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Mini CRISPR screen of Interferons and interferon-like genes in search of ISAV resistance in SHK-1 cell line.

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Abstract

Infectious salmon anemia (ISA) is a bothersome epidemy in commercially farmed Atlantic salmon (*Salmo salar*), caused by the influenza-like Infectious salmon anemia virus (ISAV). It causes severe anemia in farmed Atlantic salmon, resulting in high death rates and great economical losses if left untreated.

The infectious disease has demanded a lot of resources in the science and aquaculture communities since its discovery. Many countermeasures, such as infection control guidelines and vaccines, have been tried out, but with varying result in the field. To this day ISAV outbreaks are still common in most counties commercially farming Atlantic salmon, though not as frequent and severe as before.

Previous research on the salmon immune system indicates that interferons play an important role in the salmons first line of defense against virus infections. Salmon has a great repertoire of such interferons and interferon-like genes, that have been studied to varying degree. These interferons and interferon-like genes are therefore of great interest in the search for better virus immune response in farmed Atlantic salmon, both for ISA resistance and for virus resistance in general.

The aim of this thesis was to shed some light on what genes contribute to the natural immune response of Atlantic salmon. Specifically, which, if any, interferons or interferon-like genes contribute to ISA virus resistance. To achieve this, it will be attempted to utilize the high sequence similarity of Atlantic salmon interferons and interferon-like genes to design a CRISPR knockout experiment, intended to knock out multiple high-similarity genes.

Abbreviations

2ME	2-Mercaptoethanol
bp	Base pairs
FBS	Fetal Bovine Serum
gRNA	Guide RNA
Ifna1	Interferon alpha 1
Ifnb	Interferon beta*
Ifnc	Interferon c**
Ifne	Interferon epsilon
Ifng	Interferon gamma
ISAV	Infectious salmon anemia virus
Kb	Kilobase, 1000 base pairs
P.P	Primer pair
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pen.strep	Penicillin-streptomycin
SHK-1	Salmon head kidney cell line
SNP	Single nucleotide polymorphism
WT	Wild Type

*Ifnb in Atlantic salmon is just named LOC101448042 on the NCBI database. I have chosen to follow the general trend of naming used and resulted in calling it “Interferon beta” in this thesis.

**Ifnc is named LOC101448043 in the NCBI database, and the Greek alphabet the other interferons are named after does not contain the letter c. I have therefore chosen to call it “Interferon c” in this thesis.

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1 Introduction

1.1 Virus infections in farmed Atlantic Salmon

Virus infections and epidemics pose great threats to the commercial fish farming industry, in Norway and around the world, due to the lack of effective means to defeat them. Today viral infections in aquaculture are somewhat kept at bay using vaccination programs, disease spreading restrictions and immunostimulant feeds, all of which pose different challenges. Vaccinations, often injections, of farmed fish is both very labor intensive, expensive, inefficient and cause stress and trauma on the fish, often resulting in necrotic tissue and in some cases death. Due to the natural pecking order of fish in captivity, immunostimulant feed is an inefficient way of delivery, as the fish will consume unequal amounts of enriched feed. The uptake is also less than optimal, and a certain amount will sieve into the surrounding water column, polluting fragile marine ecosystems (Kibenge et al., 2004).

1.1.2 Infectious salmon anemia virus

Infectious salmon anemia virus (ISAV) is one of the most economically damaging virus infections in aquaculture, classified as a notifiable disease by the World organization for Animal Health since 1990 (Kibenge & Godoy, 2016). The infectious disease was first detected in Norway in 1984, and the virus causing it was isolated in 1995 (Dannevig et al., 1995). After the initiation of the 'Stop ISA campaign' from 1989 to 1993 (Håstein et al., 1999), implementing restrictions regarding transportation of salmon, smolt production and quarantining of affected areas, there has been a noticeable decline in serious ISAV epidemics in Norway. Many severe cases have since been reported in other countries with ocean-based, commercial salmon production, causing Chile to downscale its salmon production by 75% from 2009 to 2010, and the Faroe Islands to cull its entire domestic salmon population and "re-start" its aquaculture industry in 2005 (Aamelfot et al., 2014). Today, prophylactics and vaccinations are commercially available, but their effect in the field is disputed, and the method of delivery is as mentioned above questionable (Kibenge et al., 2012).

