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Optimization of exogenous gene expression in Atlantic Salmon fibroblast

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Master in Genome Science

Acknowledgements

The completion of my master's degree in Genome Science at the Norwegian University of Life Sciences (NMBU) is marked by this thesis. It was undertaken as a collaborative project with AquaGen at the Center for Integrative Genetics (CIGENE), with the aim of achieving pluripotent stem cell culture in Atlantic salmon. The laboratory work spanned from September 2022 to May 2023.

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Abstract

The Atlantic salmon is crucial to multiple ecosystems, including the Norwegian ecosystem, and is an important driver of the Norwegian economy (Aandahl & Hestvik, 2023). To address the need for understanding its anatomical mechanisms and potential for genetic improvement, gene editing in fibroblast cells can be performed. These cells are derived from the organism's skin and serve as replication of the salmon's cellular functions. Gene editing techniques, such as fibroblast transfection for exogenous gene expression investigation, have been mostly optimized for mammalian cells so far. However, there is a lack of information regarding transient mRNA transfection in Atlantic salmon. To address this gap, two chemical transfection methods were evaluated, with lipofection proving to be more efficient than calcium phosphate transfection, which resulted in no observable transfected cells under light microscopy. Additionally, incubating the transfected cells at 25°C, slightly above their typical incubation temperature, and supplementing with yeast tRNA enhanced transfection efficiency. This study revealed the possibility of achieving exogenous gene expression in Atlantic salmon fibroblasts through mRNA transfection, which is a very important target that can be used for pluripotent stem cell culture in the Atlantic salmon.

Sammendrag

Den atlanterhavslaksen er en stor del av flere økosystemer, inkludert det norske økosystemet, og er avgjørende for norsk økonomi (Aandahl & Hestvik, 2023). For å forstå dens anatomiske mekanismer og potensial for genetisk forbedring, kan genredigering i fibroblastceller utføres. Disse cellene er avledet fra organismens hud og fungerer som replikering av laksens cellulære funksjoner. Genredigeringsteknikker, som fibroblast transfeksjon for eksogen genekspresjonsundersøkelse, har for det meste blitt optimalisert for pattedyrceller så langt. Det er imidlertid mangel på informasjon om mRNA-transfeksjon hos atlanterhavslaksen. For å fylle dette gapet ble to kjemiske transfeksjonsmetoder evaluert, hvor lipofeksjon som viste seg å være mer effektivt enn kalsiumfosfat transfeksjon, noe som resulterte i ingen observerbare transfekterte celler under lysmikroskopi. I tillegg forbedret inkubering av de transfekterte cellene ved 25 °C, litt over deres typiske inkubasjonstemperatur, og supplering med gjær-tRNA forbedret transfeksjonseffektiviteten. Dettee studiet avslørte muligheten for å oppnå eksogent genuttrykk i atlantiske laksefibroblaster gjennom mRNA-transfeksjon, som er et svært viktig mål for bruk til pluripotent stamcellekultur hos atlanterhavslaksen.

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I. Introduction and literature review

A. Atlantic salmon stem cells

Atlantic salmon (Salmo salar) is a vital aquatic specie that plays a crucial role in several ecosystems, particularly the Norwegian ecosystem. It is not only an essential part of the ecosystem but also a key contributor to the Norwegian economy. The salmon farming industry has been growing significantly in recent years and has become a significant factor in the country's gross domestic product. In 2022, the industry recorded its best year yet, generating over 100 billion Norwegian kroners in export revenue. (Aandahl & Hestvik, 2023).
Conducting research on Atlantic salmon cells holds immense importance due to the significant role this species plays in global human nutrition. Atlantic salmon is highly regarded for its nutritional value, as it serves as an excellent source of minerals, vitamins, and macronutrients (E. Barasa et al., 2022), including fats.
Any instances of infections or diseases in Atlantic salmon can have a direct impact on both the economy and the availability of nutritional resources. There have been notable occurrences where Atlantic salmon has been affected by viral infections or rendered unsuitable for consumption, leading to a decline in production, causing economic disadvantages. Examples of such infections include infectious pancreatic necrosis, infectious salmon anemia, and furunculosis (E. Barasa et al., 2022).
Countries with a strong reliance on the salmon industry face substantial challenges when such phenomena arise. Therefore, conducting research on Atlantic salmon is vital not only for the sustainability of the salmon farming industry but also for a part of the human nutritional resources.

Modern scientific research primarily focuses on human and mammalian subjects, employing a variety of in vitro and in vivo techniques to study molecular mechanisms relevant to their health and wellbeing. Several model organisms have also been established; however, none of these models do not adequately represent the unique biology of Atlantic salmon. Furthermore, conducting experiments with Atlantic salmon cells as present inherent challenges, primarily attributed to their specific environmental requirements for survival. Notably, their temperature tolerance is limited, with survival temperatures not exceeding 20°C (Garside, 1973). This is very low compared to mammalian cells that generally thrive at 37°C.
Additionally, the lipid membrane bilayer of Atlantic salmon exhibits a higher degree of saturation compared to mammalian counterparts, which can impede efficient transfection processes (Lopez et al., 2001). Membrane fluidity is for example crucial for successful passage of nucleic acids into the cell during transfection.
In addition to these barriers, the long life cycle of Atlantic salmon hinders their suitability as in vivo research models. Combined with the fact that typically females can only breed once throughout their lifetime (E. Barasa et al., 2022), use of salmon in research can become very challenging.

These are some of the reasons why salmon research lacks validated experimental methods readily applicable for direct applications to address specific questions.
In Norway, there is an increasing interest in applying novel methodologies such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and induced pluripotent stem cells (iPSCs) to the field of Atlantic salmon research. These innovative techniques offer the potential to conduct comprehensive studies and gain deeper insights into the biology of Atlantic salmon.
The challenges inherent to working with Atlantic salmon, such as its unique biology and the difficulty in adapting protocols designed for model organisms, have created significant methodological obstacles. To address these challenges, the primary objective of this study is to overcome these obstacles by developing optimal conditions for mRNA transfection into salmon fibroblasts to enable the transient production of exogenous proteins in Atlantic salmon.
Stem cells, particularly iPSCs, have revolutionized research across various biological disciplines. Despite their extensive applications in mammalian studies, iPSCs have yet to be generated in Atlantic salmon, making them a current focal point in salmon research. The interest in generating iPSCs in Atlantic salmon stems from their immense potential that has been demonstrated in other species in advancing regenerative medicine, disease modeling, genetic engineering, and drug and toxicity testing (Singh et al., 2015). Disease modeling using iPSCs would facilitate a deeper understanding of the underlying causes of specific conditions, enabling the development of targeted treatments to address them (Singh et al., 2015) Additionally, iPSCs of Atlantic salmon would serve as valuable tools for gene therapy, allowing for the correction of genetic defects and the improvement of disease resistance in the species.
As mentioned, the goal is to enable transient production of exogenous proteins whereby transfected genetic material is expressed for a limited duration. Transient transfection will generate iPSCs at a much faster pace (Chatterjee et al., 2011) and offers the added benefit of preventing the integration of foreign genetic information into the host cell's genome (Kim & Eberwine, 2010). Thus, it provides an opportunity to specifically investigate exogenous gene expression (Wang et al., 2021).

1. From somatic cells to induced pluripotent stem cells

Stem cells possess the ability to undergo self-renewal and exhibit the potential to differentiate into various specialized cell types within any given lineage. These cells are classified into distinct developmental potency categories (Zakrzewski et al., 2019). These potency classes determine the range of lineages that stem cells can differentiate into, spanning from totipotent to unipotent. It is important to note that as stem cells progress along the differentiation pathway, their potency diminishes, resulting in a reduced capacity to generate diverse cell types. (Singh et al., 2015)
Totipotent cells, such as zygotes, hold the capability to develop into any cell type, including both embryonic and placental cells. Pluripotent cells, on the other hand, can differentiate into cell types pertaining to the ectoderm, mesoderm, and endoderm, but not structures specifically associated with embryonic development.

In comparison to pluripotent cells, multipotent cells exhibit a narrower potential for differentiation, generating a lesser number of cell lineages. The utilization of stem cells in disease modeling or genetic therapy has been somewhat challenging posed due to the distinction between embryonic stem cells, which are pluripotent, and adult stem cells, which are multipotent and considerably more limited in their capabilities. This limitation has made the use of stem cells for research purposes questionable. Not only does it necessitate the sacrifice of embryos, which raises ethical concerns, but it also gives rise to technical complications post-transplantation, such as the potential for immune rejection (Singh et al., 2015).

Over time, extensive research in stem cell biology has focused on the generation of pluripotent stem cells from already differentiated cells. One prominent area of investigation revolves around understanding the underlying mechanisms that enable the conversion of differentiated cells into pluripotent states. Stem cells possess a distinct set of genes that are actively expressed and indispensable for maintaining their stem cell characteristics. In contrast, these same genes are suppressed in differentiated cells. This unique gene set encodes transcription factors, known as the Yamanaka factors, which play a crucial role in activating downstream genes and orchestrating the reprogramming process to establish pluripotency. (Takahashi & Yamanaka, 2006).

The discovery of Yamanaka factors has revolutionized stem cell biology. This breakthrough has not only addressed the ethical concerns associated with the utilization of embryos for obtaining stem cell cultures, (Singh et al., 2015) but it has also overcome technical challenges by enabling the generation of autologous cells, thereby reducing the risk rejection from the body (Zakrzewski et al., 2019). The Yamanaka cocktail of transcription factors includes Oct3/4, Sox2, Myc, and Klf4. Notably, Myc and Klf4 are oncogenes that play pivotal roles in the reprogramming of differentiated cells into iPSCs (Zakrzewski et al., 2019). On the other hand, Oct3/4 and Sox2 are essential for maintaining the characteristic properties of stem cells.

By introducing this combination of transcription factors into fibroblast cultures, pluripotent stem cells can be generated efficiently and without the requirement of surplus embryo. (Takahashi & Yamanaka, 2006).

2. Role of transfection

In the context of the cell reprogramming, transfection emerges as a crucial technique. Transfection refers to the process of introducing nucleic acids into cells to obtain genetically modified cells (Kim & Eberwine, 2010). It represents a safer alternative to viral transduction, as it minimizes the risk of fortuitous effects such as cytotoxicity, genome alterations or cell death. Transfection is employed to investigate gene function or products, by enabling the alteration of gene expression in the target cells, resulting in the production of recombinant proteins that can be further studied (Wurm, 2004). Hence, transfection plays a significant role in the cell reprogramming process, where a cocktail of Yamanaka factors is transfected into somatic cells cultured under appropriate conditions, inducing their pluripotency and transforming them into induced pluripotent stem cells.

B. Transfection methodology

When developing a transfection protocol for Atlantic Salmon fibroblast cells, several key considerations must be evaluated to ensure an optimal approach. The initial decision revolves around the choice of employing a viral vector or not (Chong et al., 2021). This would then lead to the choice of which type of transfection method to be used, either chemical or physical for a non-viral process or biological for virus-mediated transfection. To make this choice various factors such as cell viability, transfection efficiency, overall cell well-being, and the establishment of a reproducible protocol need to be carefully considered. These aspects play a critical role in obtaining reliable experimental results and ensuring the future success of the study. (Kim & Eberwine, 2010). The next thing to ponder upon is the cargo molecule to be used, which refers to the genetic material that will be delivered into the cells (Kucharski et al., 2021). This decision involves choosing between DNA and different classes of RNA molecules (e.g., mRNA, siRNA, miRNA). The decision regarding the choice of cargo molecule in the transfection protocol depends on the specific objective. Distinctions exist between mRNA transfection and miRNA transfection, for instance. miRNA and siRNA transfection offer a means to modulate gene expression, particularly to decrease the expression of specific genes (Khan et al., 2009). On the other hand, mRNA transfection is employed to express a desired protein and explore its functional role (*Guidelines for RNA Transfection*, n.d.).

Lastly, proper attention must be given to maintaining good cell condition throughout the culturing process. This involves utilizing suitable media, growth temperature and employing antiseptic techniques to ensure the well-being of the cells, which directly impacts cell density. By repeatedly cultivating healthy cells, it becomes possible to establish a general protocol for seeding density, aiming to achieve an optimal cell density at the time of transfection. This step is crucial for maximizing transfection efficiency and overall experimental success, as transfection efficiency of many protocols is highly dependent of cell density (*Factors Influencing Transfection Efficiency*, n.d.).

In order to ensure a comprehensive and reliable transfection protocol, it is crucial to include appropriate transfection controls. The inclusion of negative controls is essential to assess whether any reagents or experimental conditions are causing inefficient transfection. These negative controls enable the identification and optimization of potential factors impacting transfection efficiency. Examples of negative controls may involve conducting a transfection without the cargo molecule, a transfection with only the cargo molecule, or even a well with no components introduced. A positive control is just as important and would be an optimised transfection mixture, present in each experiment to ensure properly run transfection and minimize biases. (Chong et al., 2021)

Upon completion of each experiment, it is essential to conduct a thorough analysis and quantification of the transfection results. Various methods are available for this purpose, each with its own advantages and limitations. In the context of this thesis project, which explores novel variations of standard mRNA transfection methods, the expected results primarily focus on

qualitative rather than quantitative outcomes. The primary objective of the study is to investigate the feasibility of efficient mRNA transfection in Atlantic salmon.
To analyze the results, fluorescence microscopy has been selected as the preferred method. This choice is motivated by the ease and speed of fluorescence microscopy, allowing for efficient analysis of the transfected cells. However, it is important to acknowledge a drawback associated with this method. Fluorescence microscopy cannot discern between signals emitted by the cells themselves and signals emitted from the extracellular environment (Chong et al., 2021). To address this limitation, special precautions were taken during the analysis of transfection efficiency. In this thesis, photographs of the transfected cells visualized under both fluorescent light and transparent light are presented, enabling a comprehensive evaluation of the results while considering potential extracellular signals.

1. Selection of transfection methods

When considering viral-based methods, there are two types of transfections: stable and transient transfection. Stable transfections involve the use of retroviruses or lentiviruses, which are specifically employed in dividing cells. These viruses introduce their RNA genome into the target cell's genome, resulting in a long-lasting and stable expression of the desired genetic material (Chong et al., 2021).
On the other hand, the transient viral method employs adenoviruses, adeno-associated viruses or herpes viruses. Unlike stable transfections, these transient methods do not guarantee integration of the viral genome with the target cell's genome. Nonetheless, both dividing and non-dividing cells can be successfully transfected using these viral vectors (Chong et al., 2021).
These viral-mediated methods are highly efficient, easy to use and effective in vivo. However, there is a potential for unwanted events, such as inflammation or insertional mutagenesis. Insertional mutagenesis can result in disruption of essential genes, deletion of tumour-suppressor genes or activation of oncogenes (Kim & Eberwine, 2010).

Non-viral transfection methods encompass both chemical and physical approaches, with chemical methods being the most widely employed in contemporary research.
Chemical transfections relies on cationic polymers, calcium-phosphate complexes, or cationic lipids. The underlying principle of all these methods is formation of a positively charged complex between the positively charged chemical reagent and the negatively charged nucleic acid. This complex facilitates the transport of the genetic material across the negatively charged cell membrane, enabling its delivery into the target cell (Kim & Eberwine, 2010).
Reagents for chemical transfection are commercially available, affordable and easy to use. They offer high transfection efficiency and generally do not pose biohazard risks (Kim & Eberwine, 2010). However, chemical transfection efficiency is influenced by factors such as the cell type and the conditions of transfection. Additionally, there is a potential risk of cytotoxicity associated with chemical transfection (Kim & Eberwine, 2010). There are no published reports describing

chemical transfection of DNA or RNA molecules into salmon cells. Therefore, to use this approach in Atlantic Salmon cells will require extensive optimisation.

Finally, for physical transfection, there are different methods such as electroporation, laser irradiation, sonoporation and microinjection. These methods are based on physical changes made to the cell membrane to enable the passage of genetic material into the cell, except for microinjection where the genetic material is directly injected into the cell (Kim & Eberwine, 2010). For example, electroporation is the most common physical transfection method today, which uses electric voltage to disturb the cell membrane's permeability and makes holes in the membrane where the genetic material can pass and thereafter be translated or transcribed inside the target cell (Chong et al., 2021). Physical transfection methods require dedicated specialised instruments which can often be costly. An additional issue connected to physical transfection methods is a high rate of cell death that oftens result from disruption of cellular integrity (Kim & Eberwine, 2010).

An experiment has been done in the laboratory prior to this study, where electroporation was tested out in Atlantic Salmon fibroblasts. This has caused high rate of cell death, so electroporation was not to be considered for this thesis study at this point. In addition to this, salmon fibroblast cells are adherent and most of electroporation equipment requires cells that are in suspension (Potter, 2003). This can pose some additional problems such as decreased cell viability during trypsinisation.
Following careful consideration of the advantages and disadvantages associated with various transfection methods, two specific approaches were selected for testing and optimization in this study: calcium phosphate-mediated transfection and lipid-mediated transfection.
Calcium phosphate-mediated transfection was chosen based on its cost-effectiveness, historical usage, and absence of cytotoxicity concerns. Given its longstanding presence as a transfection method, it was deemed worthwhile to explore its applicability in Atlantic Salmon. The hypothesis was that this species might respond favorably to simpler transfection methods, as opposed to more complex techniques such as electroporation.
In the case of lipid-mediated transfection, the decision to use this approach was driven by its demonstrated high efficiency in various cell lines. Additionally, the availability of multiple lipid reagents in the laboratory at the time of the study further supported this choice.

2. Transfecting DNA or transfecting mRNA?

Transfection with DNA as the cargo molecule is usually the go-to method but transfecting mRNA has its own advantages. Figure 1 illustrates the differences between DNA and mRNA lipid-mediated transfection process. The lipid complex of DNA and lipid reagent goes through the cell membrane by endocytosis. Once the complex is inside the cell, it needs to enter the nucleus to get transcribed into mRNA, the generated mRNA will then leave the nucleus to code for proteins. Meanwhile, for mRNA transfection, the lipid complex of mRNA and lipid reagent is directly

delivered to the target cell's cytoplasm and translated into proteins (*Efficient mRNA transfection for gene expression of difficult-to-transfect cells*, n.d.).

