for an engineer and supervisor will be $84,000 and $117,600, respectively. Salaries were estimated to account for 70% of the total compensation for any given employee. The remaining 30% would be in the form of employee benefits. This brings the cost of labor per employee for operators, process engineers, and supervisors to $85,714, $120,000, and $168,000, respectively. Therefore, the total labor costs will equate to $2,420,571 per year.

Table 6.2.2. Breakdown of Process Labor Cost

Position	Number of Employees	Cost per Employee	Total Cost
Operator	12	$85,714	$1,028,571
Process Engineer	6	$120,000	$720,000
Supervisor	2	$168,000	$672,000
		Total Process Labor Cost	$2,420,571

6.3. Raw Materials

Price estimates for the bulk material costs of reagents and solvents were estimated from listings on SigmaAldrich and Fisher Scientific. Because the largest quotes for the DNA template, nucleotides, and cap analog still required us to buy over a million units per year each, we assumed price discounts of 90%, 90%, and 99% respectively. To meet the production specification for 19.31 kilograms of golodirsen per year, the mass requirement and bulk cost of each starting material and the final product are summarized in Table 6.3.1.

Table 6.3.1. Annual Raw Materials Cost

Compound	Requirement per year	Listed Price	Price per year
In Vitro Bioreactor			
DNA template	0.75 g	$10,067/g	$7,550,550
Beads	30 g	$16.7/mg	$501,000
RNA polymerase	7,500,000,000 U	$3.05/250 U	$915,000
$MgCl_2$	68.328 moL	$22.22 moL	$1,518
HEPES buffer	227.76 moL	$426/moL	$97,025
DTT	113.88 moL	$2919/moL	$332,456
RNase inhibitor	57,000,000,000 U	$0.02/U	$12,034,980
Spermidine	5.7 moL	$1492/moL	$8,501
Nucleotides	227.76 moL	$0.62/moL	$14,088,440
Cap Analog	57 moL	$772/mmoL	$46,683,000

TFF Steps			
HEPES buffer	9.2 moL	$426/moL	$1,116,972
1 M NaOH (aq)	150 moL	$9.00/moL	$32,400
Affinity Chromatography			
Oligo dt (25) Resin	33.6 L	$8,824/moL	$1,185,946
HEPES buffer w/ 0.15 M NaCl	7490 L	$34.08/L	$3,063,110
HEPES buffer eluant	7490 L	$34.08/L	$3,063,110
1 M NaOH (aq)	1797.5 kg	$0.14/kg	$3,020
Size Exclusion Chromatography			
Superdex 75 Prep-Grade Resin	287 L	$3,326/L	$3,818,248
NaCl	85.54 kg	$0.06/kg	$65
Na_3PO_4	69.62 kg	$2.20/kg	$1,838
1 M NaOH (aq)	11.48 kg	$1.50/kg	$207
20% Ethanol	1607.2 L	$3.50/L	$67,502
Sterile Filtration			
Sartorius Sartopore 2 High Flow Cartridges	1 cartridge	$607/cartridge	$172,995
			Total Cost: $94,102,904

6.4. Overall Operational Expenses

Profitability analysis of this process was conducted by evaluating annual operating cash flow. Operational expenses besides raw materials, utilities, and labor include costs such as maintenance, insurance, licensing fees, and income and property taxes. The operational economic analysis of the process is summarized in Table 6.4.1. Because there is no current competitor on the market that can treat muscular dystrophy below $300,000 per year, we decided to aim for a yearly revenue that was 170% the cost of our raw materials each year. This number was picked because gross margins for large scale pharmaceutical companies

range from 10-42% (DeAngelis, 2016). When our desired revenue is plugged into the equation for gross margin we get 41.2%, which is within the target range.

$$Gross\ Margin\ = \frac{Revenue - Cost\ of\ Inputs}{Revenue} = \frac{(1.7-1)\ Cost\ of\ Inputs}{1.7*Cost\ of\ Inputs} = \frac{0.7}{1.7} = 41.2\% \quad (6.4.1)$$