ISAV is a virus in the *Orthomyxoviridae* family, the same family as the influenza virus affecting humans. It is an enveloped virus with single-stranded RNA. Signs of infection are highly variable, and may include lowered appetite, sluggish swimming at the surface, pale gills, hemorrhaging of the abdomen and vital inner organs, but above all; severe anemia. Often, the fish can be asymptomatic for the entire period of infection, until they suddenly die, without any explanation. Diagnosing depends largely on postmortem dissection and reverse transcriptase identification of the virus (Kibenge et al., 2004). Untreated ISA infection develops slowly, from a low mortality of 0.05% to over 80% over the next few months.

The HPRO-variant of the ISA virus seems to be very common across the world but does not result in the disease and death. Experiments using reverse transcriptase suggests that the more detrimental, disease-causing strains originate from this harmless "wildtype" HPRO variant. Although the spread if ISAV is not yet fully understood, it seems to spread from wild migrating fish, seemingly resistant to ISA infection, to the pens of farmed fish.

The SHK-1 cell line has been chosen for this experiment, as doing a genomic knockout-study in whole, live fish would be inefficient, time consuming and labor intensive, not to mention unethical regarding the salmon welfare. SHK-1 cells have also already been used for ISAV infection (Dannevig et al., 1995) and interferon studies (Robertsen, 2018).

1.2 Interferons in Atlantic salmon

Interferons type I has already shown to inhibit early stages of ISAV in Atlantic salmon (Svingerud et al., 2013), which lead to them being of interest for this study.

Interferons are a type of cytokines, a group of proteins functioning as an intercellular communication devise. They are so named for their tendency to "interfere" with virus infection, causing nearby cells to increase their natural immune response. Atlantic salmon interferons are structurally and functionally very alike mammalian and avian interferons, with their 5 α-helices, but contain both exons and introns, unlike those of birds and mammals (Robertsen, 2018). Interferons may have many different pathways and effects, depending on the type of cell culture or tissue they are expressed in (Robertsen, 2018). This means that results received in SHK-1 cell culture could be different than that of entire living fish, and any potentially interesting results should later be tried out in test subjects to investigate systemic results.

Due to the high portion of genome duplication in salmon (Christensen & Davidson, 2017), there is reason to believe that many of these genes have highly similar function, that gives salmon a unique repertoire of variation in virus immunity. A few Atlantic salmon interferons have been thoroughly studied, among them ifna1 and ifna2, but many of their close relatives are still barely annotated or even named (Robertsen, 2018). Therefore, it is interesting to examine all interferons, and interferon-like genes. These less studied interferons and interferon-like genes might have important biological function in the salmon immune system. Therefore, we have chosen to try designing a "mini CRISPR screen" of interferons.

1.3 CRISPR as gene-mapping tool

CRISPR knockout screening is an increasingly common tool for studying genes and their function, due to its adaptability, attainability and accuracy. CRISPR screens mostly utilize lentiviral delivery of gRNA vectors to knock out entire gene libraries, but there seems to be some issue with infecting Atlantic salmon cells with the commonly used lentiviruses. Our cell lab-colleagues and my fellow master-students are currently working on ways to use lentivirus in salmon cell culture, but until then, a mini-screen utilizing well-established methods of salmon cell-line transfection will be used.

Due to the high sequence similarity in salmon interferons, it is difficult to design gRNAs to knock out single interferons at a time without significant off-target effects. This would be time consuming, labor heavy work needing much competence on gRNA design and intensive charting of off-target effects. Therefore, we have chosen to try and knock out multiple interferons using one single gRNA per interferon group. Ideally, this experiment would knock out every single one of the interferons and interferon-like genes in the respective groups.

The gene editing of each individual gene in each group will still need to be mapped using gene-specific primers and sanger sequencing.