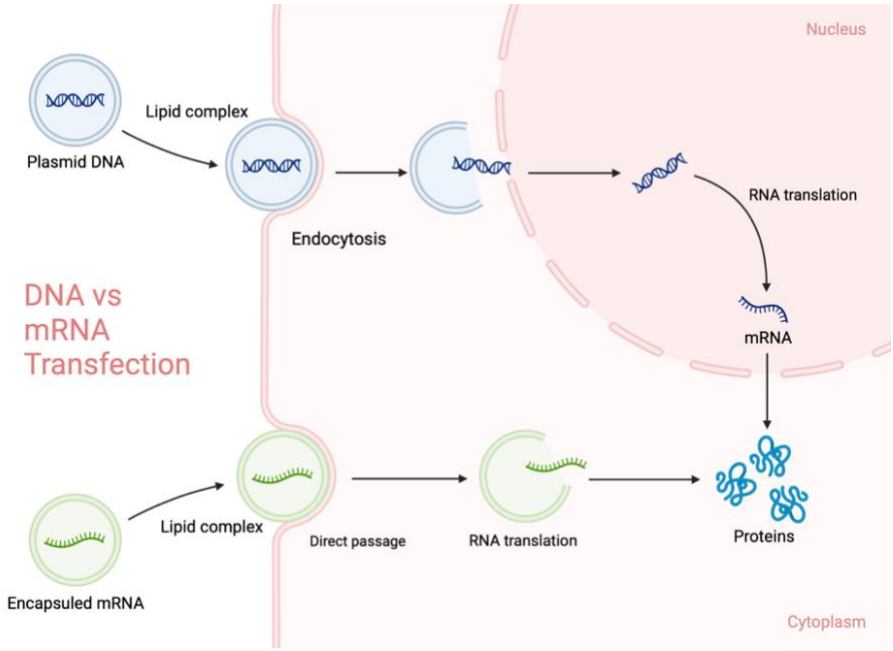

Figure 1 Comparison of lipid-mediated transfection with DNA and mRNA (Figure created with Biorender.com)

This direct passage of mRNA through the cell membrane in addition to the ability to express in the cytoplasm without needing to go through the nucleus presents a list of advantages over DNA transfection. Consequently, it makes it a suitable method for the transfection of hard-to-transfect cell types, such as stem cells (*Efficient mRNA transfection for gene expression of difficult-to-transfect cells*, n.d.). These advantages are presented in Table 1.

Table 1 Differences between DNA and mRNA transfection

DNA transfection	mRNA transfection	Reference
Lower transfection efficiency (dependent on cell line)	Higher transfection efficiency (dependent on cell line)	(Chong et al., 2021)
Multiple days for protein expression	Few hours for protein expression	(Chong et al., 2021; Kim & Eberwine, 2010)
Necessary nucleus entry for expression	Expressed in cytoplasm	(Kim & Eberwine, 2010)
Cell survival can decrease due to disrupted nuclear envelope	High cell survival rate	(Tolmachov & Tolmachova, 2015)
Possibility for mutagenic insertions	No mutagenic genomic insertions	(Tolmachov & Tolmachova, 2015)
Difficulty to use DNA transcriptome	Use of mRNA transcriptome	(Kim & Eberwine, 2010)
Design of plasmid DNA does not go through nucleoside modifications	Caution when handling mRNA due to nucleoside modifications	(Kim & Eberwine, 2010)

Transfection of mRNA has been extensively used in numerous mammalian cells. It is common in stem cell biology and used for inducing pluripotency.
On the other hand, there has been very little research done on mRNA transfection in fish cells, most specifically Atlantic salmon cells. There is virtually no published information about its successful application in fish cells.
As mentioned in Table 1, mRNA transfection can use a transcriptome, referring to a group of several mRNAs, which allow introduction of many gene products at the same time (Tolmachov & Tolmachova, 2015). This is well-suiting for the future of this study as it is necessary to transfect several pluripotency drivers in Atlantic Salmon fibroblasts to induce pluripotent stem cells.
Originally Yamanaka used viral transduction to introduce Yamanaka factors in fibroblasts. Viral vector can integrate into the genome, and cause damage so alternative ways are preferred, like RNA which does not integrate into the genome.
In addition to this, analysis of reprogramming efficiency reports shows that a non-integrating method with mRNA has the highest efficiency amongst other methods for the introduction of Yamanaka factor (Singh et al. 2015).
After evaluation of the pros and cons of RNA transfection, it seems that mRNA can offer good efficiency and diminish cell death compared to DNA transfection. Therefore, mRNA was the chosen cargo molecule for the experiments of this study.

3. mRNA alterations for efficiency

mRNA has several structures of great importance for its translation and stability (Kim & Eberwine, 2010). These structures include the cap, untranslated regions at the 5' and 3' end, open reading frame, and the poly-A tail. Alterations can be made to these structures to enhance the mRNA's expression, such as altering the size of the poly-A tail or choosing specific untranslated regions to provide high ribosome loading (Linares-Fernandez et al., 2021).
In this study, it was chosen to synthesize both capped and tailed mRNA for transfection, so it has a Cap-0 and a poly-A tail included in the reverse primer sequence used for amplification. This poly-A tail will help stimulate the translation process. Additionally, the poly-A tail has been proven to be important for the mRNA stability and translation efficiency in a study aiming for an alternative lipofection method for fish cell transfection (Tan et al., 2020).
mRNA that have not been capped have phosphate group residues that are very immunogenic. Dephosphorylation can used to remove these phosphates. It has been shown that dephosphorylating mRNA can help achieving high protein expression (Linares-Fernandez et al., 2021) and it can also prevent "the recircularization in a self-ligation reaction" of mRNA (Avci-Adali et al., 2014) . Therefore, in this study mRNA was additionally dephosphorylated before transfection.
To generate mRNA, an in vitro transcription kit of the anti-reverse-cap analogue (ARCA) variety has been used. ARCA has demonstrated the capacity to enhance translation efficiency and mRNA stability, attributable to the cap modifications and elongated phosphate bridges it engenders

(Strenkowska et al., 2010). At the same time, ARCA substantially reduces the likelihood of cap incorporation in the reverse orientation. This is essential because a reverse cap cannot be recognized by the translation initiation factor 4E, which precludes translation. (Stepinski et al., 2001) Therefore, ARCA is a crucial component in ensuring proper cap orientation and subsequent translation, its chemical composition is shown in Figure 26.

4. Calcium phosphate transfection & Lipid-mediated transfection procedures

a) Calcium phosphate-mediated transfection

The chemical principle underlying calcium phosphate-mediated transfection is neutralisation of the mRNA charges. The cell membrane is negatively charged, for that reason the negatively charged mRNA will be repelled by the membrane. Therefore, neutralisation of negative charges is necessary as calcium phosphate enables the binding of mRNA to the cell membrane. Neutralization happens by binding of the positively charged calcium ions to the negatively charged mRNA phosphate backbones (Chong et al., 2021; Raman, 2016).
Neutralized, the mRNA in a $CaCl_2$ solution, is then added in a dropwise fashion to a phosphate buffer. This will form a fine precipitate that will carry the mRNA's way into the target cells (*Calcium Phosphate Transfection Kit*, 2011). During incubation of the cells with the transfection mixture, the complex including the calcium phosphate and the mRNA will enter the cell by endocytosis, , and end up inside endosomes in the target cells. After the mRNA is released from the endosomes it will be translated in the cytoplasm as it is shown in Figure 2 (*Deliverance: Understanding Transfection Complexes*, 2022).

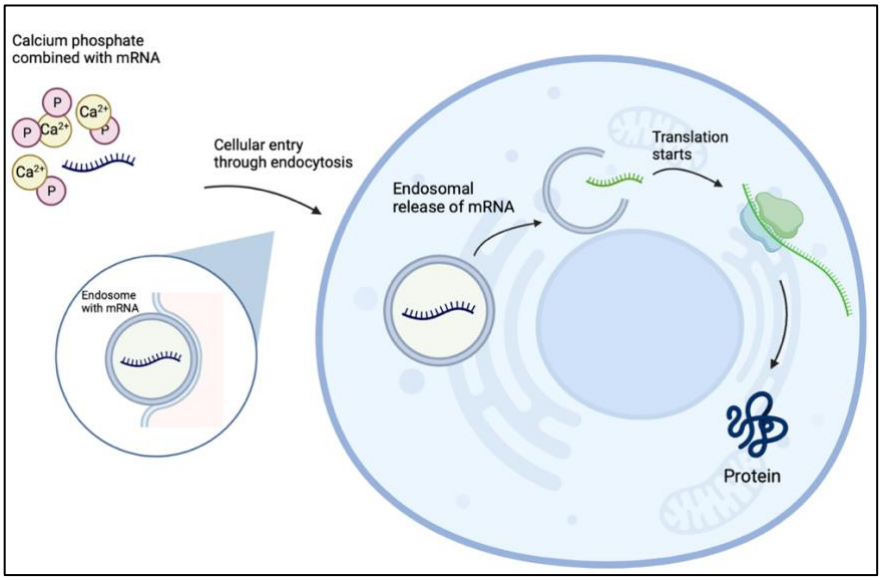

Figure 2 Calcium phosphate-mediated transfection of mRNA *The combination of phosphate and calcium forms positively-charged particles that surround the negatively charged mRNA. Cellular entry of the complex happens through endocytosis as it*

binds to the negatively charge lipid membrane. Thus, the mRNA shall be released from the endosome to the cytoplasm and start translation (Figure created with Biorender.com)

There are several factors that have to be defined as they influence the transfection efficiency. For example the volume of Calcium chloride in the transfection and the amount of mRNA have to be experimentally defined for higher transfection efficiencies. Different amounts of mRNA need to be tested out as it can in some cell types cause cell death, while for other types would need large amounts of mRNA to be transfected (Raman, 2016). In addition to the factors mentioned above, the cell density is also important to control, the less cell to cell contact there is the lower the efficiency but having too confluent cell populations will reduce uptake of any foreign genetic material. In theory for calcium phosphate transfection the cell density should not be above 50% (*Calcium Phosphate Transfection Kit*, n.d.)

b) Lipid-mediated transfection

For lipid-mediated transfection the the principle and the procedure are the same as described in I.B.4.a), the only difference would be the lipid complexes that get endocytosed into the cell. The lipid complexes are also composed of the mRNA but with positively charged heads of synthetic lipids which can easily fuse with the phospholipid bilayer of the target cell (*Cationic Lipid Transfection*, n.d.). The positively charged head group of the lipids enable the smooth entry of mRNA into the negatively charged cell membrane through endocytosis as described in Figure 3.

The endosomal release of mRNA is essential because if the mRNA does not get released, it will go through lysosomal degradation which will affect the transfection efficiency. ("Transfection Methods: Chemical Transfection," n.d.)

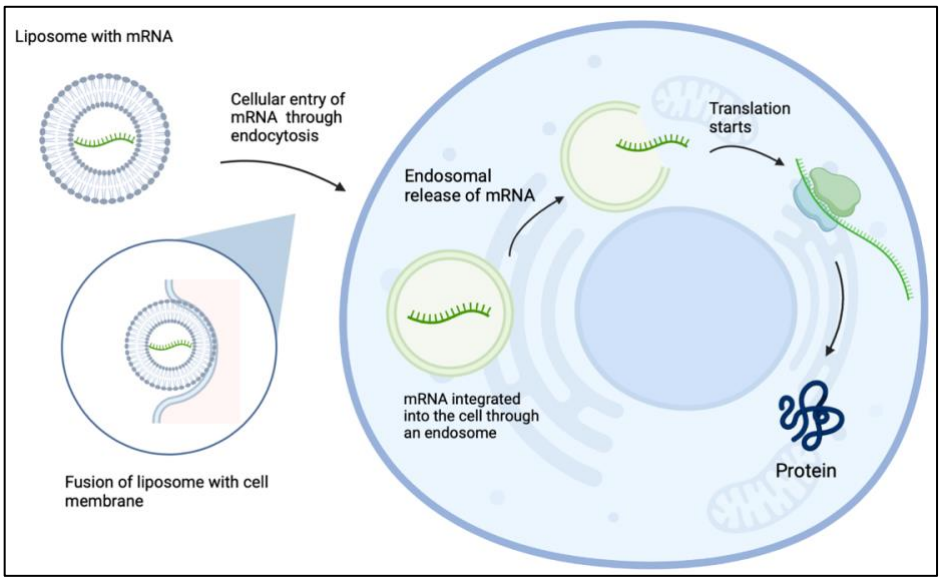

Figure 3 Lipid-mediated transfection of mRNA *The liposome consists of synthetic lipids surrounding the mRNA. It enters the cell through endocytosis where the lipids will facilitate the fusion of the liposome content into the endosome. The endosome will release its mRNA content to the cytoplasm so translation can begin, and the target protein is synthesized. (Figure created with Biorender.com)*

Lipid-mediated transfection also requires some optimizations to achieve high transfection efficiency. For example different ratios of mRNA to lipid reagent shall be tested out to find an optimal ratio for specifically Atlantic salmon fibroblasts (McLenachan et al., 2013).
A study has found that 1.5µg of mRNA and 2µL Lipofectamine 2000 appeared to work best for the transfection experiment conducted in human cells, such as BJ fibroblast (Avci-Adali et al., 2014). Therefore this could then be a starting point for our optimizations of the mRNA to lipid reagent ratios. Additionally, different mRNA to lipid reagent ratios have been proven to give different transfection efficiencies in different cell types (Avci-Adali et al., 2014).
Just as for the Calcium chloride based transfections, different cell seeding densities should also be tested as the confluence plays an important role in lipid-mediated transfection. The impact of confluence varies from cell type to cell type, but in general the confluence of cells at time of transfection should be between 70% and 90% for high transfection efficiency (*Cationic Lipid Transfection*, n.d.). This range of confluence allows the cell-to-cell contact and eases the fusion of liposomes into the membrane.
Incubation time of cells with transfection mixture, usually does not play a role in lipid-mediated transfection. However, it is possible to encounter apoptosis with sensitive cell types when they are incubated in the transfection mixture for too long (Raman, 2016). For that reason it might be necessary to change the media of the cells already 4 to 6 hours succeeding time of transfection (*Lipofectamine 2000*, 2006).

5. Further optimization of the chosen transfection methods

Other than the optimizations steps mentioned in I.B.4, there are additional techniques that can be included to achieve higher transfection efficiency in Atlantic salmon cells. In this study, tRNA supplement and incubation temperature alteration were chosen as additional optimization techniques.

a) Transfer RNA (tRNA)

tRNA are abundant RNA species that are often used as a carrier or a decoy for RNAses (Alberts et al., 2002). In previous studies that used calcium phosphate-mediated transfection of RNA, calf liver tRNA was included in together with mRNA to form calcium phosphate-bound RNA complexes for transfection (Kleinschmidt & Pederson, 1990). However, this study does not report any specific increase in efficiency due to the use of tRNA .
Although research on the impact of tRNA use on transfection efficiency is limited, multiple studies of CRISPR gene editing have demonstrated the efficacy of tRNA supplementation as a useful tool to promote gene editing (Qi et al., 2016; Song et al., 2018).
Based on these factors, it has been hypothesized that the inclusion of tRNA could enhance in some way the transfection efficiency.

b) Incubation temperature

In accordance with the principles of thermodynamics, a decrease in temperature brings liquids to solidification and an increase in temperature brings solids to liquefaction. Lipids in the organism are a form of liquid at the organism's body temperature (*How is the cell membrane affected by temperature?*, 2022). Atlantic salmon, for instance, exhibit optimal cellular functioning at temperatures no higher than 20°C, beyond which survival rates decline precipitously. Lethal temperatures for this species fall within the range of 27°C to 32°C, as has been documented by prior studies. (Elliott & Elliott, 1995; Garside, 1973)

It is widely recognized that the lipids present in the cell membrane of Atlantic salmon are predominantly saturated. This characteristic feature is attributable to the organism's homeoviscous adaptation, which is a cellular response to thermal stress (Ernst et al., 2016). As a result, the cell membrane of Atlantic salmon is notably more rigid, which poses a significant barrier to the successful penetration of foreign nucleic acids (Lopez et al., 2001).

Transfection protocols are often optimized for mammalian cells that thrive at higher temperatures than aquatic organisms. This is why incubation temperature should be taken into consideration, given the differing rigidity of cell membranes, which may impact the efficiency of transfection (Farkas et al., 2001).

Raising the incubation temperature of Atlantic salmon cells can result in an increase in the solubility of phospholipids within the cell membrane. This effect leads to an increase in membrane permeability, which can facilitate the passage of foreign nucleic acids into the cytoplasm (Singh, 2021). Between the optimal and lethal temperature ranges mentioned above, the optimal temperature for improving transfection efficiency is estimated to be around 25°C.

This temperature is thought to promote efficient fusion of liposomes with endosomes, as the cell membrane approaches its melting temperature. Such enhanced fusion of lipids may hypothetically lead to improved transfection efficiency, making an increased incubation temperature a favorable choice.

C. Aim of thesis

The genetic modification of Atlantic salmon requires an efficient transfection method to introduce genetic material into cells. To enable experiments towards developing methodology for induced pluripotency it is essential to deliver nucleic acids to fibroblasts .

Defining an optimised transfection protocol for Atlantic salmon will provide an opportunity to achieve successful reprograming which in turn will allow the pursuit a wide range of scientific questions relevant to Atlantic salmon health and well being.

Therefore, the main focus of this thesis is optimizing exogenous gene expression in Atlantic salmon fibroblasts. The first step is to identify a suitable mRNA transfection method and then optimize it for efficient use in Atlantic salmon. The research objectives involve testing various mRNA transfection methods in Atlantic salmon fibroblasts, followed by several optimization steps to identify the most effective transfection protocol. Finally, the thesis presents a comparison of transfection efficiency in relation to cell viability.

II. Methods and Materials

A. Workflow overview

The transfection of fibroblast started with polymerase chain reaction (PCR) to amplify the gene coding for green fluorescent proteins (GFP). This was used as a reporter gene in transfection. The PCR product was quantified in gel electrophoresis, then cleansed and finally quantified again in Nanodrop. From this PCR product, mRNA was generated with in vitro transcription. The generated mRNA was also dephosphorylated and lastly purified. Following the quantification of mRNA in Nanodrop, different transfection experiments took place. The results of these transfections were analysed through fluorescence microscopy.

B. Fibroblast and HEK cells culture

To be able to conduct any experiments with fibroblasts, the processes of cell culturing is key and need to be respected. Specifically for Atlantic salmon fibroblasts, lack of media change or high trypsin volumes, can easily affect the cells' health.
The fibroblast was developed from Atlantic Salmon's skin cell line and then cultured in culture flasks with appropriate media. Cell culture and experiments were performed in a flow hood, where antiseptic routines were followed, such as spraying hood and gloves with 70% ethanol.

1. Fibroblast

Fibroblast cells were cultivated in 75-cm^2 cell culture flasks in a culture media consisting of Leibovitz's L-15 media without L-glutamine (Sigma-Aldrich, L5520), complemented by 20% fetal bovine serum (FBS) (Sigma-Aldrich, F7524), and 1% Pen-Strep (Gibco, 15140122).
The culturing media for fibroblast did not contain L-glutamine, it has been detected that the cells' growth was faster and just as healthy as the same culturing media with L-glutamine.
Fibroblasts were kept in an incubator at 20°C and the media was changed every 3 days. Changing of media consisted of filtering the old media through a syringe filter, Filtropur S (Sarstedt, 83.1826.001) with a pore size of 0.2 μm, which gives the conditioned media.
Half of the newly changed media consisted of the conditioned media and the other half of new culture media.
The subculture was made whenever the cells were at a high confluence (over 80%) following the below steps:

1) Washing cells with 5mL of Phosphate-buffered saline (PBS)
2) Trypsinization with 1mL of trypLE (Thermo Fisher)
3) Add 9 mL of new culture media.
4) Pipette mix the detached cells with the culture media.
5) Keep a 1:10 to 1:2 of cells in the culture flask and discard the rest of the cells.