Therefore, the annual revenue from sales was estimated to be little less than $160,000,000. This would correlate to a cost of $4103/g golodirsen. Utility and labor costs were calculated as described in Section 6.2 and 6.3. Administration, maintenance, insurance, licensing fees, plant overhead, and operating supplies were calculated as described in Turton. Depreciation was calculated by assuming a straight line seven-year depreciation schedule. The corporate income tax rate in Boston of 8% was added to the federal corporate income tax rate of 21% in order to determine the combined annual corporate income tax burden. Property taxes were determined by estimating the plant size and then using the median price per square foot and property tax in Boston. The plant size was conservatively estimated to be around 12,000 square feet and the average price per square foot in Boston is $1,650. The plant would therefore have a property value of $19,800,000. The corporate property tax in Boston is 1.12% of earnings before taxes and depreciation. This in turn equates to an annual property tax burden of $221,760. Our final profit after tax was calculated to be slightly below $43,000,000 per year. Total invested capital is the sum of working capital and fixed investment capital. As per Turton, working capital was estimated at 20% the value of the fixed investment capital. The return on investment (ROI) for this process after a single year was calculated by dividing the profit after tax (PAT) by the total invested capital (TIC). After a single year of operating the plant, the return on investment was calculated to be 980%.

$$ROI\ = \frac{PAT}{TIC} = \frac{\$43{,}000{,}503}{\$4{,}386{,}720} * 100 = 980\% \quad (6.4.2)$$

Table 6.4.1. Operating Cash Flow Analysis and Return on Investment

Revenue from Sales	$159,974,937
Raw Materials	($94,102,904)
Utility Costs	($17,173)
Labor Costs	($2,420,571)
Administration	($395,541)
Maintenance	($219,336)
Insurance	($116,979)
Licensing Fees	($109,668)
Plant Overhead Expenses	($1,582,163)
Operating Supplies	($32,900)
Earnings before Taxes and Depreciation	$60,977,702
Depreciation	($104,446)
Federal and Corporate Income Tax	($17,653,244)
Property Tax	($221,760)
Profit after Tax	$42,998,252
Total Invested Capital	$4,386,720
Return on Investment	980%

6.5. Discounted Cash Flow Model

To fully demonstrate the profitability of this process, a case flow model was constructed. The construction of the plant is expected to take approximately one year with the plant operating fully all following years. We set the discount rate to 8%, which is a low estimation of pharmaceutical discount rates (Avance, 2020).

Table 6.5.1. Discounted Cash Flows

Year	Cash Flow	Discounted Cash Flow	Cumulative Cash Flow
0	($4,386,720)	($4,386,720)	($4,386,720)
1	$42,712,423	$39,548,540	$35,161,820
2	$42,712,423	$36,619,018	$71,780,838
3	$42,712,423	$33,906,498	$105,687,337
4	$42,712,423	$31,394,906	$137,082,243
5	$42,712,423	$29,069,357	$166,151,600
6	$42,712,423	$26,916,072	$193,067,672
7	$42,712,423	$24,922,289	$217,989,960
8	$42,712,423	$23,076,193	$241,066,153
9	$42,712,423	$21,366,845	$262,432,999
10	$42,712,423	$19,784,116	$282,217,115