The following ISA virus challenge of the edited cell lines is intended to indicate if even one of the interferons in the knocked-out group have a noticeable effect on the cell's immune response to ISA virus, and thus give information on which group to further investigate for ISA virus resistance. This could significantly reduce the number of relevant genes, narrowing it down to just a couple candidate genes for further investigation

1.4 Thesis aims

The main goal of this thesis is to design, conduct and optimize a CRISPR knockout mini screen of interferons and their closely related genes in SHK-1 cell lines, to establish a better understanding of interferons' role in natural virus resistance. Secondary aims include conducting an ISAV titration and establishing a baseline for virus concentration needed for observable infection of the wildtype SHK-1 cell line. It also includes designing gRNA for CRISPR knockout of selected genes, conducting the knockout experiment and sequencing and analyzing the results for further optimization. The last aim of this thesis is to design and conduct an ISAV challenge experiment to determine if the CRISPR-initiated gene-edits has had any effect on the cell lines immune response to ISAV.

2 Methods

Shown below is a flowchart giving an overview of the intended and preformed methods of this thesis, where the blue color indicates steps that were completed, and the red indicates steps that were cut short due to time issues.

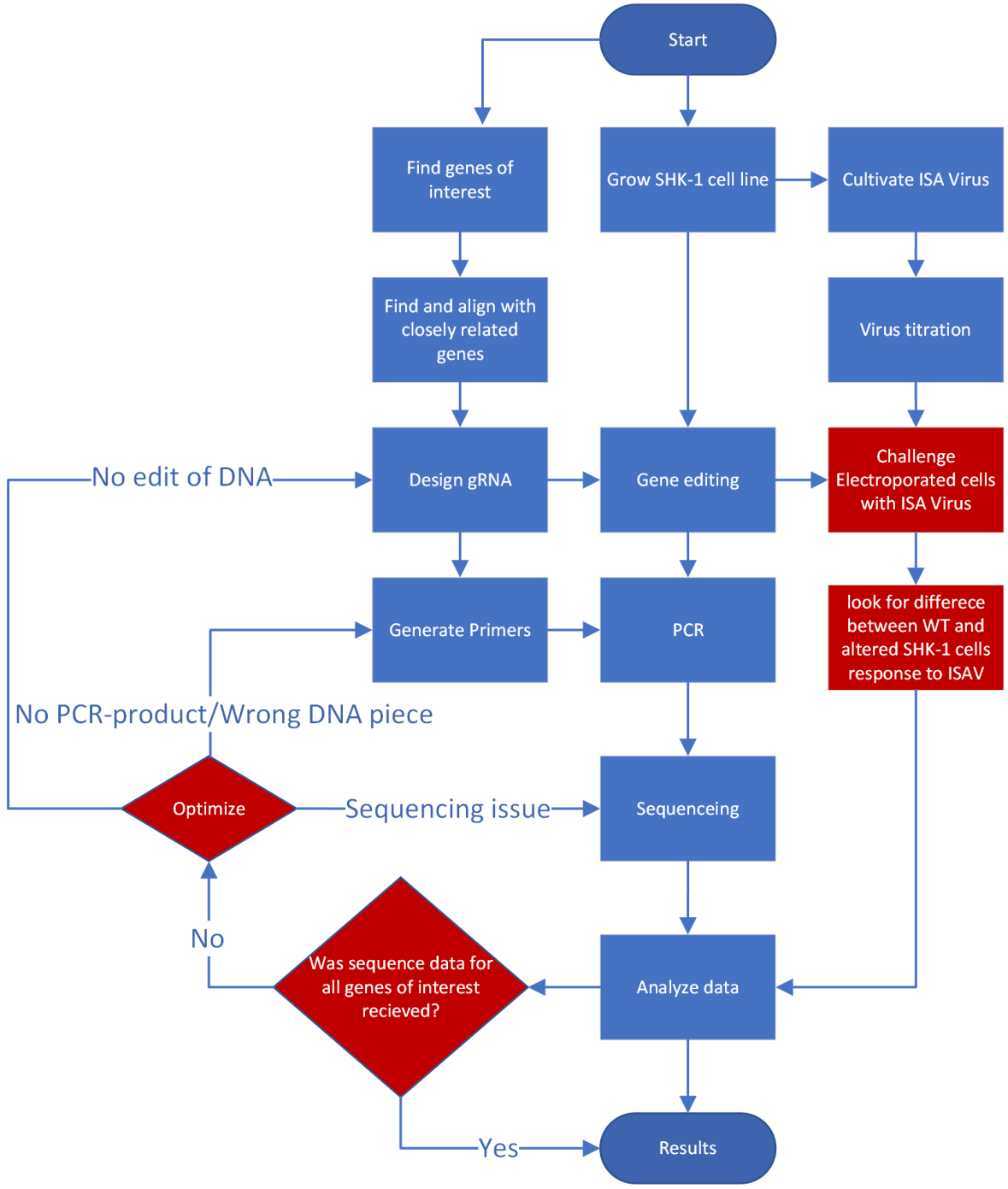

FIGURE 2.0: FLOWCHART OF PLANNED AND COMPLETED STEPS IN METHOD

2.1 Pre-electroporation procedures

2.1.1 Bioinformatics

Four type 1 interferons and an outlier consisting of a single type 2 interferon were decided upon for further study: Interferons a1 (gene DI 100137019), b (gene ID 101448042), c (gene ID 101448043) and e (gene ID 106600961) of type one, and interferon g (gene ID 100136413) of type two.

As Atlantic salmon is known to have a high proportion of duplicated genes, it was chosen to try and knock out interferons in groups, according to sequence similarity and relatedness. https://salmobase.org/ was used to find potential functional and non-functional copies and close relatives of each gene (see table 3.2.1 in results for summary and Appendix II: Additional information and raw data for all data used). The gene IDs for each individual gene were then located on https://www.ncbi.nlm.nih.gov/, where the FASTA file for the sequences were obtained (see appendix III: Genomic data).