6) Add 5 mL of conditioned media to the culture flask and add new culture media to have in total 10mL of media and cell in flask.
7) Incubate at 20°C.

For the 24-well plates that were used under transfection experiments, only new culture media was utilized.

2. Human embryonic kidney (HEK) cells

HEK293 cells were used in this project as control cells before transecting fibroblasts, as they are easy to transfect cells.
These cells were used to check if the synthesised mRNA was working properly and to note to which extent the transfection protocol needs to be optimised.
HEK293 cells were cultivated in 75-cm^2 cell culture flasks in a media consisting of Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, D6429) with 10% FBS (Sigma-Aldrich, F7524), 1% Pen-Strep (Gibco, 15140122), and 2 mM L-glutamine (Thermo Fisher, 25030024). HEK293 cells were incubated at 37°C with 5% CO_2. Cells were passaged every 2 to 3 days, by trypsinization with 1 mL of trypLE (Thermo Fisher) followed by reseeding of 1/3 of the original cell numbers in the fresh culture media pre-warmed to 37°C .

C. DNA template

The GFP sequence used in Polymerase chain reaction (PCR) to generate a template for in vitro transcription was from the pTagGFP2-N plasmid. The DNA plasmid was previously purified in the laboratory and its map is shown in Figure 4. 800ng purified plasmid DNA was used directly in PCR reactions to generate templates for in vitro transcription.

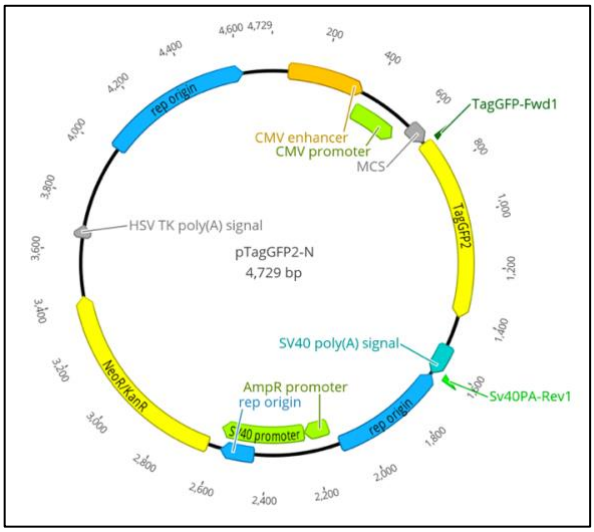

Figure 4 pTagGFP2-N plasmid sequence *The sequence includes the CMV promoter, the origin of replication and gene of interest: TagGFP, The figure is retrieved from Geneious Prime.*

D. Polymerase chain reaction

For in vitro transcription PCR product needs to include a promoter for the RNA polymerase. T7 RNA polymerase was specifically selected for this task due to its extensive usage and its ability to rapidly synthesize RNA. Moreover, T7 RNA polymerase features a compact promoter that can be seamlessly integrated into PCR primers, leveraging its high selectivity. (Tabor, 2001)
PCR amplification was done according to the protocol (ThermoFisher, 2015) for Platinum™ Hot Start PCR Master Mix 2X (Invitrogen) with minor alterations.
The GC enhancer was not included in the PCR reaction as it is optional and was not considered necessary for the reaction that amplifies a relatively short fragment from a low complexity template, such as plasmid DNA.
The primers including the T7 promoter (ref. green sequence in the "Forward primer") and the poly-A tail, used in the reaction, are presented in Table 2, 1μL of a mix containing both primers was used for a 50μL reaction. The reverse primer contains an extended series of nucleotide T repetitions, which serves as the encoding sequence for the poly-A tail that will be added to the mRNA.

Forward primer	ACGTCTTAATACGACTCACTATAGGGGTCGCCACCATGGTGAGCAAGGGCGAG
Reverse primer	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACTTGTACAGCTCGT

Table 2 Forward and reverse primers for PCR The highlighted sequence in green represents the T7 promoter sequence

For the incubation in a thermal cycler, 35 cycles were run to ensure the production of a sufficient yield, the reaction started with the initial denaturation that took place at 94°C for 2 minutes. Each cycle consisted of: first denaturing at 94°C for 30 seconds then annealing at 55°C for 30 seconds and finally extension at 72°C for 1 minute per kb.
Gel electrophoresis was done after the reaction, to ensure a properly run PCR by checking the GFP gene's weight and checking the presence of any primer dimers as their presence can affect negatively in vitro transcription.
PCR clean-up was processed by following the QIAquick PCR Purification Kit (Qiagen, 28104) protocol and then quantified in Nanodrop to note the concentration for in vitro transcription use.

E. mRNA synthesis with In vitro transcription

In vitro transcription was conducted after PCR to make mRNA encoding the chosen reporter gene, green fluorescent protein (GFP), which will be used in transfection to induce protein expression in fibroblast.
This took place by using the HiScribe T7 ARCA mRNA Kit (New England BioLabs Inc (NEB), E2060S), following the kit's protocol Standard mRNA Synthesis (NEB, E2060).
For a 20μL reaction, 10μL of 2X ARCA/NTP mix, 2μL of T7 RNA polymerase mix and 8μL PCR product were assembled. Nuclease-free water was not supplemented to the reaction because the PCR product was added to 20 μL reaction and had a concentration between 70 ng/μL and 100 ng/μL.

Usually, 1µg of DNA template is used for in vitro transcription, but when using PCR product, a range of 0.1 to 0.5µg can be employed for this kit according to the manufacturer's recommendation. The choice of 0.8µL of PCR product was therefore made so that the addition of nuclease-free water could be neglected, and a high yield would be ensured.
The first incubation at 37°C endured 35 minutes and after the DNase treatment, the 5' ends of mRNA were dephosphorylated. The dephosphorylation was made with Quick CIP (NEB, M0525), 2µL was added to the 20µL reaction and incubated at 37°C for 10 minutes.
The Poly(A) tailing reaction was not done, as the reverse primer used in PCR already codes for a poly-A tail.
Finally, mRNA was purified with Monarch RNA Cleanup Kit (NEB, T2050) following its protocol without any alterations other than warming nuclease-free water at 55°C before elution for elution efficiency. Quantification was completed with Nanodrop for the final concentration. The product was then stored in a freezer at -20°C as it is a small aliquot.

F. Methods of transfection

1. Protocols

a) Calcium phosphate transfection

The first chemical transfection tested out was calcium phosphate transfection. The transfection protocol was first optimised for HEK cells, then tested in fibroblast and optimised.
The protocol mainly followed the Calcium Phosphate Transfection Kit protocol (Invitrogen, 250067) but with alterations.

<u>Pre-transfection preparations:</u>

- The cells were seeded in a 24-well plate the night before transfection and cultivated in 0.5 mL culture media. Cells were seeded the night before to ensure that they were at their highest proliferating rate and translation would takeplace at the time of transfection.
- For HEK cells, it was important to vortex the cell suspension between trypsinization and cell counting for seeding, so when they are not clumped and would grow individually instead of in colonies.
- The cell's density was at a range of 25000 to 37500 cells/well to have a confluence under 50% the day of transfection.
- The media in each well was changed with new culture media 3 hours prior to transfection.
- $CaCl_2$ was made fresh weekly, where 0.294g of calcium chloride (Sigma-Aldrich, C4901) was mixed with 1 mL of H_20. This was stored in a fridge at 4°C.
- HEPES Buffer Solution 1X (Gibco, 15630056) was used in $CaCl_2$ transfection and diluted to 1:2 ratio with H_20 beforehand.

- mRNA was kept on ice throughout the transfection and was vortexed and centrifuged before use.
- At the time of transfection, some HEK cells can still be in suspension but if the adhered cells showed enough confluency the media was changed with new pre-warmed media by pipetting it slowly through the well's wall.

Transfection process (for each transfection sample)
- Two Eppendorf tubes of 1.5 mL were labelled A and B
- The appropriate amounts to experiment with of $CaCl_2$ and mRNA were supplemented to tube A and sterile H_20 was used to fill up the total desired volume of transfection.
- The same total volume as tube A of diluted HEPES Buffer Solution was added to tube B.
- The solution in tube A was slowly added to tube B while bubbling the solution in tube B until tube A's solution is depleted.
- This mixture was then incubated at room temperature for half an hour.
- The 24-well plate was then taken out of the incubator and the precipitate was added dropwise all over its respective well.
- The plate was swirled gently and incubated overnight at 20°C for fibroblast and 37°C for HEK cells.
- The next day, the cells were assayed with fluorescence microscopy.

b) Lipid transfection

The second chemical transfection tested out was lipid-mediated transfection. The transfection protocol was optimised for fibroblast and the mRNA was initially tested out in HEK cells to check for proper transfection ability and for no defect with its coding sequence.
Four different lipid reagents had their protocol optimised to determine which reagent and what conditions would give us the most efficiency.
The followed protocols were Lipofectamine® 2000 Reagent (ThermoFisher, 2006) and Lipofectamine® MessengerMAX® Reagent (ThermoFisher, 2015) and Lipofectamine® 3000 Reagent (ThermoFisher, 2016) but with a small alteration.

Pre-transfection preparations:
- Fibroblast was seeded in a 24-well plate the day before transfection and cultivated in 0.5 mL culture media, to ensure that the cells were at their highest proliferating rate and translation is taking place at the time of transfection.
- The cell's density was at a range of 80 000 to 95 000 cells/well to have a confluence between 70% and 90% on the day of transfection.
- The lipid reagents were stored in a fridge at 4°C and stored in an ice box throughout the transfection.

- mRNA was stored in an ice box throughout the transfection and was vortexed and centrifuged before use.

Transfection process: (for each transfection sample)

1. In a first Eppendorf tube, mRNA was diluted in 50µL of Opti-MEM I 1X (Thermo Fisher, 31985-070) for Lipofectamine 2000.
   For Lipofectamine MessengerMAX and Lipofectamine 3000, mRNA was diluted in 25 µL of Opti-MEM I 1X.
2. This dilution was vortexed for 2 to 3 seconds.
3. In a second Eppendorf tube, the lipid reagent was then diluted in 50µL of Opti-MEM I 1X for Lipofectamine 2000 and 25µL of Opti-MEM I 1X for Lipofectamine MessengerMAX and Lipofectamine 3000.
4. For Lipofectamine 2000, this second dilution was incubated at room temperature for 5 minutes.
5. The diluted mRNA was then implemented into the diluted lipid reagent. For Lipofectamine 3000, the diluted mRNA was combined with P3000 before combining it with the diluted Lipofectamine 3000.
6. The diluted Lipofectamine 2000 was incubated for 20 minutes at room temperature, the diluted Lipofectamine MessengerMAX was incubated at room temperature for 5 minutes and the diluted Lipofectamine 3000 for 10 minutes.
7. Lastly, the lipid complexes were supplemented to each well and the plate was rocked back and forth to gently mix the complexes into the culturing media.
8. The next day, the cells were assayed with fluorescence microscopy.

For the TransIT reagent, the protocol followed was *Trans*IT®-LT1 Transfection Reagent (Mirus, n.d.).
The general workflow of these protocols is described in Figure 5.
A small alteration was tested out from the followed protocol for Lipofectamine 2000, where the transfection volume of 100µL could affect the efficiency as the other lipid reagents followed a protocol that used a total volume of 50µL instead of 100µL. For that reason, an experiment was conducted where the volume of Opti-MEM to be diluted in for the mRNA and Lipofectamine 2000 was halved to 25µL.

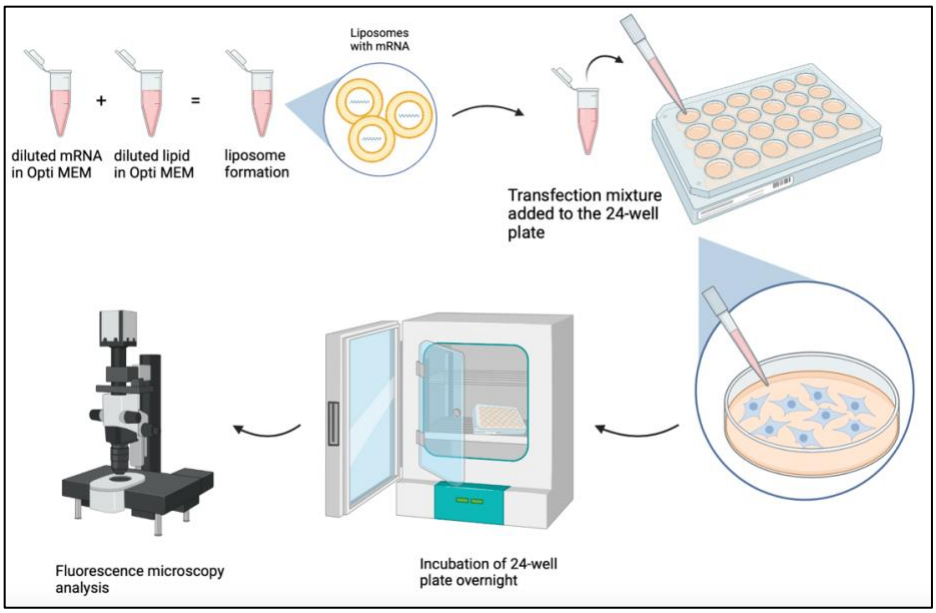

Figure 5 Workflow of lipid-mediated transfection. *(Figure created with Biorender.com)*

2. Optimizations for Calcium phosphate-mediated transfection

Transfection optimization was achieved by setting up a matrix in a 24-well plate to determine optimal conditions for transfection efficiency and cell viability.

a) mRNA to $CaCl_2$ ratio

In the first round in HEK cells, a range of 0.2 to 0.5µg mRNA and a range of 12 to 14% of the solution A of $CaCl_2$ was used. This was done in order to see if low amounts of mRNA would be able to transfect our cells, as it is what we would need in theory to transfect the Yamanaka factors, or if a higher amount is necessary.
This first range of $CaCl_2$ that was tested was based on the fact that the Calcium Phosphate Transfection Kit protocol (Invitrogen, 250067) uses the solution containing of 12.9% of $CaCl_2$. Therefore, a range that goes both slightly lower and higher to this amount seemed to be appropriate for the first try. In a second round, much higher $CaCl_2$ concentrations were tested. They were in range of 14 to 17% of $CaCl_2$ in solution In initial test HEK cells were transfected to see if it had a better effect on transfection with a slightly higher mRNA range than the previous one going from 0.5 to 0.9µg. In a third round, it was determined to stay in a range of 13 and 14% $CaCl_2$ of solution A but much higher values of mRNA were also tried out including 1 to 1.8µg.
As several papers used an mRNA value of 2µg with other transfection methods, a new round of transfection was completed with 1.5, 2.0, and 2.5µg mRNA complexed with 14% $CaCl_2$ in solution A
For a final transfection in HEK cells, a total of 2µg of mRNA and 14% $CaCl_2$ in solution A was used. In fibroblasts, transfections were done with mRNA amounts in a 2 to 2.5µg range and 13

and 14% $CaCl_2$ in solution A. All these rounds of transfection, included a negative control where solution A consisted of only $CaCl_2$ and H_2O to be able to control how $CaCl_2$ affects autofluorescence the cells.

b) Transfection mixture volume

Different protocols of calcium phosphate transfection have different transfection volumes for the same plate. Consequently, the volumes of transfection mixtures were also experimented with to find the optimized volume, in case different volumes affect efficiency in different ways.
The Calcium Phosphate Transfection Kit protocol (Invitrogen, 250067) came with an important guideline stating that the final volume of solution A should not be higher than 10% of the culturing media's volume.
In that protocol, solution A was 3% of the culturing media volume therefore it was determined to start with the same volume but also test a range that is close to this volume.
A first try was made with the same percentage in HEK cells and then an optimisation of this was performed.
In the first round of transfection, solution A had a volume representing 4 to 6% of the culture media.
Later, with the same mRNA to $CaCl_2$ ratios, solution A represented 8 and 10% of the culture media to compare with 4 and 6% of the culture media.
From that, different wells of fibroblast were transfected with solution A being 6, 8 and 10% of the culturing media's volume.

c) Seeding density

The cell density is an important factor for achieving high transfection efficiency as noted in several studies. The different seeding densities that were tested with were based on ThermoFisher's recommendation for seeding density in a 24-well plate. For fibroblast, different densities were tested to find optimal confluence at the time of transfection, so this could be used in future experiments. In a 24-well plate, each well was seeded with a different density. The next day confluency was then calculated using automated "Cell culture" counter on the EVOS M5000 Imaging System.
Cell counts for calculation of seeding density was done by trypan blue staining using Countess 3 automated cell counter (Invitrogen).

d) tRNA application

As reported in the literature, addition of tRNA can increase transfection efficiency while allowing use of lower amounts of mRNA. Therefore, it has been decided to include tRNA to some of the optimization steps for calcium phosphate transfection.

The tRNA (Sigma-Aldrich, R8508) used in optimization experiments is derived from baker's yeast (S.cerevisiae) and provided from the manufacturer at a concentration of 1μg/μL.
tRNA has been used in a first round, in both HEK cells and Salmon fibroblasts, according to the method described in *RNA processing and ribonucleoprotein assembly studied in vivo by RNA transfection* (Kleinschmidt & Pederson, 1990).
Subsequently this procedure has been changed to simply adding the desired amount of tRNA to solution A in fibroblasts. For this, the different mRNA-to-tRNA ratios listed in Table 3 were tested in order to see which ratio gave the highest efficiency.

Table 3 mRNA to tRNA ratios transfected in HEK cells and fibroblast with Calcium phosphate

Cell type	mRNA:tRNA ratio	mRNA(μg)	tRNA(μg)
HEK	1:0.25	2.0	0.5
HEK	1:0.5	2.0	1.0
HEK	1:1	2.0	2.0
Fibroblast	1:5 Values inspired from (Kleinschmidt & Pederson, 1990)	2.0	10.0
Fibroblast	1:1.3	1.5	2.0
Fibroblast	1:1	2.0	2.0
Fibroblast	1:0.25	2.0	0.5
Fibroblast	1:3	1.0	3.0
Fibroblast	1:2	1.0	2.0

3. Optimizations for Lipid-mediated transfection

a) mRNA to reagent ratio

Based on published protocols, 1.5μg of mRNA and 2μL Lipofectamine 2000 was selected as the starting point of the mRNA to lipid reagent ratio optimization. It was also the first ratio tested for transfection in fibroblasts. Subsequently it was further optimised by both increasing and decreasing the mRNA mass and lipid reagent volume.
Notably the original recommendations from the reagent's manufacturer for mRNA to lipid reagent ratio were 1:2 and 1:3. These ratios were also tested in experiments with Lipofectamine 2000. Later, higher amounts of mRNA were transfected with the same ratios of mRNA to lipid reagent to determine if that increases the amount of expressed protein.
For Lipofectamine MessengerMAX, TransIT, and Lipofectamine 3000, experiments were conducted in a similar way where the standard ratios that are recommended by the manufacturer were tested first. Then, the amount of mRNA and the volume of lipid reagent were increased to check which amount would give the best efficiency.

b) Seeding density

In a 24-well plate, 120 000 cells/well density gives full confluence (*Useful Numbers for Cell Culture*, n.d.). This number is for human HeLA cells, and the densities suggested by ThermoFisher can differ from cell type to cell type. Full confluence for Atlantic salmon fibroblast was then estimated to be around 100 000 cells/well.
Thereby, it was necessary to find an optimal seeding density for lipid-mediated transfection. A test on a 24-well plate was performed, where each well was seeded with a different number of cells with the goal of getting approximately 80% to 90% confluency the next day.
Just as it was done for Calcium phosphate transfection (II.F.2.c), the day after seeding the cell culture, confluence in each well was qauntified with the "Cell culture" counter on the EVOS M5000 Imaging System.
Cell counts for calculation of seeding density was done by trypan blue staining using Countess 3 automated cell counter (Invitrogen).

c) tRNA implementation

As tested with in $CaCl_2$ transfection, the addition of tRNA could increase the lipid transfection efficiency. Therefore, tRNA was also included in optimization of the lipid based transfection method with the goal of increasing efficiency.
The tRNA (Sigma-Aldrich, R8508) used in optimization experimetns is derived from baker's yeast (S.cerevisiae) and provided from the manufacturer at a concentration of 1μg/μL.
For transfection tRNA was simply added together with mRNA at the Opti-MEM dilution step. Different mRNA-to-tRNA ratios from Table 4 were tested to see which ratio gave the most efficiency.