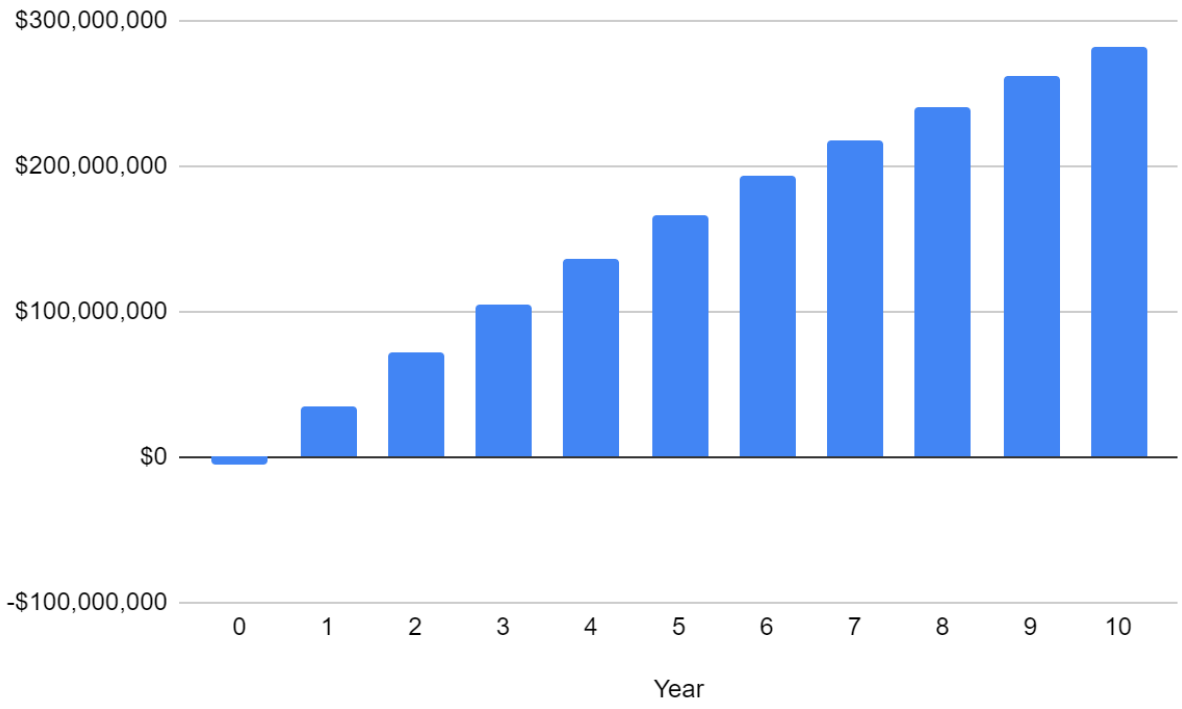

Figure 6.5.1: Cumulative Cash Flow over 10 Years of Operation

As demonstrated by Figure 6.5.1, the process is highly profitable and offers attractive returns due to a fairly low initial investment and strong profit margin. The cumulative (summed) discounted cash flow 10 years after the initial investment otherwise known as the net present value of the investment (NPV) is shown to be approximately $280,000,000.

VII. Conclusions and Recommendations

Oligonucleotides therapeutics are a relatively new technology that has gained considerable traction due to their use in vaccines against SARS-CoV-2. As such, there was a need for many assumptions in completing this capstone project. As more research and development is conducted in the field, many of these assumptions could be supported with solid scientific evidence. Additionally, more pharmaceutical companies entering the oligonucleotide market would provide many more patents to study and learn from. This would have been particularly beneficial to us, since Moderna and Pfizer were the first two companies to establish large-scale manufacturing plants in this field and each had little information publicly available.

By synthesizing information from available patents and research, a process was designed to produce Vyondys 53 for 6,250 patients each year. The process includes an *in vitro* bioreactor, three tangential flow filtration units, an affinity chromatography column, a size exclusion chromatography column, and a sterile filtration unit. Each unit was completely specified to describe the exact performance of the unit operation. Reactor R-101 will use immobilized DNA templates to produce and cap the desired strand of mRNA. Filter F-101 will then remove all small molecules present in the reaction and introduce the desired chromatography buffer. Column C-101 will remove unused nucleotides and incomplete mRNA strands. The eluent will be concentrated by filter F-102 and subsequently fed into column C-102, which will remove all remaining undesired mRNA strands. Filters F-103 and F-104 will formulate the product for delivery and ensure the product is sterile, respectively. Thus, the output of the process will be prepared for vial filling and intravenous delivery to the patient.

Future work would include addressing many of the assumptions made. Applying kinetic models to optimize the reactor design would aid in strengthening the upstream portion of the process design. Given the complexity of co-transcriptional capping and the chemical structure of the cap analog of choice, we decided to approach synthesis for our RNA therapeutic with a recipe-like method. When designing the chromatography units, an assumption was made that many of the same models and methods of designing protein chromatography units could be applied to RNA based systems. Furthermore, the affinity chromatography resin that we chose to use is a new proprietary technology with little publicly available information. Given this

assumption, we could have drastically overestimated or underestimated our yields and chromatographic resolution. Negligible concentration gradient was another major assumption that may have had an impact on this process design. Due to the lack of scientific publications on flux as a function of oligonucleotide concentration based on molecular weight, we were left to use empirical correlations for proteins. However, given the linear nature of oligonucleotides compared to globular proteins, this may have incorrectly assumed near-ideal performance of our tangential flow filtration units.