The gene sequences were aligned in groups A, B, C, E and G using the alignment tool on https://benchling.com/. gRNA for knockouts was created for each gene-group in https://chopchop.cbu.uib.no/, using the original gene of interest (ifna1, ifnb++) as input, and choosing gRNAs in the areas of high sequence similarity within each group.

Primer pairs were designed using the primer wizard tool in benchling.com. They were set to not produce PCR products bigger than 1kb, as that is the limit to the sanger sequencing available, and a melting temperature of 60°C. This resulted in the primer pairs showed in table 2.1.1. All PCR-products are estimated at around 700-800 bp. M13 (GTAAAACGACGGCCAG) tails were added to the 5' end of all forward primers, to be able to prepare the samples for sequencing with ease, using only one sequencing primer. The table below contains all gRNAs and primers designed for the experiment. Reverse primers are written in red and forward primers are written in blue, with a * indicating the M13 tail. Another primer pair available in our lab, from an earlier CRISPR experiment on the SHK-1 cell line, was used as primer for positive controls, as it had already been demonstrated as functional.

TABLE 2.1.1: THE TABLE CONTAINS THREE CHOSEN GRNA FOR EACH SELECTED INITIAL GENE OF INTEREST, AND THEIR RESPECTIVE FORWARD AND REVERSE PRIMERS. *INDICATES M13 TAIL ADDED TO ALL FORWARD PRIMERS; **GTAAAACGACGGCCAG**

Gene of interest	gRNA nr.	gRNA sequence	Primer sequence	Primer pair name
Ifna1	1	GACTGGATCCGACACCACTA	*GTTGAAAGCAAAGCTCGCCAA TGGGGCATCCTGCTTTGTGATA	A1
	2	GGAATATGAAATCTGTCACC	*ATTGCTGTGACTGGATCCGACA ACTGGGGTTGGAGTGAAAACCT	A2
	3	CACCTGGGACAAGAAAAAGC		
Ifnb	1	GGTGGACGCAGTTTAGGTTG	*AGGGAAGCGAAAGTCTGAACCA TATCGCATAGCCTCCAATGCCA	B1
	2	CAGGTGGACGCAGTTTAGGT		
	3	AAGCTGAACGATGTGAGCAT		
Ifnc	1	TTGCCAGCTACAAGGACAGC	*ATATCCGGTCTTGCGGTTCACT TAGGCAGGTCGTCAGCTTCAAA	C1
	2	TAGCTGGCAAGGCATGGGCA		
	3	GAAAATTCTCCAACTTCTGT	*CTGGTGCGAATAACCCACAACC GCCCACAGAAGGCTATCAGAGT	C2
Ifne	1	AACTCTTAACTTGTCCTTTG	*TACACGTTCCATCCCTTGGGAG GGGATACAGCCTCTTGGTCTCC	E1
	2	CTTTTGAAGTAGTTCTTAAT	*GGAGACCAAGAGGCTGTATCCC AAGTCGTTGGTTGCATCACACC	E2
	3	TTTCAGCGCGTGCTCATGGG		
Ifng	1	TTTCCCCAAGGACACGTTTG	*CCTTCCTAATGGGAGGCCAACA TTTTGCAACAGAGCATGGGGTC	G1
	2	TCCGTTGAACAGCTGGTCCT		
	3	CGGAAAACCTGTTTTCCCCA		