Table 4 Overview of different mRNA to tRNA ratios transfected in fibroblast with lipid

mRNA:tRNA ratio	mRNA(μg)	tRNA(μg)
1:1	2.0	2.0
1:2	1.0	2.0
1:3	1.0	3.0
1:4	1.0	4.0
1:5	1.0	5.0
1:2	2.0	4.0
1:10	0.2	2.0
1:4	0.5	2

d) Incubation temperature

The plates containing salmon fibroblasts were usually incubated at 20°C. In some instances, the incubation temperature was increased to 25°C. In those cases, the transfected plate was put in a zip-lock bag and stored in a different incubator set at 25°C for the desired period of time.

4. Cell viability post-transfection

Successful introduction of nucleic acids into a living cell can significantly affect its viability. For every transfection, cell viability was monitored to make sure that a specific condition does not kill cells even if there is efficient transfection. The cell viability was then checked at three timepoints: 24 hours, 48 hours and 72 hours post-transfection. All images of transfection results presented in the thesis are taken 22 to 24 hours post transfection unless stated otherwise. Figures with several images show different areas of the same transfected well.

When the transfection efficiency was estimated to be over 10% with fluorescence microscopy, the cells were trypsinized and gone through cell count on Countess 3 automated cell counter (Invitrogen) with a GFP cube installed to additionally count both the total cell numbers and the numbers of GFP positive cells representing successful transfection events.

In addition, an experiment was done using several different controls to determine what transfection components drive cytotoxicity. This led to further optimization of either reagent or mRNA amount would be done.

The negative controls were the following, where different volumes and masses were employed:

- Transfection mixture with mRNA only
- Transfection mixture with tRNA only
- Transfection mixture with lipid reagent only

III. Results

The results presented in this chapter, are not of the quantitative type as this project is focused on determining if it is possible to successfully transfect mRNA into Atlantic salmon's fibroblasts. Here the experiments were designed to identify which method and what reagent ratios gave us the most live transfected cells. When there was a high enough efficiency: cell viability and percentage of transfection efficiency were also considered.

A. Calcium phosphate transfection

The protocol for calcium phosphate-mediated transfection was first optimised in HEK cells to then be further optimised in fibroblasts. This was done in order to make it easier to determine which specific reagents were to be optimised.

1. Transfection with different mRNA to $CaCl_2$ ratios in HEK cells

The first round, with a range of 0.2 to 0.5µg mRNA and a range of 12 to 14% of the solution A was $CaCl_2$ in "Experiment A" done in HEK cells. The results of Experiment A were negative as there were no cells that expressed GFP indicating that they were not transfected.
From this and other studies, it was deduced that a higher amount of mRNA could be necessary. Optimising the proportion of $CaCl_2$ seemed also necessary as several papers and protocols were recommending different ratios of $CaCl_2$.
The wells were transfected with different concentrations of $CaCl_2$: 14%, 15%, 16% and 17%, all combined with 0.6, 0.7, 0.8 µg mRNA. In addition and both 15% and 17% of $CaCl_2$ were tested with 0.9 µg of mRNA. In this experiment again, the results were negative as most of the cells did not survive the transfection. Nevertheless the cells that were transfected using 14% $CaCl_2$ solution had notably less cell death.
In a third round, "Experiment C" 13 and 14% $CaCl_2$ in solution A was initially used with 0.5, 0.6, 0.7µg mRNA. Subsequently, then with much higher amounts of mRNA: 1, 1.2, 1.5 and 1.8 µg, were tested. The results of Experiment C were mostly negative. One well had 2 bright green cells indicating that they were transfected even though the efficiency was extremely low. This well's cells were transfected with 1.5µg mRNA and 14% $CaCl_2$ of solution A. The total transfection mixture in this well was of volume 60µL. The duplicate of this condition confirmed the transfection success as it also had a few green cells.
A final transfection made in HEK cells "Experiment D" was done with 2µg mRNA and 14% $CaCl_2$ of solution A, where the total transfection mixture was 100µL. This gave even more transfected cells than Experiment C, as we can see in Figure 6.

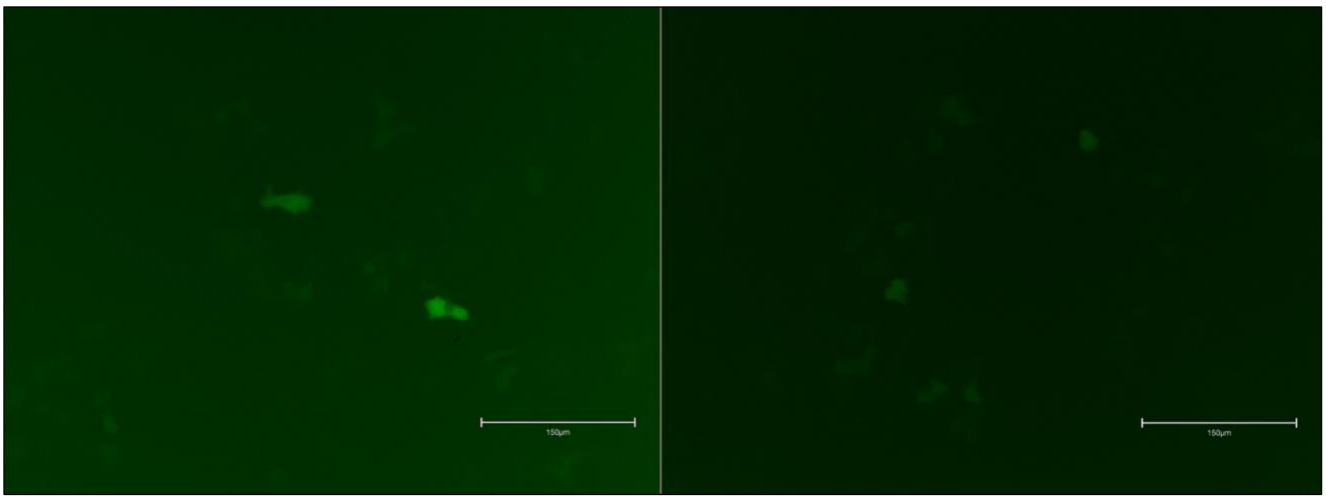

Figure 6 Transfected HEK cells with 2µg GFP mRNA and 14% CaCl2 of solution A in a 100µL transfection mixture

2. Optimizing transfection method with cell density

For Experiments A and B, the HEK cells were seeded at a density of 50 000 cells/well, this gave a confluency higher than 50%, between at 60% to 70% at the time of transfection. The seeding density was then decreased by half to 25 000 cells/well. In Experiment C when several transfected cells were onserved, the confluence at the time of transfection was around 40% (± 5%). In fibroblasts, a different seeding density was necessary to get suitable confluence, as they have a different growth rate from HEK cells. In a 24-well plate, the different densities from VI.Appendix A were tested out to find the right seeding density to give a confluence of 40-50% at the time of transfection.
It is important to note that the EVOS M5000 Imaging System gives a confluence percentage with some bias to it, it is usually higher than what it is according to manual inspection.
The results presented in VI.Appendix A show that a suitable seeding density is at 37 500 cells/well which gives around 40% confluence, so it was determined to henceforth use this density for the calcium phosphate transfections in salmon fibroblasts.

3. Optimizing transfection method with transfection volume

As seen in III.A.1, Experiment C, the same ratios of mRNA to CaCl2 were tested out in different volumes, 60µL and 40µL of the transfection mixture. The transfection was successful with a 60µL transfection mixture whereas the same ratios and conditions in a 40µL transfection mixture did not give any transfected HEK cells.
After noticing that a higher volume of transfection mixture gave transfected cells, it was decided to try the same mRNA to $CaCl_2$ ratios but with even higher volumes.
Experiment D from III.A.1 was done with a 100 µL transfection mixture which worked even better.

Increasing the volume of the total transfection mixture in HEK cells resulted in higher transfection efficiency. However, in fibroblasts, this optimization did not generate any transfected cells, even though they were transfected with 60, 80 and 100 µL transfection mixture as those in III.A.4.

4. Transfection with different mRNA to CaCl2 ratios in Atlantic salmon fibroblasts

In the initial tests, CaCl2 does not cause any additional autofluorescence in fibroblast in fibroblasts. In "Experiment E", the fibroblasts were transfected with the optimal ratios and volumes identified in HEK cells, which were 1.5 and 2.0µg mRNA. A higher amount of 2.5µg mRNA was also tested. For all mRNA amounts a 14% $CaCl_2$ in solution A was used. For the transfection different volumes were tested: 60, 80 and 100µL as those were the volumes that gave us some efficiency in HEK cells.
In addition, some wells were transfected with the same amounts of mRNA but with 13% CaCl2 of solution A in 80 and 100µL transfection mixtures.
The results of Experiment E were negative, no cell was successfully transfected. The cell viability was high and only some cell debris formed 24 hours post-transfection.

5. Transfection efficiency after adding tRNA in HEK cells and Atlantic salmon fibroblasts

Inclusion of tRNA in the transfection mixture did not work as anticipated therefore the optimization of the published method (Kleinschmidt & Pederson, 1990) had to be done first in HEK cells and then tested again in fibroblasts.
After inclusion tRNA in the transfection mixture, the transfection efficiency was highly improved in HEK cells. In "Experiment F" the HEK cells were transfected with a range from 0.5 to 2 µg of tRNA in a mixture consisting of 2 µg mRNA and 14% $CaCl_2$ of solution A.
The cells that were transfected with 2µg of tRNA showed the highest efficiency while a ratio of mRNA to tRNA of 1:0.5 did not work as efficiently and 1:0.25 showed some efficiency.
The number of transfected cells was increased as illustrated in Figure 7 and Figure 8 in comparison to the tRNA-free transfection from Figure 6.

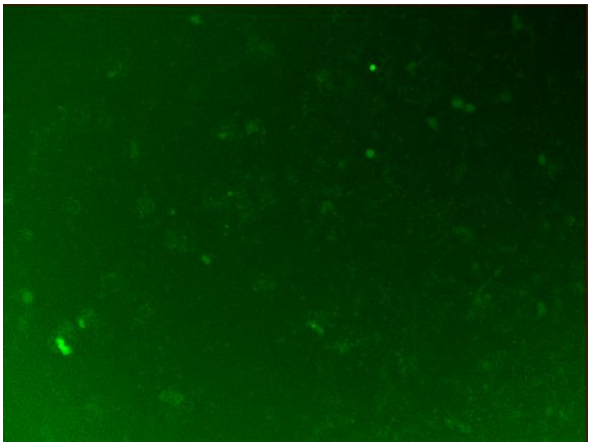

Figure 7 HEK cell transfected with 2µg mRNA, 0.5µg tRNA in combination with 14% Calcium chloride

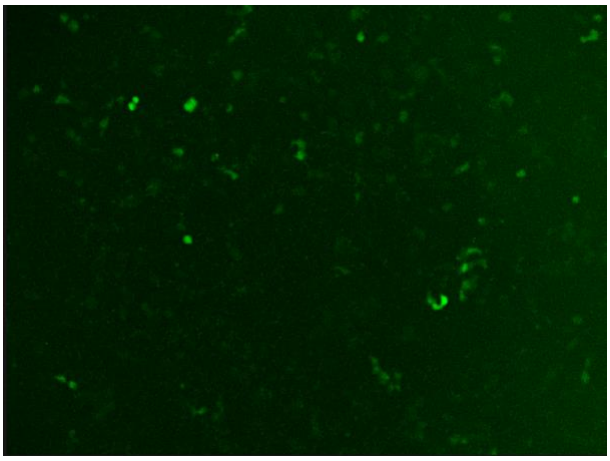

Figure 8 HEK cell transfected with 2µg mRNA, 2µg tRNA in combination with 14% Calcium chloride

Incorporation of tRNA into mixtures at the same ratios and amounts that efficiently transfected HEK cells, when used for transfection of fibroblasts did not produce any GFP positive cells.

B. Lipid-mediated transfection

1. Positive control of mRNA in HEK cells

Before starting the experiments on lipid-mediated transfection, a pilot test was done in HEK cells. Lipid-mediated transfection has been extensively used in HEK cells in contrary to $CaCl_2$-mediated transfection, so optimization of the method was not necessary. A single transfection was run to check the efficiency of the mRNA for cell transfection. HEK cells were transfected with 0.5µg mRNA and 0.75µL Lipofectamine 2000. This ratio has been recommended by the manufacturer on their official website (*Lipofectamine 2000*, 2006).
Figure 9 shows on the left the HEK cells that were successfully transfected when lit by GFP excitation wavelength, and on the right the same field is lit by the white light.

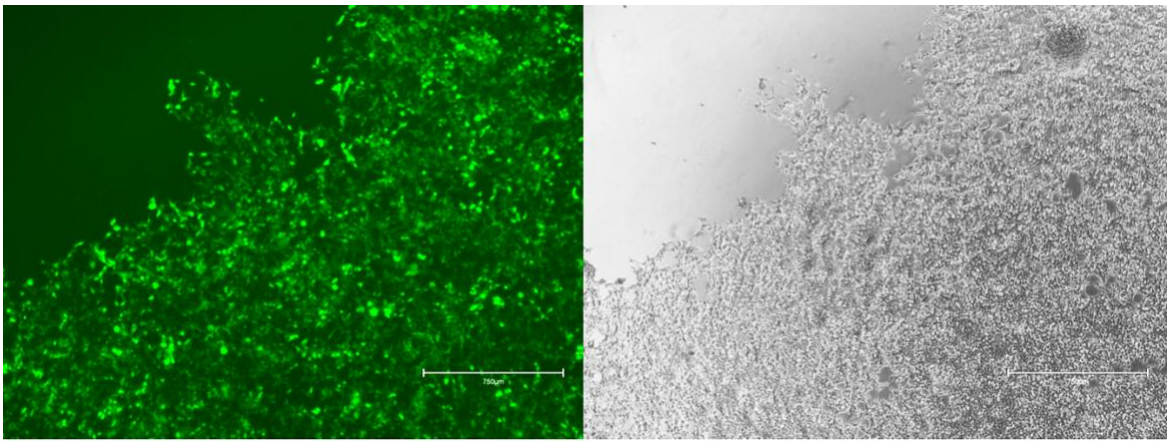

Figure 9 HEK cells transfected with 0.5µg mRNA and 0.75µL Lipofectamine 2000 The images are under 4X magnification

2. Transfection with different mRNA-to-lipid reagent ratios

In the first attempt, "Experiment 1" the reagent ratios shown in Table 5 were used to transfect the fibroblasts.
In addition to this, a negative control was set up to see if Lipofectamine 2000 alone causes autofluorescence in fibroblasts.
The negative control did not show any fluorescence coming from the cells themselves, but cell debris would light up under 488nm light, this is noticeable in Figure 10 and was taken into account when interpreting the experimental results.

Table 5 The different mRNA to Lipofectamine 2000 ratios tested under Experiment 1

mRNA:lipid reagent ratio	mRNA(µg)	Lipofectamine 2000(µL)
1:3	1	3
1:1.6	1.25	2
1:2	1.25	2.5
1:1.3	1.5	2
1:1.6	1.5	2.5
1:3	1.5	4.5
1:4	1.5	6
1:1.25	2	2.5
1:2	2	4
1:3	2	6

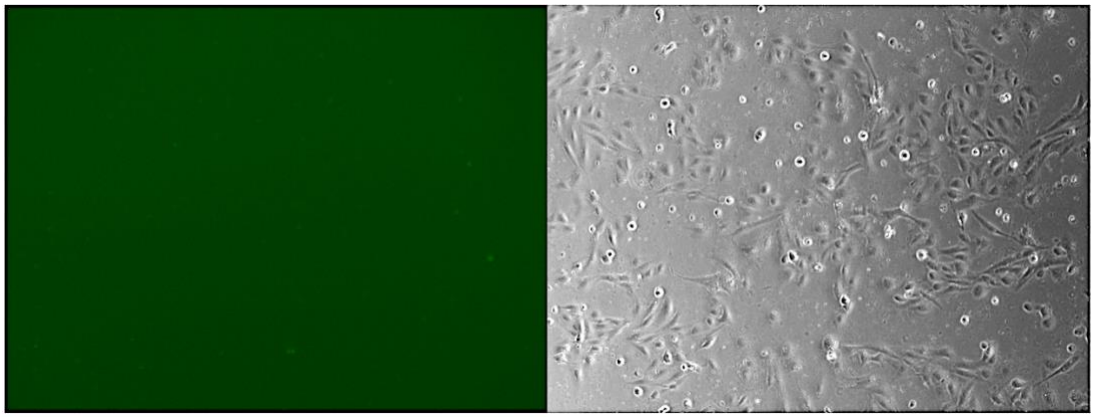

Figure 10 Negative control with Lipofectamine 2000 alone transfected in Atlantic salmon fibroblast *The cell debris lights up weakly as shown on the left image , the images are under 4X magnification*

Experiment 1 resulted in some transfection successes and some failures. The wells that had very low transfection success meaning a pair or so of transfected cells, were the cells transfected with 1.25µg mRNA in combination with 2.0µL Lipofectamine 2000, in addition to those that were transfected with 1.25µg, 1.5µg and 2.0µg mRNA in combination with 2.5µL Lipofectamine 2000. Higher transfection rates, estimated at around 10% by analysis of the microscope images, was found in cells that were transfected with 2.0µg mRNA in combination with 3.0µL Lipofectamine 2000 (Figure 11), and cells that were transfected with 1.5µg mRNA in combination with 2.0µL Lipofectamine 2000 (Figure 12).