VIII. Acknowledgements

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X. Appendix

Appendix A. Size exclusion chromatography (SEC) modeling

1. Selectivity curves of Superdex 75 prep grade resin (*Superdex prep grade and prepacked HiLoad columns*, 2020).

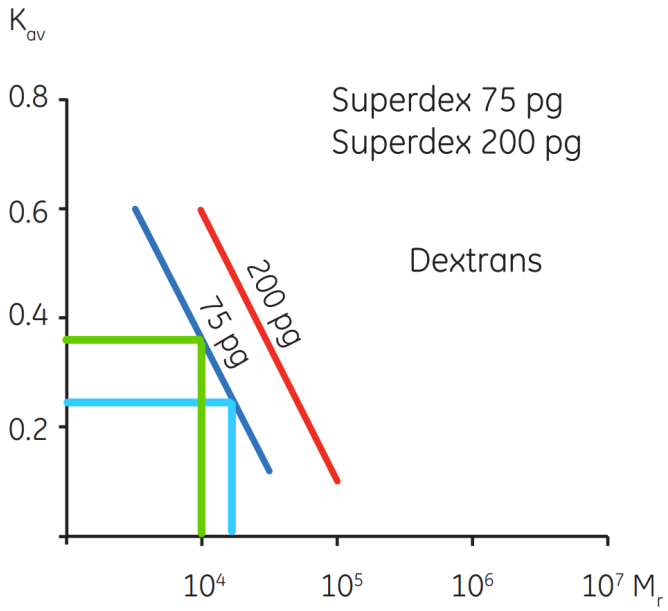

Figure A.1.1. Selectivity curves of Superdex 75 prep grade resin. The green line shown gives the partition coefficient (K_{av}) of golodirsen, 0.36. The blue line shown gives K_{av} of elongated mRNA strands, 0.24. Elongated strands were assumed to have the M_r of golodirsen dimers, 26 kDa.

2. Modeled SEC chromatogram and input parameters used to generate the curves

Input Paramters		
C_{FA} ==>	0.11	(A feed conc., g/L)
C_{FB} ==>	4.88	(B feed conc.,g/L)
m_A ==>	0.24	(linear isotherm slope for A)
m_B ==>	0.36	(linear isotherm slope for B)
ε =	0.35	(extraparticle porosity)
d_p ==>	34	(particle diameter, µm)
D_{eA} ==>	1.00E-07	(effective pore diffusivity of A, cm^2/s)
D_{eB} ==>	1.00E-07	(effective pore diffusivity of B, cm^2/s)
t_F ==>	200	(duration of feed injection, s)
t_{cycle} ==>	4320	(cycle time, s)
u ==>	50	(superficial velocity cm/h)
L ==>	60	(column length, cm)
Number of cycles ==>	1	

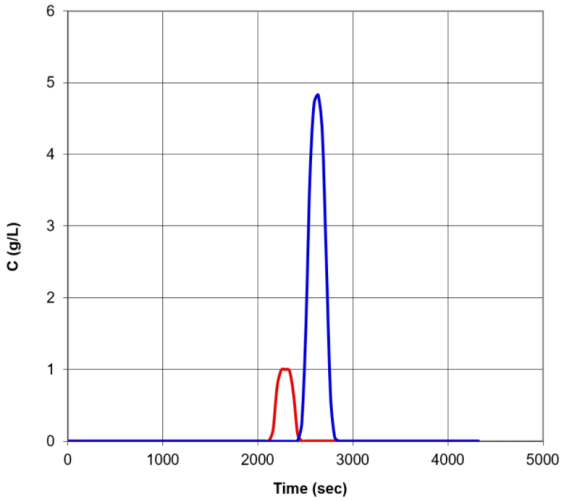

Figure A.2.1. SEC chromatography model. m_A and m_B are the partition coefficients determined in Appendix A.1. Elongated mRNA elutes first (red peak) and golodirsen elutes last (blue peak).

3. Calculations to determine the concentration of the solutions exiting the SEC column (C-102)

Table A.3.1. Calculation of golodirsen concentration from chromatographic peak area

	Impurities	Product				
Elution Start	2160	2419		total buffer in	298.76	L
Elution End	2482	2851		total mRNA in	58.8	g
Peak Width	322	432				
Peak Height	1	4.8				
Peak Area	161	1036.8				
% of buffer out	0.134	0.866				
Vol buffer out	40.16	258.60	L			
Mass of mRNA	3.06	55.7	g			
Conc out	0.0762	0.2154	g/L			

Appendix B: Operating Pressure of Equipment

Table B.1. Operating Pressure of Equipment

Unit	Inlet Pressure (bar)	Outlet Pressure (bar)
V-101	1.01	1.01
R-101	1.01	1.01
V-102	1.01	1.01
F-101	2.00	0.50
C-101	3.51	1.01
V-103	1.01	1.01
F-102	2.00	0.50
C-102	3.09	1.01
V-104	1.01	1.01
F-103	2.00	0.50
F-104	2.00	0.50