2.1.2 Maintaining SHK-1 cell line

The SHK-1 cell line was kindly gifted from Øystein Evensen and Amr Ahmed Abdelrahim Gamil. All cell work was completed in cell lab, in sterile laminar flow hood. Cell culture was maintained based on the protocol from the cell culture provider (ECACC, s.a.), with some minor alterations.

Cells were grown t75 flasks, in media based on Leibovitz's L-15 media (L15), with 10% fetal bovine serum (FBS), 1% Penicillin-streptomycin (Pen.Strep) and 40µM 2- Mercaptoethanol (2ME) and kept in incubator at 20°C with ambient CO_2 levels. When cells grew 80-100% confluent they were split into multiple culture flasks as follows:

Media was discarded* and flasks were washed twice with Phosphate buffered saline (PBS). 1-3ml 0.05% Trypsin was distributed across the flask to detach the adhered cells from the flask surface. The cells were observed in microscope for morphological change. Once the cells obtained a circular shape and were floating around in the liquid, 1-3ml of freshly combined media was added to stop trypsinization. The liquid was collected from the flasks into a falcon tube and centrifuged at 200g for 5 minutes.

10µl was not centrifuged but combined with 10µl 0,4% Trypan Blue and analyzed in the TC-20 Automated cell counter. One 90% confluent flask typically contained 5-6 x10^6 live cells, enough to seed 2-3 new culture flasks.

After centrifugation, supernatant was discarded, and pellet was re-suspended in freshly prepared media, with volume according to cell count. Cells were distributed in new t75 flasks, each containing about 2 million cells and 10ml fresh media. Within the next few days media with dead cells were discarded, the flasks were washed twice with PBS and fresh media was added.

*The cell culturing started out using conditioned media, but due to bacterial contamination it was decided upon to use 100% fresh media. Due to this contamination, the flasks were also washed twice with PBS and the media was exchanges every few days, or at any sign of bacterial growth in the culture.

2.1.3 ISA virus production

ISA virus was cultivated following the protocol by (Dannevig et al., 1995), with minor adjustments, as it was not intended to keep producing ISA virus over a longer period of time.

ISA virus was kindly provided Øystein Evensen and Amr Ahmed Abdelrahim Gamil and added to confluent t75 flasks of SHK-1 cells with L15 media containing 1% FBS, 40µM 2ME and 1% Pen.Strep. Cell cultures were incubated at 15°C until most cells were dead, around 20 days. Media was collected and filtered using 50ml syringes and filter tips. Filtered media containing virus was divided into 1ml Eppendorf tubes and stored in -80°C until use. This was done a total of three times, resulting in three batches of virus, dated and kept separately.

2.1.4 ISAV titration

Virus titration procedure is based on the protocol by (Dannevig et al., 1995). In this experiment the virus titration was conducted to determine a prudent virus concentration for infection of the wildtype SHK-1 cell-line, and how long the infection would need to noticeably damage the cells. This was done to establish a baseline for later virus infection, to know what virus concentration to use and how often to observe the cells in the downstream experiment, when it would be needed to challenge the genetically edited cell lines with ISAV to determine if their resistance has shifted from the baseline.

Approximately 10 000 SHK-1 cells were seeded in 64 wells of 94 well plates. After cells had grown confluent in each well, original media was removed, and the wells were washed two times using FBS. A dilution series of virus (from frozen stock) was created with fresh media (1%FBS, 40µM 2ME and 1%Pen.strep.) with 6 replicates, ranging from 10^0 (undiluted postproduction concentration) to 10^{-7}. Media with varying virus concentrations were applied to wells as shown in the figure below, where the red numbers indicate the concentration as 10^N and C indicates control wells without any virus.