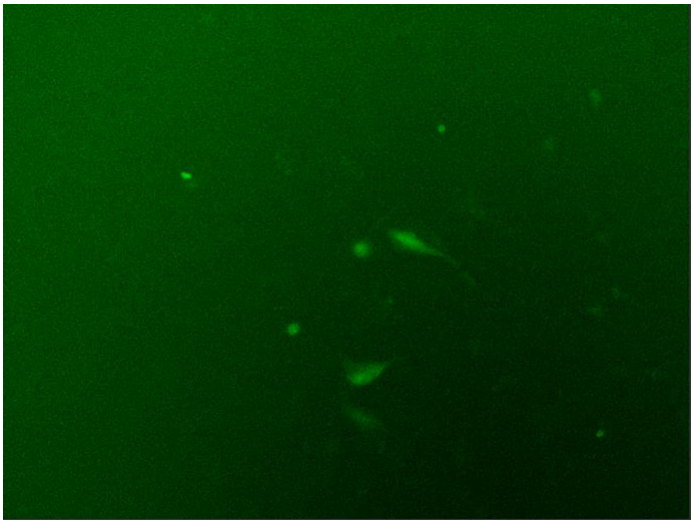

Figure 11 Fibroblast transfected with 2µg mRNA and 3.0µL Lipofectamine 2000 *The image is under 4X magnification*

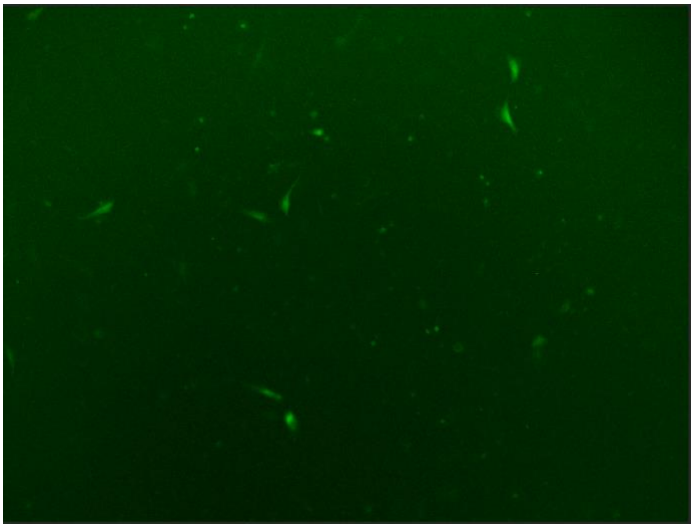

Figure 12 Fibroblast transfected with 1.5µg mRNA and 2µL Lipofectamine 2000 The image is under 4X magnification

3. Optimizing transfection efficiency with seeding density and time of transfection

When it comes to seeding time prior to transfection, different timepoints were tested. For the same mRNA to lipid reagent ratio, the cells were transfected 24 hours after seeding and a different plate was transfected 48 hours after seeding using the same ratio of reagents. This gave different results that were observed in Experiment 2.

In "Experiment 2", two reagents were evaluated with several mRNA to lipid reagents ratios. The ratios in Table 6 were tested out with Lipofectamine 2000 and the ones in Table 7 with Lipofectamine MessengerMAX. The two first ratios in Table 6 were replicated from Experiment 1 as a positive control to verify the experiment's results.

As 2.0µg mRNA seemed to have worked well in Experiment 1, this mass was tested out with different Lipofectamine 2000 volumes to evaluate the ratio giving the best efficiency.

Table 6 The different mRNA to Lipofectamine 2000 ratios tested under Experiment 2

mRNA:lipid reagent ratio	mRNA(µg)	Lipofectamine 2000(µL)
1:1.3 (Positive control)	2	3
1:2 (Positive control)	1.5	2
1:2	2	4
1:2.5	2	5
1:3	2	6

Table 7 The different mRNA to Lipofectamine MessengerMAX ratios tested under Experiment 2

mRNA:lipid reagent ratio	mRNA(µg)	Lipofectamine MessengerMAX(µL)
1:2	0.5	1.2
1:3	1	1.2
1:4	2	1.2
1:1.3	1.5	2

Experiment 2 resulted in no transfected cells at all, except for the well that was transfected with 2.0µg mRNA in combination with 5.0µL Lipofectamine 2000 which yielded only one transfected cell, as shown in Figure 13.

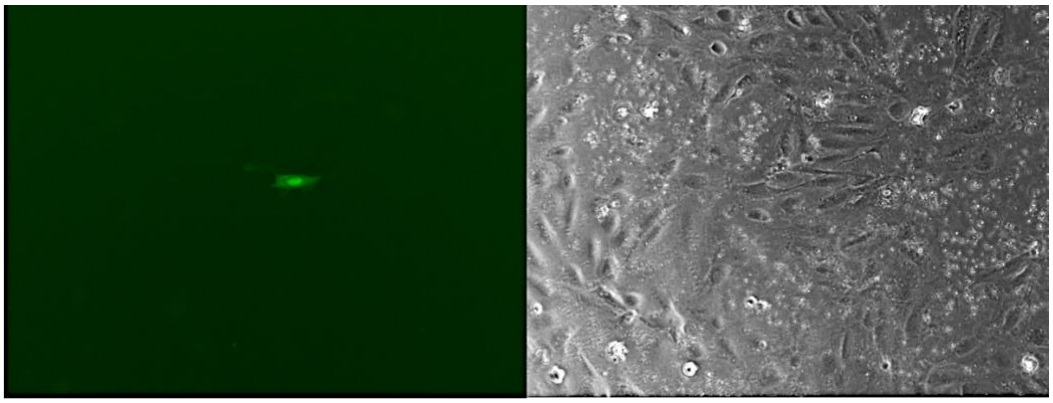

Figure 13 Cells transfected with 2.0µg mRNA with 5.0µL Lipofectamine 2000 The images are under 10X magnification

The positive controls from Table 6 did not give any transfected cells in Experiment 2, therefore assuming the other tested ratios to be non-efficient would be a biased assumption.
What differed from Experiments 1 and 2 was the transfection time, in Experiment 1 the cells were transfected less than 24 hours post-seeding and in Experiment 2 the transfection was conducted 48 hours post-seeding. From these results, it was concluded that it is necessary to perform transfection no more than 24 hours post-seeding.
Later, a pair of transfection experiments were completed by adhering to the timeframe for transfection post-seeding, but the efficiency was not improved as much as desired even if different mRNA to lipid reagents ratios were tested out including the positive controls. The cells were very confluent 48 hours post-seeding, which seemed to affect the transfection efficiency as it was quite low and again assuming the transfection ratios to be non-efficient would be a biased assumption.
Thus, in addition to transfection time, the seeding density seemed to also need optimization, as the cells have been so far seeded at a range from 107 500 to 95 000 cells/well for lipid-mediated transfection. In this instance, different seeding densities were tested in a 24-well plate, the density giving approximately an 80% confluency 24 hours post-seeding was 75 000 cells/well as shown in VI.Appendix A . From the results in VI.Appendix A , an optimal seeding density was determined to be between 90 000 and 75 000 cells/well. All subsequent experiments had a seeding density between 82 500 cells/well and 87 500 cells/well.

4. Optimizing mRNA to lipid reagent ratio with different reagents

In "Experiment 3", the cells were seeded with a seeding density of 82 500 cells/well and transfected 22 hours post-seeding with confluence around 70% at the time of transfection.
Lipofectamine 2000, Lipofectamine MessengerMAX and TransIT, were evaluated to determine which reagent gives the best efficiency and at what optimal ratio.
First, the manufacturer's recommendations for Lipofectamine MessengerMAX and TransIT were followed and the ratios for Lipofectamine 2000 were determined by Experiment 1 results in III.B.2. Lower amounts of mRNA were used as it was hypothesised that too much RNA could be detrimental to the cells.
Table 8 shows the mRNA-to-lipid reagents ratios that were transfected, "Well ID" in Table 8 indicates the number of the well on the 24-well plate which is later used to refer to the transfected ratio.

Table 8 The mRNA-to-lipid reagents ratios transfected under Experiment 3 A) shows ratios with Lipofectamine 2000, B) shows ratios with Lipofectamine MessengerMAX and C) shows ratios with TransIT

A)

mRNA:lipid reagent ratio	mRNA(µg)	Lipofectamine 2000(µL)	Well ID
1:1.3 (Positive control)	1.5	2	A1
1:1.6	1.5	2.5	A2
1:3	1.5	4.5	A3

B)

mRNA:lipid reagent ratio	mRNA(µg)	Lipofectamine MessengerMAX(µL)	Well ID
1:1.5	1	1.5	B1
1:2	1	2	B2
1:2.5	1	2.5	B3
1:2	1.5	3	B4
1:2	1.0 (+ 2.0µg tRNA)	2.0	B5
1:2.5	1.0 (+ 3.0µg tRNA)	2.5	B6

C)

mRNA:lipid reagent ratio	mRNA(µg)	TransIT(µL)
1:2	1	2
1:3	1	3
1:4	1	4
1:1.25	1.5	2
1:2	1.5	3
1:2	2	4

24 hours post-transfection, Atlantic salmon fibroblasts transfected with TransIT reagent did not have any GFP positive cells. The cell viability was not affected by TransIT as cells continued

regular growth. Unexpectedly, Lipofectamine 2000 also had low transfection efficiency, only a pair of the cells in A2 expressed GFP. At the same time, it was noted that cell viability was especially low when 4.5µL of Lipofectamine 2000 was transfected in the cells.
Out of all reagents, Lipofectamine MessengerMAX showed best transfection efficiency which was nevertheless very still low. In well B1(ref. Table 8), there were hardly any cells that expressed GFP as shown shown in Figure 14.

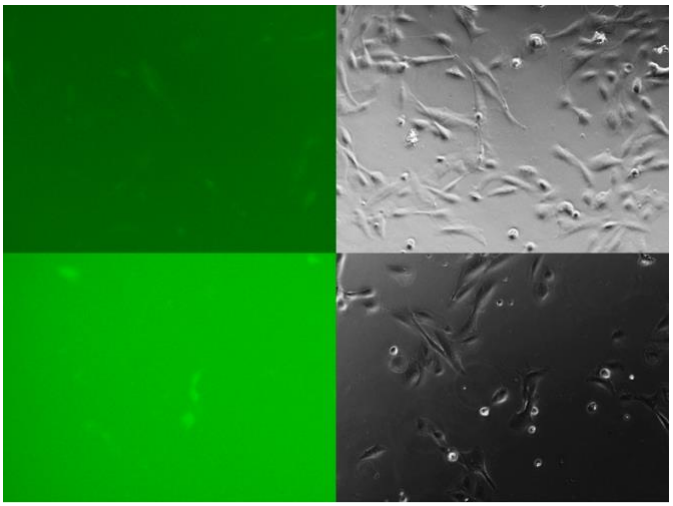

Figure 14 Fibroblast transfected with 1µg mRNA and 1.5µL Lipofectamine MessengerMAX The images on the left show the B1 well (Table 8) under green fluorescent light and on the right the corresponding image under transparent light

When it comes to the transfected cells in B2 and B3 (ref. Table 8), there was not much difference between them, at most a few cells were transfected (ref. Figure 15 for B2 and ref. Figure 16 for B3).

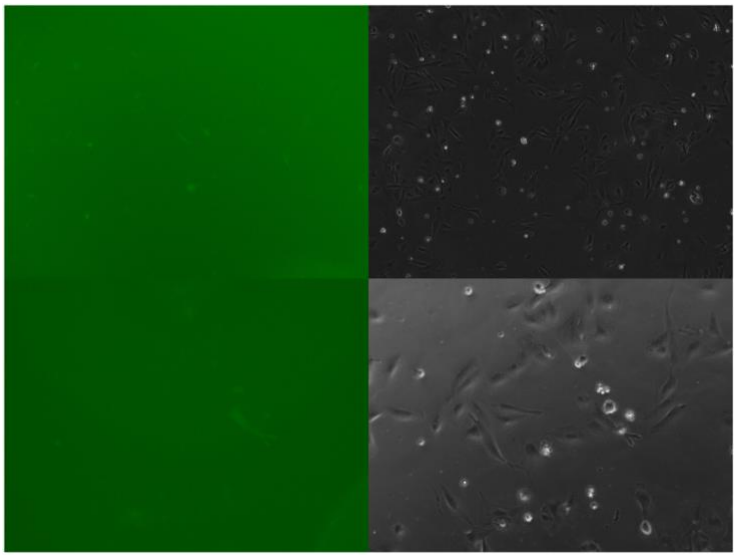

Figure 15 Fibroblast transfected with 1µg mRNA and 2µL Lipofectamine MessengerMAX The images on the left show the B2 well (Table 8) under green fluorescent light and on the right the corresponding image under transparent light. The images at the top row are under 10X magnification and below under 20X magnification.

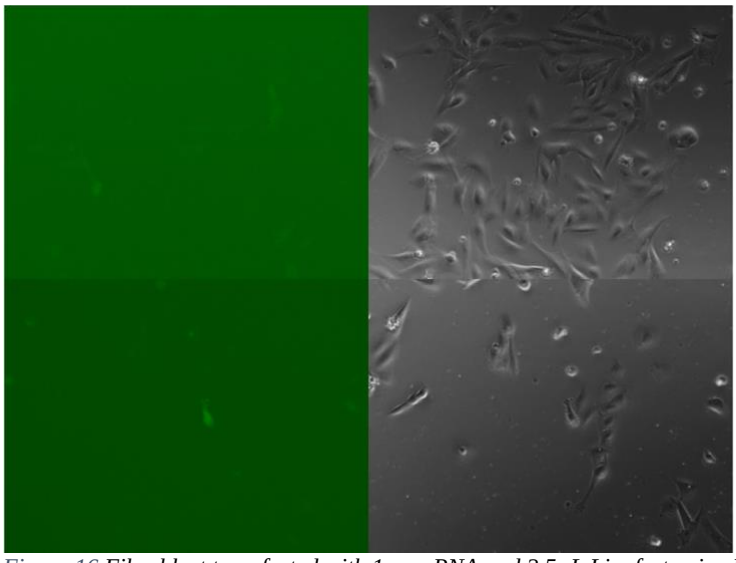

Figure 16 ***Fibroblast transfected with 1µg mRNA and 2.5µL Lipofectamine MessengerMAX*** *The images on the left show the B3 well (**Table 8**) under green fluorescent light and on the right the corresponding image under transparent light. The images are under 20X magnification.*

In B4 (ref. Table 8), no cells were expressing GFP, but the cell death was significantly high here again.

5. Optimizing transfection efficiency with tRNA implementation and incubation temperature

For the transfection ratios with Lipofectamine MessengerMAX in Experiment 3 III.B.4, two of the wells had tRNA added to their transfection mixture to compare the efficiency with this lipid reagent. Figure 17 shows B5 (ref. Table 8) that was transfected with 1µg mRNA, 2µg tRNA and 2µL of Lipofectamine MessengerMAX. Figure 18 shows B6 (ref. Table 8) that was transfected with 1µg mRNA, 3µg tRNA and 2.5µL of Lipofectamine MessengerMAX. As observed Lipofectamine MessengerMAX with mRNA alone (ref. Figure 15 and Figure 16) did not have as high of the transfection efficiency as when tRNA was included (ref. Figure 17 and Figure 18), where there was a noticeable improvement. Notably the cells that were transfected with 2.5µL of Lipofectamine MessengerMAX seemed to have low cell viability already 24 hours post-transfection.

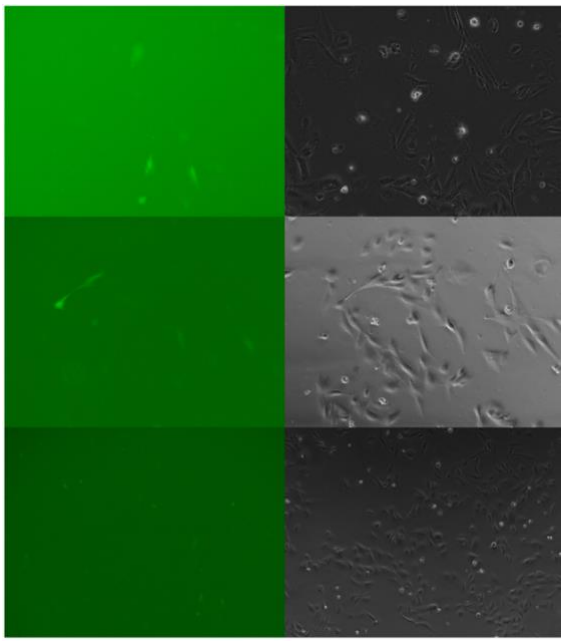

Figure 17 Fibroblast transfected with 1µg mRNA, 2µg tRNA and 2µL Lipofectamine MessengerMAX *The images on the left show the B5 well (**Table 8**) under green fluorescent light and on the right the corresponding image under transparent light. The images at the top and middle row are under 20X magnification and below under 10X magnification.*

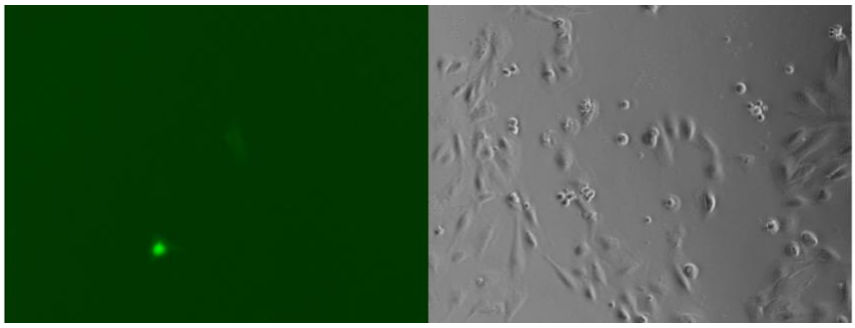

Figure 18 Fibroblast transfected with 1µg mRNA, 3µg tRNA and 2.5µL Lipofectamine MessengerMAX *The image on the left shows the B6 well (**Table 8**) under green fluorescent light and on the right the corresponding image under transparent light. The image is at 20X magnification.*

In a second plate under Experiment 3 (ref. III.B.4), a well was transfected with the earlier defined positive control ratio: 1.5µg mRNA with 2µL of Lipofectamine 2000. This was incubated at 25°C for 22 hours. These conditions resulted in higer transfection efficiency was higher than the positive control that was incubated at 20°C.

The efficiency was estimated at around 10% with the fluorescence images from the microscope, as shown in Figure 19.

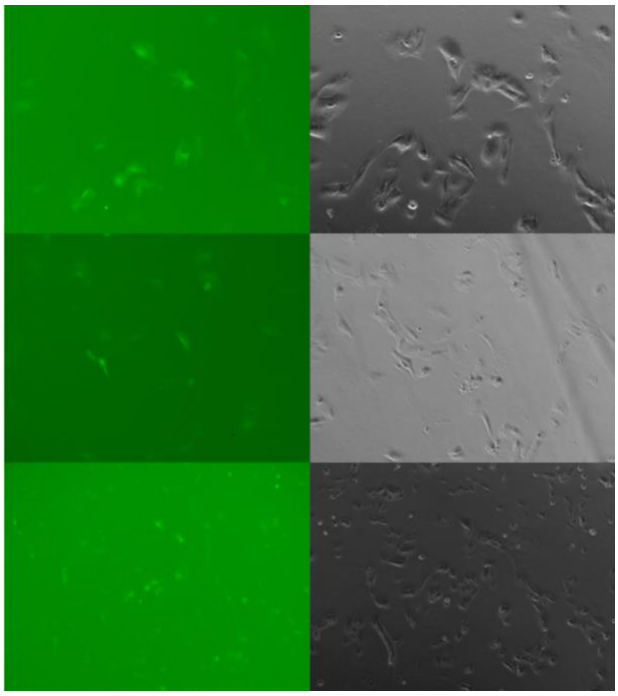

Figure 19 Fibroblast transfected with 1.5μg mRNA and 2μL Lipofectamine 2000, incubated at 25℃ for 22 hours The images at the top and middle are under 20X magnification and at the bottom under 10X magnification

Overnight incubation at 25°C resulted in similarly high efficiency. Under these conditions GFP positive represented 4% of the total number of alive cells. In "Experiment 4" tRNA was included in addition to 25°C incubation to test if Lipofectamine 2000 transfection is also improved under conditions thested with Lipofectamine MessengerMAX. The cells were seeded at a density of 87 500 cells/well and were transfected 22 hours post-seeding.
The two ratios of mRNA to tRNA from Table 9 were transfected in the cells in combination with Lipofectamine 2000.

Table 9 mRNA to tRNA ratios transfected under Experiment 4

mRNA:tRNA ratio	mRNA(μg)	tRNA(μg)	Lipofectamine 2000(μL)	Well ID
1:2	1	2	3	A5
1:3	1	3	3	A6

Based on analysis of microscope images, shown in Figure 20 and Figure 21, the transfection efficiency for A5 (ref.Table 9) was estimated to be at 15% and for A6 (ref.Table 9) to be at 20%. These transfected cells were afterwards counted using automated Countess 3 cell counter and 10% of the alive cells from A5 (ref.Table 9) expressed GFP while only 3% of the alive cells from A6 (ref.Table 9) did.

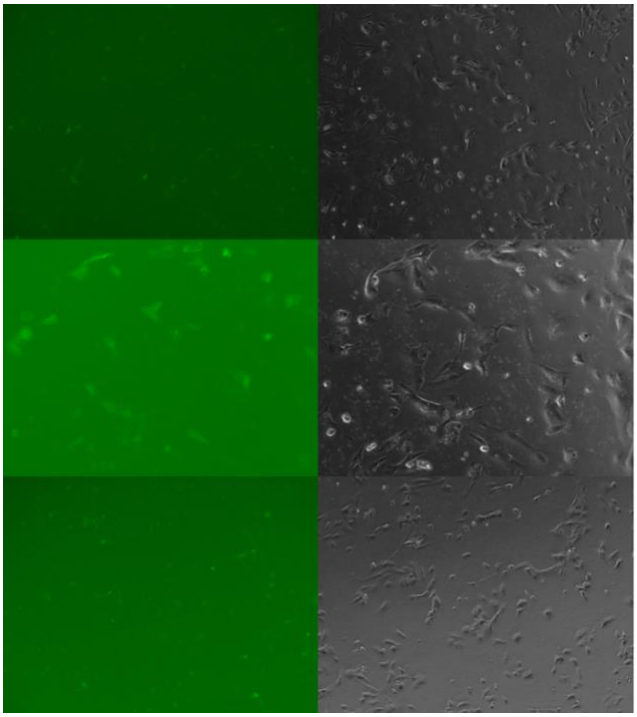

Figure 20 Fibroblast transfected with 1μg mRNA, 2μg tRNA and 3μL Lipofectamine 2000, incubated at 25℃ for 22 hours *The images show the well A5 (ref.**Table 9**), at the top and bottom are images under 10X magnification and at the middle under 20X magnification*

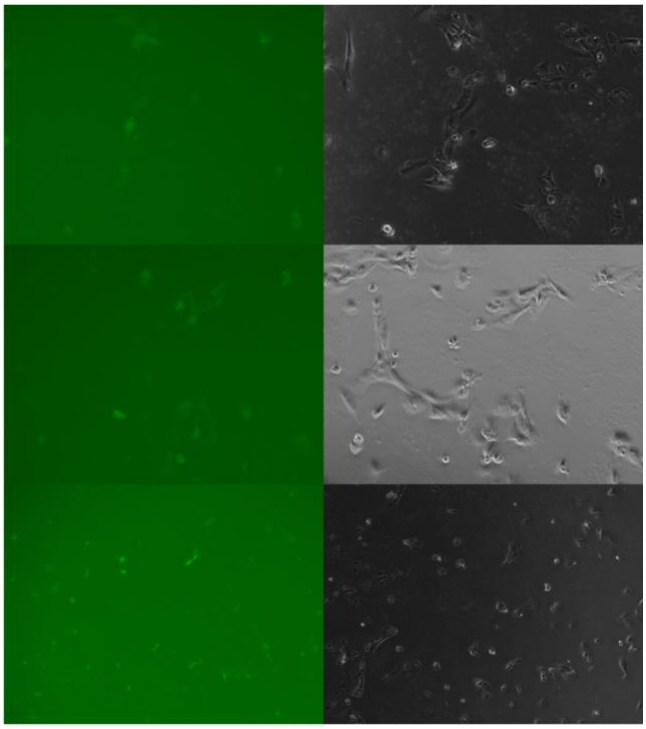

Figure 21 Fibroblast transfected with 1μg mRNA, 3μg tRNA and 3μL Lipofectamine 2000, incubated at 25℃ for 22 hours *The images show the well A6 (ref.**Table 9**), at the top and middle are images under 20X magnification and at the bottom under 10X magnification*

"Experiment 5" was set up with the ratios in Table 10 to investigate further refine the results from both Experiment 3 (ref. III.B.3) and 4 (ref. III.B.4) and see if other ratios can improve transfection efficiency.
Lower amounts of mRNA were also used in Experiment 5, to test the impact on transfection efficiency and cell viability. The cells were transfected 22,5 hours post-seeding, and seeding density was at 86 000 cells/well, which gave a confluence of 80% at transfection time. The 24-well plate was incubated at 25°C for 22 hours.

Table 10 mRNA to tRNA ratios transfected under Experiment 5 with Lipofectamine 2000 and MessengerMAX

Lipofectamine type	mRNA:tRNA ratio	mRNA(µg)	tRNA(µg)	Volume of reagent (µL)	Well ID
2000	1:4	1.0	4.0	3.0	A1
2000	1:4	0.5	2.0	3.0	A2
2000	1:10	0.2	2.0	3.0	A3
2000	X	X	2.0	2.0	A4
2000	X	X	1.0	2.0	A5
2000	1:3	1.0	3.0	2.0	B1
MessengerMAX	1:4	0.5	2.0	2.0	B2
MessengerMAX	1:2	1.0	2.0	2.0	B3

A1 from Table 10 had high cell death, and only a few cells were transfected (ref. Figure 22). A2 (ref. Table 10) produced a good number of transfected cells (ref. Figure 23) but not as much as A5 from Table 9.
While A3 (ref. Table 10) did have a few transfected cells (ref. Figure 24) but the transfection efficiency was not as high as A2 (ref. Table 10).

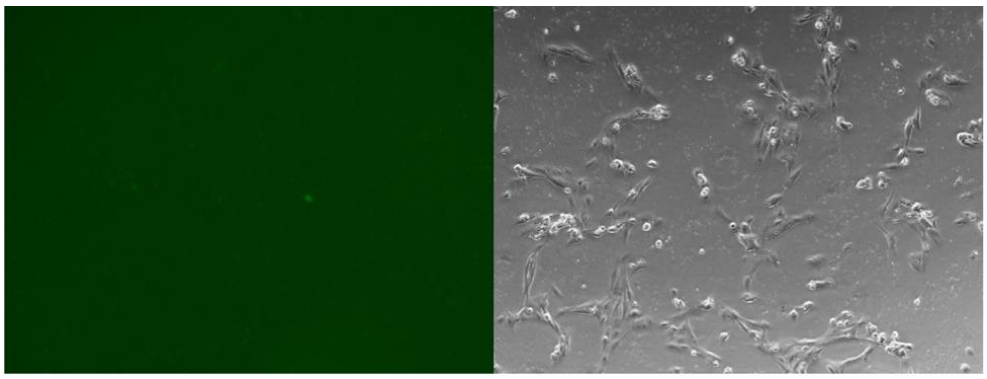

Figure 22 Fibroblast transfected with 1µg mRNA, 4µg tRNA and 3µL Lipofectamine 2000, incubated at 25°C for 22 hours The images show the well A1 (ref.Table 10), the images are under 10X magnification

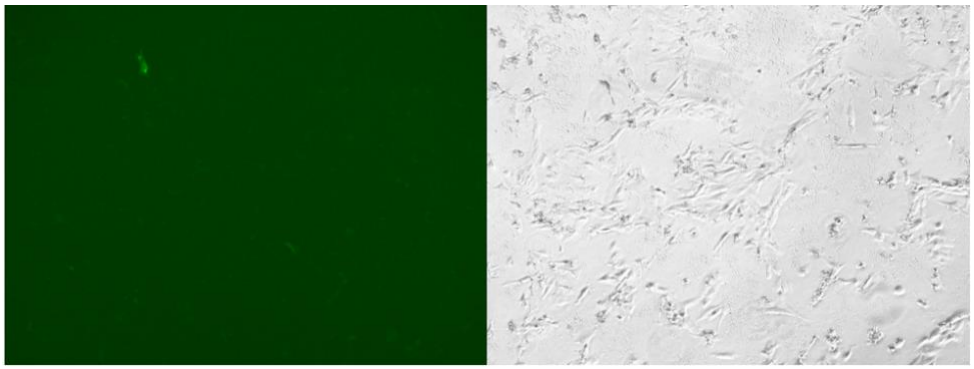

Figure 23 Fibroblast transfected with 0.5μg mRNA, 2μg tRNA and 3μL Lipofectamine 2000, incubated at 25℃ for 22 hours The images show the well A2 (ref.Table 10), the images are under 10X magnification

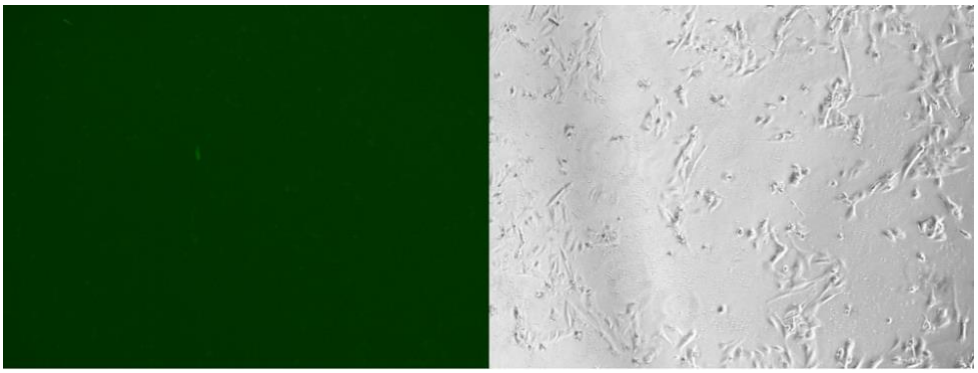

Figure 24 Fibroblast transfected with 0.2μg mRNA, 2μg tRNA and 3μL Lipofectamine 2000, incubated at 25℃ for 22 hours The images show the well A3 (ref.Table 10), the images are under 10X magnification

When it comes to A4 and A5, those were negative controls to test if tRNA had any effect on cell viability in combination with a lipid reagent. The cells were not that much affected, only some cells died after 24 hours from transfection.

B1(ref.Table 10) was transfected with 50μL for the total volume of transfection mixture, as an alteration to the original protocol to investigate if the efficiency would increase if the same volume was employed as in transfection with Lipofectamine MessengerMAX. The well had fewer transfected cells than A6 from Table 9.
Transfection with Lipofectamine MessengerMAX was less efficient than Lipofectamine 2000 when a low mass of mRNA is employed, in B2 (ref.Table 10) barely a few cells were transfected as shown in Figure 25.
B3 (ref. Table 10) was a duplicate of the transfection done in B5 (ref.Table 8) to compare the transfection efficiency when the cells are incubated at 25°C and 20°C. In this case, B3 (ref. Table 10) had no cell expressing GFP.

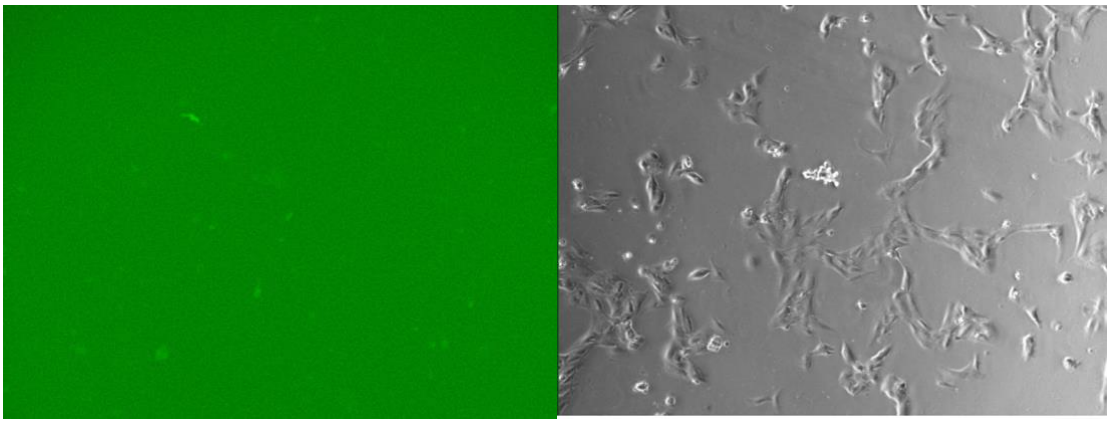

Figure 25 Fibroblast transfected with 0.5μg mRNA, 2μg tRNA and 2μL Lipofectamine MessengerMAX, incubated at 25℃ for 22 hours The images show the well B2 (ref.Table 10), the images are under 10X magnification

6. Negative controls for cause of high cell death and last optimizations

Cell viability has been low under conditions of the Experiment 3. In Experiment 3 (ref. III.B.3), the cells that were transfected with 2.5μL Lipofectamine 2000 showed some cell death already 24 hours post-transfection and those that were transfected with 4.5μL Lipofectamine 2000 showed a large number of dead cells 24 hours post-transfection. To determine which component is responsible for increase in cell death a 24-well plate was set up, with different reagents tested as shown in Table 11. From this, it was concluded that high volumes of the lipid reagent negatively affect cell viability, henceforth the transfection experiments conducted had lower volumes of Lipofectamine.

Table 11 Overview of negative controls tested out for reason of low cell viability

Negative control	**Condition transfected**	**Result – 42 hours**
Lipid reagent	3μL Lipofectamine 2000	Very high number of cell death
Lipid reagent	4μL Lipofectamine 2000	Very high number of cell death
Lipid reagent	2μL Lipofectamine MessengerMAX	High number of cell death
mRNA	2μg mRNA	Low number of cell death
Lipid reagent	0.75μL Lipofectamine 3000	Low number of cell death
Lipid reagent	1.0μL Lipofectamine 3000	Low number of cell death
Lipid reagent	1.25μL Lipofectamine 3000	High number of cell death
Lipid reagent	1.5μL Lipofectamine 3000	Very high number of cell death
Negative control	**Condition transfected**	**Result – 24 hours**
Lipid reagent	2μL Lipofectamine 2000	Low number of cell death
Lipid reagent	1.5μL Lipofectamine 2000	Low number of cell death
Lipid reagent	2.5μL Lipofectamine 2000	Moderate number of cell death

"Experiment 6" was thus performed to test out ratios (ref. Table 12) that could ensure higher cell viability and potentially higher efficiency, by additionally using Lipofectamine 3000.
Plate A had cells seeded transfected 26 hours post-seeding, where the resulting confluency at time of transfection was 90%. Plate B had cells also transfected 26 hours post-seeding, where the resulting confluency at time of transfection was 70%.

Each transfection condition in Plate A had its duplicate, and all duplicates had their media changed 4 hours post-transfection and incubation period at 25°C was of 22 hours. Likewise for Plate B, were all conditions had media change 4 hours post-transfection but incubation at 25°C for only 4 hours. This was performed as it was hypothesized that a shorter incubation period of the transfected cells could decrease the amount of cell death.
Unfortunately, this experiment did not yield any transfected cells, only one green cell was achieved that got transfected with 1µg mRNA 2µg tRNA and 1µL Lipofectamine 3000 from Plate B. From Plate A, one green cell was achieved which was also transfected with the same ratios.

Table 12 mRNA to tRNA ratios transfected under Experiment 6 with Lipofectamine 2000 and Lipofectamine 3000

Plate A:

Lipofectamine type	mRNA: reagent ratio	mRNA(µg)	tRNA(µg)	Volume of reagent (µL)
2000	1:1	1.0	2.0	1.0
2000	1:1.5	1.0	2.0	1.5
2000	1:2	1.0	2.0	2.0
2000	1:2.5	1.0	2.0	2.5
2000 (positive control)	1:3	1.0	2.0	3.0
3000	1:0.75	1.0	2.0	0.75
3000	1:1	1.0	2.0	1.0
3000	1:1.25	1.0	2.0	1.25
3000	1:1.5	1.0	2.0	1.5
3000	1:1.25	1.0	X	1.25

Plate B:

Lipofectamine type	mRNA:reagent ratio	mRNA(µg)	tRNA(µg)	Volume of reagent (µL)
3000	1:1	1.0	2.0	1.0
3000	1:1.25	1.0	2.0	1.25
3000	1:2	1.0	2.0	2.0
3000	1:1.5	1.0	2.0	1.5

IV. Discussion

Atlantic salmon is an aquatic specie of great economic and ecological significance in Norway. Nevertheless there has not been much progress in basic research using specifically Atlantic salmon cells. Moreover there is a lack of validated methodology for salmon cell transgenesis and genetic manipulation. Up to now, DNA transfection is the only approach that have been tested and used in Atlantic salmon. There has been no reports so far for using mRNA transfection . The aim of this thesis research was to identify a specific method that can be used to transfect mRNA in Atlantic salmon fibroblasts and to determine its efficiency. The ultimate goal is to optimize mRNA transfection protocols make them sufficiently efficient for further use in inducing pluripotent stem cells.
The results of this research indicate that Calcium phosphate-mediated transfection is not achievable in Atlantic salmon. On the other hand, lipid-mediated transfection shows higher efficiency in these hard-to-transfect cells, and with some optimizations the efficiency can be further increased. However, lipid reagents appear to be toxic in Atlantic salmon cells reducing transfected cell viability. This would create a problem for inducing fibroblast into pluripotent stem cells, as the cell needs to survive long enough to undergo deprogramming.

This chapter discusses first the results of calcium phosphate transfection, then the results of lipid transfection in combination with further optimizations that can be done. The chapter then ends with the discussion of alternative transfection methods that could be tested to further optimize a transfection protocol in Atlantic salmon fibroblasts.

A. Calcium chloride transfection

The results presented in III.A indicate that calcium phosphate transfection has moderately good efficiency of introducing mRNA in HEK cells but cannot transfect salmon cells. There are three key variables in this protocol that are crucial to transfection efficiency.
First, transfecting low amounts of mRNA with calcium phosphate resulted in no transfected cells. Some transfection was achieved in later experiments where higher amounts of mRNA ranging from 1.5 μg to 2.0μg were used. From this, it was concluded that at least 2.0μg of mRNA is necessary for efficient calcium phosphate transfection. Low amounts of mRNA not failing to produce any transfected cells could be due to the cells not being able to respond when encountering high amounts of mRNA showing that efficiency is limited by the size of the nucleic acid information.
In addition to this, changing the mRNA amounts was not sufficient by itself for increasing transfection efficiency. The results of Experiment B and C (ref. III.A.1) where different ratios of CaCl2 of the solution A were tested out ranging from 14% to 17% were tested and suggests that 14% is the concentration proportion of calcium-chloride which was confirmed in Experiment C where this concentration was compared to 13% of CaCl2.

Collected data suggests that high CaCl2 concentrations in the transfection mixture is causing cytotoxicity with 14% has showing the least toxicity for HEK cells in Experiment B.

Other factors that played a role in optimizing calcium phosphate transfection, was the cell density and transfection volume. After running several experiments, transfection efficiency was shown to be conditional on the cell density at the time of transfection.Different seeding densities were used for the same transfection mixtures, which gave different transfection results. Efficiency was not achieved when the cells were 60% to 70% confluent at the time of transfection, while starting with 40% confluence indeed gave transfected HEK cells.
The effect of the volume of the total transfection mixture was evaluated as well. The efficiency of the transfection seemed to also correlate with the total volume, when the transfection mixture was 100 µL there was most efficiency in HEK cells. Although this led to increase in number of transfected HEK cells, it did not lead to any improvement in efficiency in fibroblasts. This data suggests that optimization of the transfection mixture volume is necessary for each specific cell line. Transfection mixture being a tenth of the culture media volume, suggests that cytotoxicity might be the cause of low efficiency. However, even with lower volumes of transfection mixtures, there are no Atlantic salmon cells transfected.

tRNA was an additional reagent introduced product combined into the calcium phosphate transfection mixture, it increased efficiency in HEK cells. This supports the theory of increased efficiency by including tRNA that has been reported in the literature. A ratio of 1:1 of mRNA to tRNA worked the most efficiently, suggesting that an equilibrium between these two types of nucleic acids is optimal. Additionally, this ratio also proves that high amounts of tRNA does not affect the cells negatively, meaning that increasing the tRNA proportion can be used as further optimization.

Far from expectations, Atlantic salmon cells were not transfected no matter which variation of the method for $CaCl_2$-mediated transfection was tested. Changes in neither the ratio of mRNA to CaCl2, the seeding density, transfection volume, nor even tRNA were able to produce transfected fibroblasts. This could be due to an incompatibility between the fibroblasts cell membrane and calcium chloride complexes. For example, CaCl2 could be causing disruption of the cell membrane's pH level. It is known that lipids of the cell membrane have acido-basic properties giving them the ability to be affected by pH, and the change can induce lipid deformation (Angelova et al., 2018). If the pH of the cell membrane is altered from the normal 7-value, the permeability of the membrane is decreased, making the passage of molecules into the cytoplasm more difficult (Singh, 2021). Calcium chloride is an acidic salt, and acids have the opportunity to lower the pH level by incorporating hydrogen ions (*Buffers, pH, Acids, and Bases*, n.d.). Therefore, there is room to speculate that the CaCl2 reagent was not helping the mRNA to pass the cell membrane because it alters the pH level of the fibroblast membrane.
The pH level of the HEPES buffer that was combined with Solution A, was measured once and found to be at 7.1 which is the norm. The culture media did also not have its pH measured; this

could be critical for transfection efficiency as this was observed in a study where primary hippocampal neurons were transfected with calcium phosphate and the pH value of both the HEPES saline buffer and culture media were kept consistent for successful transfection (Sun et al., 2013). Additionally, it is important to note that in this study HEPES buffer solution was used and not HEPES buffer saline 2X, as mentioned in other protocols (*Calcium Phosphate Transfection Kit*, 2011; Raman, 2016). This could have played a role in the inefficiency of transfection observed in fibroblasts. The difference between the buffer saline and the buffer solution is that the buffer saline contains natrium chloride and disodium phosphate in addition to 50 mM HEPES (Raman, 2016), while the buffer solution does not contain these salts. The HEPES buffer solution was diluted using a ratio of 1:2 to approximate a concentration closer to 50 mM of HEPES in the HEPES buffer saline. This could have also decreased the pH of the diluted HEPES buffer solution, as the pH was initially at 7.1 and adding water would have slightly decreased it (*How does dilution affect the pH of a basic solution?*, 2022). However even with only using diluted HEPES buffer solution, it was still possible to get transfected HEK cells and not transfected salmon fibroblasts, so it did not affect the mRNA complex formation. Therefore, it is recommended for future studies to test out transfection with 2µg mRNA and 14% of CaCl2 in solution and combine it with 50µL of HEPES buffer saline 2X in fibroblasts, to ensure that the inefficiency is not originating from the type of HEPES buffer.

Other improvements can be done to try achieving transfected fibroblast cells, such as performing Dimethyl Sulfoxide (DMSO) shock or glycerol shock post transfection. Generally, it is a recommended procedure for higher efficiency depending on the concerned cell type as these products are toxic for cells (*Calcium Phosphate Transfection Kit*, 2011), therefore it was not tried out in the first place as Atlantic salmon cells fibroblast are already sensitive. In any way it is important to optimize the DMSO to be supplemented, it has been previously shown that 2% DMSO caused massive cell death while 1% DMSO increased transfection efficiency (Villa-Diaz et al., 2010). This increase in efficiency is not yet well understood but it can be explained with 2 reasons, the first being the cell membrane getting chemically altered which facilitates the uptake of the genetic information (*Calcium Phosphate Transfection Kit*, 2011). Secondly, it could be that DMSO is stabilizing the cell membrane just like when it is used to freeze cells (Melkonyan et al., 1996).

An additional improvement of the calcium phosphate transfection for RNA delivery is using a nanoparticle system developed through sonification. It has been done in a study where the goal was to achieve high miRNA delivery to tumors without damage induced to the RNA nanoparticles, the most successful method was found to be generating calcium phosphate-based nanoparticles of 700 nm through ultrasonic pulverization combined with PEG blending. (Wu et al., 2021)

Often while generating calcium phosphate precipitate making, aggregates can form under the crystallization (Wu et al., 2021) which will affect the transfection efficiency as they can lead to cytotoxicity (Sun et al., 2013). The AFA sonification treatment comes in to stop the aggregation of carbone apatite particles which gives super carbonate apatite of 10 to 20 nm in size. These super

carbonate apatite nanoparticles are then loaded with small RNAs to deliver in cells. In addition to sonification PEG blending is utilized to control the size of the nanoparticles. (Wu et al., 2021)
The data of the calcium phosphate transfection show that calcium chloride itself does not cause cytotoxicity or cell death. Another study has also shown that calcium phosphate transfection does not cause as much death in mesenchymal stem cells as with lipid-mediated transfection, and electroporation (Lo et al., 2019). Therefore, this method could possibly have great potential to induce pluripotent stem cells from Atlantic salmon fibroblast, if the problem of mRNA cytoplasmic entry is overcome.

B. Lipid mediated transfection

Lipid mediated transfection has shown better results in Atlantic salmon fibroblast than those observed with Calcium phosphate transfection has proven.

1. Optimizing the method with mRNA-to-reagent ratios and implementing tRNA

From the results of Experiment 1 (ref. III.B.2), it is clear that there were more transfected cells when the transfection mixture contained 1.5µg mRNA in combination with 2.0µL Lipofectamine 2000 than 1.25µg mRNA in combination with 2.0µL of the same lipid reagent. This data is suggesting that there could be correlation between the mass of mRNA transfected and the number of transfected cells. However, based on the other findings of this experiment, where 2.5µL Lipofectamine 2000 was transfected in combination with higher amounts of mRNA, there were not more transfected cells than with the 1.5µg mRNA and 2.0µL Lipofectamine 2000 mixture. In addition to this, 2.0µg mRNA combined with 3.0µL Lipofectamine 2000 gave a good number of transfected cells but less than what was achieved with 1.5µg mRNA and 2.0µL Lipofectamine 2000, as shown in Figure 11 and Figure 12. This provides a new insight into the relationship between the transfection efficiency and the combination of both mRNA amounts and lipid reagent volume, suggesting that it is not the amounts of mRNA alone that affects the number of transfected cells. This suggests that specific amounts of mRNA are dependent on the proportional volume of lipid reagent to transfect Atlantic salmon cells.
The results of Experiment 3 are the most informative for optimization of the mRNA to lipid reagent ratio. These results contradict the findings of Avci-Adali et al. (2014) as higher volumes of Lipofectamine than what was recommended by the manufacturer did not give much higher efficiency as they have found in their study. Besides, these high volumes are compromising the cell viability, giving a high number of dead cells after 24 hours.
This is most likely due to the cytotoxicity effect these lipids can easily induce when present in larger quantities, this is valid for the use of large quantities of mRNA as well (*Examples of Cells Transfected Successfully*, n.d.).

TransIT in Experiment 3 did not transfect any cells at all no matter the ratio of mRNA to lipid reagent, which indicates that the reagent is only functional with DNA as the manufacturer claims. However, the cells were unaffected by incubation with TransIT-LT1 suppletion, suggesting that the reagent might not be toxic to Atlantic salmon fibroblast. This data demonstrates that it is worth a try to either use another type of TransIT reagent to transfect mRNA or use TransIT-LT1 to transfect DNA as other options to keep high cell viability post-transfection, which was proven to be an issue with Lipofectamine in Experiment 3 and 5.

Our study has also shown that Lipofectamine MessengerMAX is not as efficient as Lipofectamine 2000 in transfecting Atlantic salmon fibroblast. Generally, the number of transfected cells achieved was much inferior to those with Lipofectamine 2000. This contradicts the theory of Lipofectamine MessengerMAX being a better reagent for this study as it is targeted for mRNA transfection in primary cells. Furthermore, the cells were not thriving after addition of Lipofectamine MessengerMAX was supplemented, as they were even more sensitive to high volumes of the reagent compared to Lipofectamine 2000.

However, Lipofectamine 3000 has the potential for increasing transfecting efficiency, given that it has exhibited less fatal effects on Atlantic salmon cell viability compared to Lipofectamine 2000 (ref. III.B.6). This finding implies that exploring further optimization of the transfection ratio using Lipofectamine 3000 could prove beneficial, particularly as it was able to successfully transfect a cell under conditions where the positive control failed.

It is important to note that the discussion on mRNA transfection efficiency is constrained by the unavailability of composition information for Lipofectamine 2000 and Lipofectamine MessengerMAX. This information gap poses a challenge to determining the efficiency differences between the two reagents for mRNA transfection, thus limiting the scope and depth of this discussion.

The suboptimal performance of Lipofectamine MessengerMAX in fibroblast can possibly be attributed to the incubation temperature. Despite previous research into its effectiveness, there were no studies of the impact of lower temperatures on performance of this reagent. It is plausible that MessengerMAX was developed specifically for mammalian cells that are known to thrive at much higher temperatures than a range of 20°C to 25°C, thus accounting for the observed lack of efficacy in Atlantic salmon cells.

It has been well established that poikilothermic animals, such as the Atlantic salmon, possess the ability to perform homeoviscous adaptation (HVA) due to their inability to regulate their body temperature. This mechanism facilitates the maintenance of cellular functions in response to changes in the environmental temperature. (Ernst et al., 2016)

Specifically, in situations where there is an increase in temperature, the distance between the cell membrane lipids also increases. As a result, the HVA mechanism comes into effect by saturating the lipids' fatty acids to preserve the cellular membrane viscosity. (Yoon et al., 2022)

Therefore, another hypothesis for the suboptimal performance would be due to the differences in the cellular lipid makeup between Atlantic salmon cells and mammalian cells, as they have the HVA mechanism, making the lipid formulation of MessengerMAX is more suitable for mammals.

Incorporating tRNA improved the transfection efficiency of lipofection in Atlantic salmon fibroblast compared to calcium phosphate transfection, where it did not help.
The results of Experiment 3 and 4 are indeed in line with the hypothesis discussed in the introduction on the potential effect of tRNA (ref.I.B.5.a). It was hypothesised that implementing tRNA the transfection mixture could increase the transfection efficiency; this was the case in lipid mediated transfection. There was first a noticeable improvement in efficiency under Experiment 3 for the cells transfected with both mRNA and tRNA compared to only mRNA transfection, in combination with Lipofectamine MessengerMAX.
Interestingly, the cells transfected with 2µg tRNA and 2µL of Lipofectamine MessengerMAX were greater in number than the cells transfected with 3µg tRNA and 2.5µL of the same lipid reagent (ref. Figure 17 and Figure 18). This difference could mostly be due to two factors: the higher amount of tRNA and the higher volume of Lipofectamine. This was tested further under Experiments 4 and 5, and the efficiency improvement was confirmed with the different ratios tested (ref. Table 9 and Table 10).
The highest efficiency achieved in this study was when the cells were transfected with 1µg mRNA, 2µg tRNA and 3µL of Lipofectamine 2000. This transfection mixture achieved 10% efficiency according to Countess 3 and 15% through microscopic imagery analysis. The difference between efficiency percentages can be due to a too high volume of trypsin used to detach the transfected cells for counting. 150µL of trypsin was used for 5 minutes at room temperature, which could have killed some GFP expressing cells under the detachment.
However, the efficiency was surprisingly much lower, at 3%, when 3µg tRNA was used. These data provide a new insight into the relationship between the amount of tRNA transfected and the efficiency of transfection, there is room to hypothesize that high amounts of tRNA is consequential on transfection efficiency. The latter could be explained by overexpression of yeast tRNA causing toxicity, this has indeed been researched by Zimmerman et al. (2018). While studying acid amino misincorporation, the cause was found to be accumulation of toxic yeast tRNA (Zimmerman et al., 2018), similarly it has been proven that excess tRNA can affect negatively protein function through alteration of protein folding (Zhang et al., 2009). These research-based proofs could explain in some way the experimental findings of this study. High amounts of tRNA can be causing tRNA overabundance and at the same time toxicity which is negatively manipulating GFP translation making transfection less efficient than with lower tRNA masses. The higher amount of tRNA can also be impacting the cell membrane as molecular overload, which could affect a successful passage of tRNA into the cytoplasm.
This cell toxicity induced by a high amount of tRNA has been observed in Experiment 5, 4.0µg of tRNA was transfected in a well and caused high cell death 24 hours post-transfection.
On the other hand, negative controls were performed to see the effect of tRNA alone on Atlantic salmon fibroblast, cell viability was not highly compromised in this case.
However, for these negative controls, only 1.0µg and 2.0µg of tRNA were used in combination with 2.0µL Lipofectamine 2000, which can be considered a relatively low amount of tRNA. This

supports the previously suggested hypothesis, that indeed high amounts of tRNA cause cell toxicity.
Extensive research has demonstrated the effect of charge ratio of liposome/DNA complexes on their size and the transfection efficiency, indeed a decrease in liposome size can in fact cause increase in efficiency (Brgles et al., 2012). This suggests that the molecular weight of nucleic acids can also impact transfection efficiency, as a slight increase in the mass of nucleic acid can decrease transfection efficiency.

2. Optimizing the method by varying the seeding density and time

The seeding time and density were found to be critical factors that significantly impacted transfection efficiency in Atlantic salmon fibroblast. Experiment 2 highlighted the challenge of transfecting cells 48 hours post-seeding, resulting in no transfected cells despite the use of positive controls. The optimal seeding time for transfection is 24 hours post-seeding, as it coincides with the active dividing phase of the fibroblast, which is crucial for efficient mRNA uptake (*Factors Influencing Transfection Efficiency*, n.d.).
In Experiment 3 revealed a noteworthy factor in that the expected number of transfected cells was not achieved. The low efficiency can be attributed to the cell confluence at the time of transfection, as most wells had a confluence of only 70%, which is the minimum requirement for lipofection. This study showed that 70% confluence at the time of transfection does not provide as much efficiency as 80%. This finding is consistent with previous studies that highlighted the importance of seeding density for transfection efficiency. Specifically, some of the wells in Experiment 3, such as those in Figure 19, had 80% confluence, which resulted in better transfection efficiency, like the cells under Experiment 4. However, in Experiment 6, 90% confluence resulted in no transfected cells except for one with Lipofectamine 300. This contradicts the findings in *Liposome-mediated messenger RNA: An alternative for fish cell transfection in culture* (Tan et al., 2020), in which 90% confluency was required for transfection in primary cells.

The present study contributes to the existing body of evidence highlighting the significance of seeding density in transfection efficiency. Based on the experimental results, it is reasonable to infer that attaining a confluence of 80% at the time of lipofection is crucial for achieving high efficiency in Atlantic salmon fibroblast. Additionally, it is essential to perform transfection within 24 hours post-seeding.
These findings suggest that a confluency of 70% or lower in Atlantic salmon fibroblast may lead to insufficient cell-to-cell contact necessary for efficient transfection. Furthermore, it should be noted that attaining a confluency of 90% or higher may lead to contact inhibition, whereby the fibroblast becomes resistant to mRNA uptake. (*Transfection*, n.d.)
Lipofection is known to be dependent on the cell division rate and a high rate of endocytosis (Gresch et al., 2004). When there is cell-to-cell contact but without contact inhibition, there is sufficient space for them to divide at a high rate (de Carvalho et al., 2018), leading to an increase in lipofection efficiency.

Another study has demonstrated that an elevated cell density of macrophages can induce inflammation, while the same cells at a lower confluence do not exhibit an increased inflammatory response (Aldo et al., 2013). Thereby inflammation is negatively impacting the cell membrane and restricting its ability to uptake foreign nucleic acids, as it is known that inflammation can cause cell toxicity over a longer period of time (Six, 2022). Therefore, one could hypothesize that a high cell confluence of fibroblasts could be inducing some type of cellular response comparable to what happens in human macrophages. This could then possibly explain the lack of transfection observed in cells with a higher level of cell confluency.
As a result, for Atlantic salmon fibroblast, a confluence of 80% at the time of transfection can be considered important as it provides the necessary conditions for efficient lipofection.

3. Optimizing the method by varying the incubation temperature and time

To further optimize the transfection protocol for Atlantic salmon fibroblasts, it was necessary to conduct additional experiments to determine the optimal temperature range for their survival. The efficacy of Lipofectamine 2000 was demonstrated at high temperatures, with a notably high efficiency observed at 37°C in HEK cells (ref. III.B.1). In Atlantic salmon fibroblasts, Lipofectamine 2000 was more effective at 25°C than at 20°C, with an increase in transfection efficiency observed at 4% without tRNA supplement and up to 10% with tRNA supplement. However, the MessengerMAX reagent did not show improved efficiency at higher incubation temperatures in Experiment 4, which may be attributed in part to low mRNA amounts used in that experiment. Otherwise, this data further supports the theory of cell sensitivity towards MessengerMAX mentioned in IV.B.1.
Additionally, knowing that Atlantic salmon exhibits HVA response towards thermal stress, there is there is a lpossibility that the saturation of the cellular membrane's phospholipids, which occurs in response to an increase in incubation temperature, may limit the activity of the lipid reagent. This occurrence could potentially account for the observed decrease in the efficiency of MessengerMAX transfection in Atlantic salmon cells, when increasing the temperature.

There is indeed a correlation between transfection efficiency and the temperature of incubation, which is in line with the initial hypothesis made in I.B.5.b), but is relevant for Lipofectamine 2000 only.
Although higher temperatures have been shown to increase transfection efficiency in Atlantic salmon fibroblasts, the issue remains of incubating the transfected cells in an environment that does not compromise their cell viability.
Based on the experimental results, it is possible to optimize the lipofection protocol by increasing the incubation temperature of the transfected fibroblast without exceeding the lethal threshold of Atlantic salmon's survival (ref. I.B.5.b) and decreasing the incubation period to less than 22 hours. This could potentially improve transfection efficiency while maintaining cell viability. A potential approach could involve replicating the conditions of Experiment 6, which showed improved cell

viability by changing the media and incubation temperature 4 hours post-transfection. Further research can explore this technique to refine the lipofection protocol for Atlantic salmon fibroblast.
However, it should be noted that while Lipofectamine has been shown to improve efficiency, research conducted on European sea bass at 25°C has demonstrated that Lipofectamine 2000 is less efficient than Fugene and can cause cell death (Crespo et al., 2013). This suggests that the use of Fugene as a reagent may result in further increased efficiency in fibroblast, given that sea bass and Atlantic salmon are poikilothermic fishes.

Overall, Lipofectamine 2000 was found to be the most efficient reagent among those tested, regardless of the optimization method employed. It exhibited some efficiency even with low mRNA amounts, which Lipofectamine MessengerMAX was unable mediate mRNA trasnfection in salmon cells. The Atlantic salmon fibroblasts were found to be highly sensitive to Lipofectamine MessengerMAX and unresponsive to TransIT. The transfection efficiency declined when optimizing the method with MessengerMAX, such as by increasing the incubation temperature or reagent volume. However, the inclusion of tRNA was the only successful approach to improve transfection efficiency with MessengerMAX.

It is important to note that the passage number was not tracked in this study, which may have impacted the results. Due to the lack of data on passage numbers, the observed low efficiencies may be attributed to a high passage number, limiting the interpretation of the results. It is known that higher passage numbers can be altering the cell's viability and for example reduction in the cell growth rate can occur (Kwist et al., 2016).
Additionally, transfection of salmon fibroblasts with a low passage number was not feasible in this study, as the cells required a prolonged period to grow and attain the desired confluence for lipofection.
Furthermore, there is a correlation between transfection efficiency and the cell's well-being. A successful transfection process is likely to occur when cells are appropriately cultured and maintain their health. The maintenance of cellular health is critical for extending cell viability after the transfection process. Previous research findings have demonstrated the significance of cellular health in post-transfection viability, as healthy primary hippocampal neurons were shown to survive for up to 3 weeks after transfection, whereas unhealthy neurons failed to maintain their viability post-transfection (Sun et al., 2013).

Based on the experimental outcomes of this study, an optimized protocol this far would entail the following steps:

1) The cells shall be seeded at a density that results in 80% confluency at time of transfection.
2) The cells shall be transfected 24 hours at maximum post-seeding.
3) Lipofectamine 2000 shall be the lipid reagent of choice (further optimizations with Lipofectamine 3000 are highly recommended).
4) To supplement the transfection mixture with tRNA is highly advised.

5) Both mRNA to tRNA ratio and mRNA to Lipofectamine 2000 shall have a ratio of 1:2.
6) Incubation of transfected cells, for 22 hours post-transfection at 25°C shall be done (optimizing the incubation time period is highly advised)

C. Additional optimization techniques for increased transfection efficiency

There are additional factors that could further enhance the efficiency of transfection. While these were not included in the present study, they can be tested in future experiments as part of further optimization efforts aimed at establishing a reliable protocol for inducing pluripotent stem cells from salmon fibroblasts.

For instance, the low efficiencies observed in this study's experiments can be due to the mRNA composition, thus modifications could be made to the mRNA. In the present study, the mRNA has been synthesized with both Cap-0 and a poly-A tail, in addition to being dephosphorylated (ref.I.B.3). Research in Xenopus has demonstrated that transfected mRNA with Cap-1 exhibits greater translational efficiency compared to mRNA with Cap-0 (Kuge et al., 1998). Therefore, an mRNA modification that can be done is to methylate the Cap-0 of mRNA in vitro, which would result in a conversion to Cap-1 containing a methylated ribose (*mRNA capping* n.d.). This is shown in Figure 26.

On the other hand, nanotechnology has provided with new promising approaches to efficacious mRNA delivery, both in vitro and in vivo (Hou et al., 2021). As such, the delivery approach of mRNA could be modified to include delivery in the form of nanoparticles (Phua et al., 2013). Previous studies in human and mouse dendritic cells have proven that mRNA nanoparticles yielded superior transfection efficiency compared to carrier-lacking mRNA (Phua et al., 2013). Hence, an alternative mode of mRNA delivery could involve generating either mRNA nanoparticles (Phua et al., 2013) or lipid nanoparticles for binding to mRNA (Hou et al., 2021).

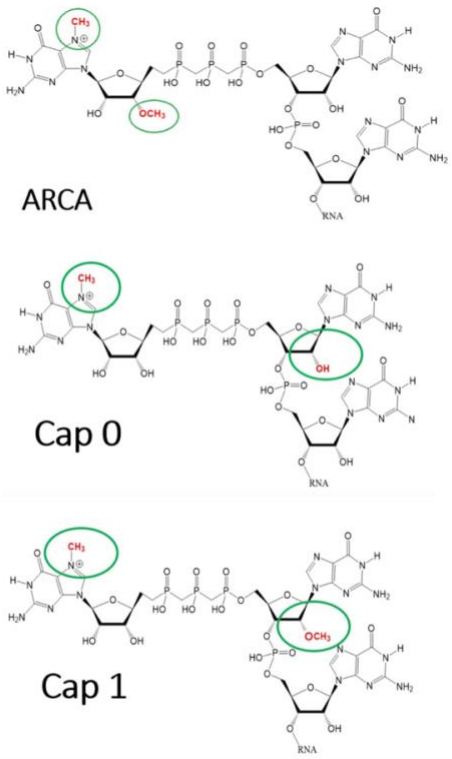

Figure 26 Overview of the differences between Cap-0 and Cap-1 compared to ARCA *The encircled structures are the differences between each Cap structures with OCH3 being methylated ribose and OH being hydroxyl. This figure is retrieved from (What is ARCA?, 2021)*

Additionally, there are other alternatives to the transfection method that could be used for effective genetic reprogramming to stem cells. One such alternative involves the utilization of mini-intronic plasmid production, which has been demonstrated to be efficient in the derivation of induced pluripotent stem cell lines from both human and mouse fibroblasts, without the need for multiple successive transfections (Diecke et al., 2015). It is noteworthy that this approach is predicated on the reduction in size of the genetic material to be transfected, which aligns with the general observation that smaller particles/masses tend to yield higher transfection efficiencies, as is also evident from the data generated in the present study (ref. IV.B.1).
Another viable transfection protocol that has been demonstrated to yield higher transfection efficiency is electroporation. For instance, in the context of Atlantic salmon's head kidney cell line, enhanced transfection has been achieved utilizing Amaxa nucleofector technology, which has been shown to result in transfection efficiencies ranging from 11.6% to 90.8%(Schiotz et al., 2011). Nonetheless, the wide range of observed efficiency values could pose certain limitations to the reproducibility of this method. Therefore, it is recommended that alternative optimization strategies, such as those aforementioned, to be explored before electroporation is considered for generation of induced pluripotent stem cells.

V. Conclusion

In this study, the objective was to enhance exogenous gene expression in Atlantic salmon fibroblasts by developing an efficient mRNA transfection method.
Results indicated that lipofection outperformed calcium-phosphate transfection in terms of efficacy for salmon fibroblasts. However, further improvements were observed by incorporating yeast tRNA into the transfection mixture and limiting post-transfection cell incubation at 25°C to a maximum of 22 hours. Despite these advancements, the study encountered challenges related to cell viability, as evidenced by an increasing cell death rate already 24 hours post-transfection. Further research is needed, to determine the causes of this increased cell death to investigate the underlying causes of increased cell death, enabling the development of appropriate interventions. These findings confirm the feasibility of mRNA transfection for transiently modified cells in Atlantic salmon, emphasizing the need for continued optimization based on the recommendations outlined in the previous chapter.

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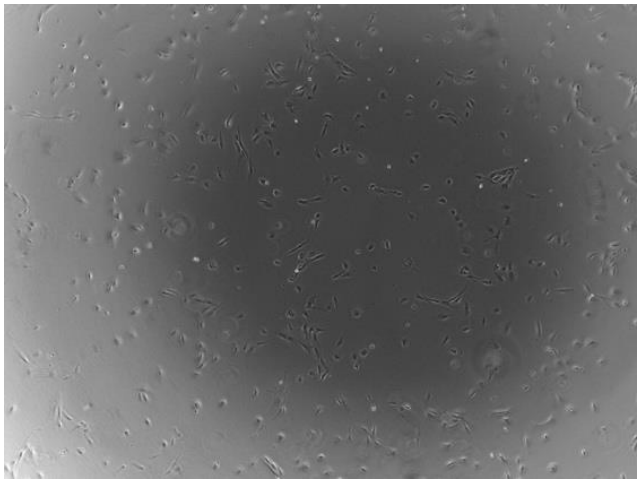
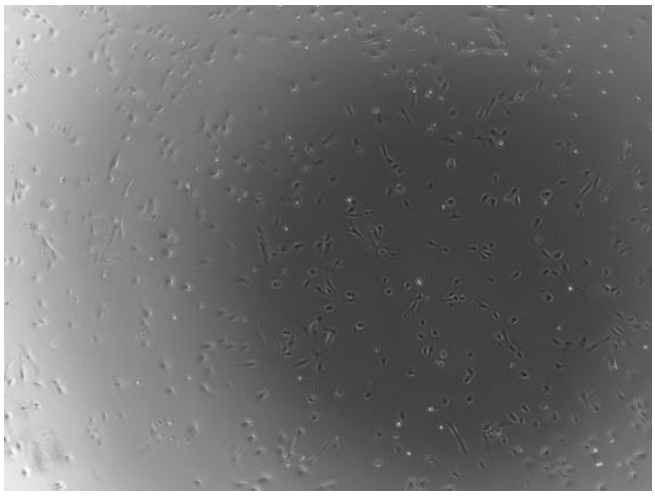
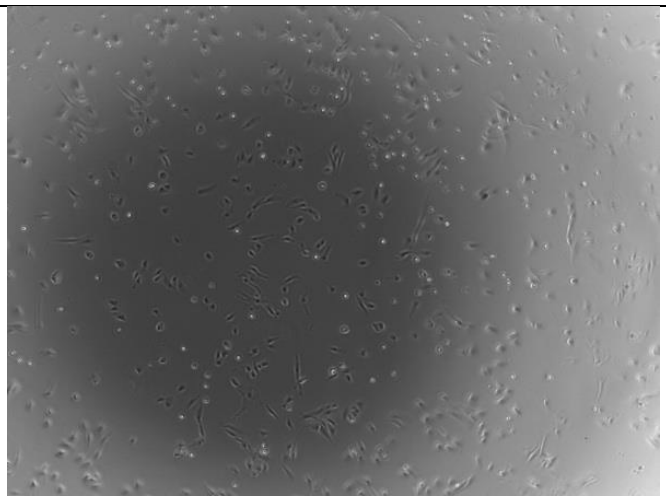
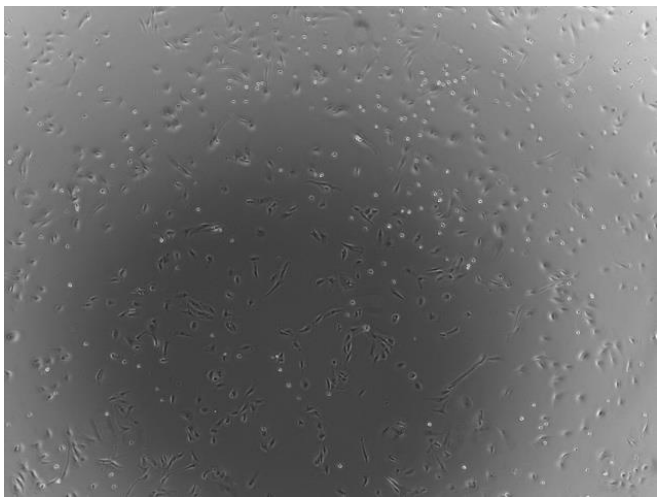
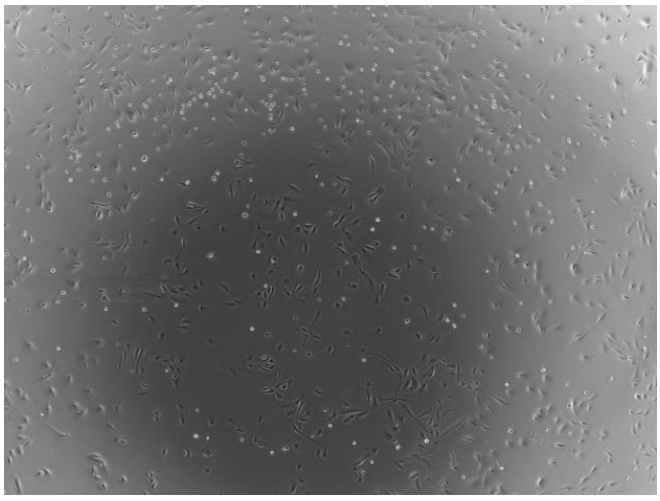
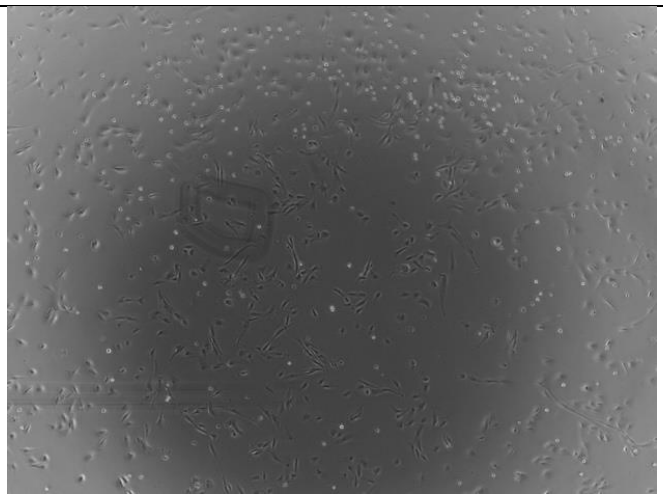
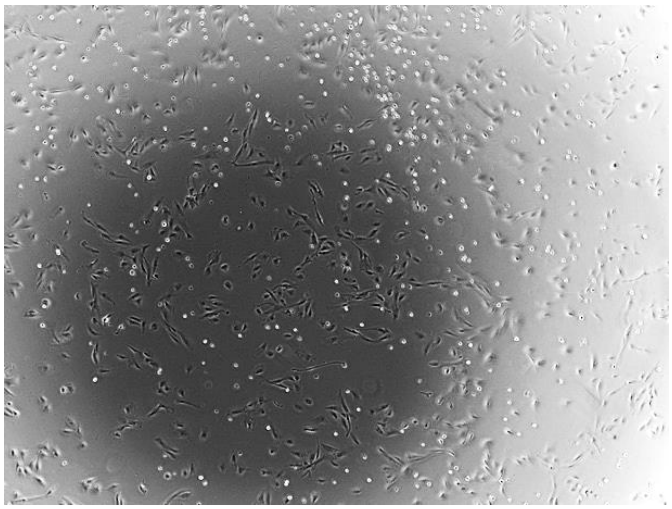
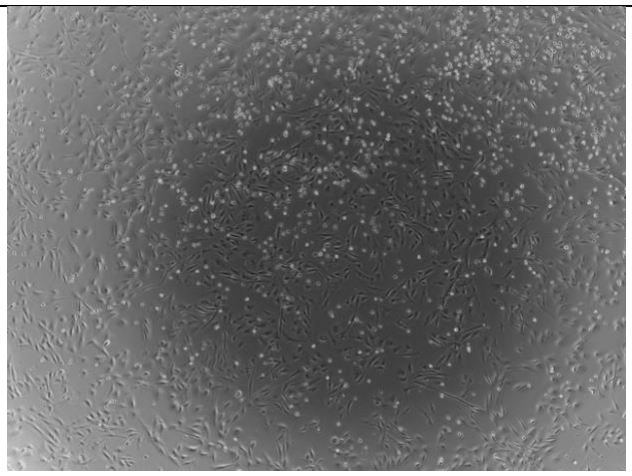
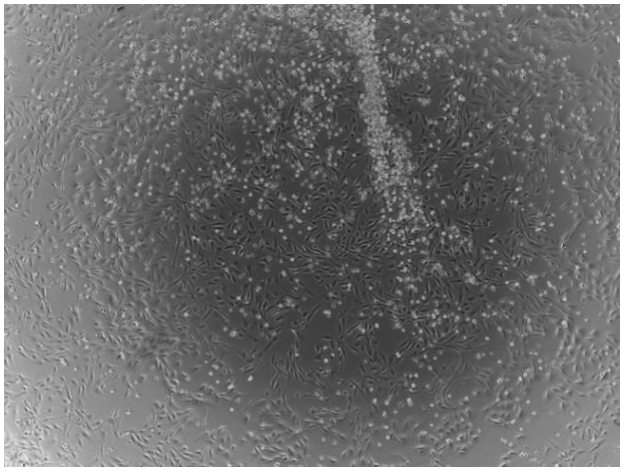
Appendix A : Overview of confluence results for fibroblast seeding densities

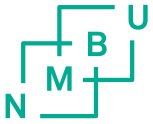
This table shows the seeding densities experimented with to find the optimal density for the transfection experiments and their corresponding confluence as percentage with its photo taken on the microscope.

Seeding density	Resulting confluence as figure	Resulting confluence as percentage
25 000 cells/well		18%
27 500 cells/well		22%

30 000 cells/well		30%
32 500 cells/well		38%
35 000 cells/well		40%

37 500 cells/well		46%
42 500 cells/well		60%

75 000 cells/well		80%
107 500 cells/well		90%



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