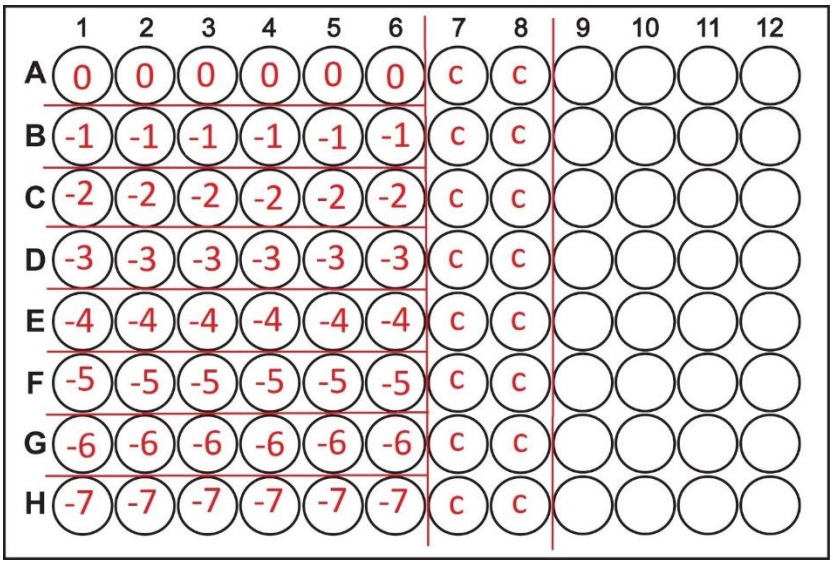

FIGURE 2.1.4: DISTRIBUTION OF DIFFERENT VIRUS CONCENTRATIONS IN 94 WELL PLATE SHOWN AS (UNDILUTED POSTPRODUCTION CONCENTRATION) *10^{N} AND C INDICATING CONTROL WELLS WITHOUT ANY VIRUS PRESENT.

The 94 well plate were observed in microscope over the next 21 days, or until control wells start showing signs of decay. The wells showing signs of virus infection were scored as described in (Dannevig et al., 1995), and presented in figure 3.1 in the results section.

The virus titration was conducted three times; once to gain experience with the method, the second time around to establish any difference in potency between "fresh" virus straight from cell culture and virus just thawed from storage. Results from the third and last virus titration were used to calculate $TCID_{50}$ as explained below.

$TCID_{50}$ was calculated using the a $TCID_{50}$ calculator (Kangro & Mahy, 1996). $TCID_{50}$ is a commonly used measure of the dilution of a virus required to infect 50% of a cell culture (Lei et al., 2020).

2.1.5 Electroporation optimization

For optimization, the Garatacap protocol (Gratacap et al., 2020) was followed with gRNA and primers from a previous experiment on the SHK-1 cell line in our lab. Pulse, time and voltage was altered during optimization, as well as the cas9 concentration used. After electroporation, the cells were grown until confluent and DNA was extracted, replicated using PCR and sequenced as explained in the following pages (2.2.2 Sequencing)

The highest indel% was achieved using the same pulse, time and voltage as used by Gratacap et al, but our results showed higher indel% with twice the cas9 concentration.

2.2 CRISPR knockout experiment

2.2.1 Electroporation

Electroporation protocol was based on the NEON user manual and (Gratacap et al., 2020) with a few minor adjustments after our own optimization experiment.

2.2.1.1 RNP preparation

gRNA was prepared by combining equal amounts of crRNA and tracrRNA, annealed by heating the samples to 95 °C for five minutes, then slowly cooling it to room temperature using the PCR-machine. The

gRNAs aimed at the same genes were mixed, and the RNA mix was combined with twice its total volume of 20µM cas9. This was left to incubate in room temperature for 15 minutes, then kept on ice until used.

2.2.1.2 SHK-1 cell-culture preparation

A 24 well plate was prepared with 1mL freshly combined media without antibiotics each well. Cell cultures were treated as described in 2.1.2 until centrifuging. After first centrifugation, supernatant was discarded, pellet was suspended in PBS and centrifuged again at 200g for 5 minutes. Supernatant was once more discarded, and cells were resuspended in OptiMEM, to the concentration of 10^7cells/mL.

Four Eppendorf tubes were labelled and filled with 40µL SHK-1 suspension and 16µL of RNP aimed at their respective genes. A fifth tube was prepared without RNP, to use as negative control.

2.2.1.3 Electroporation of cells

The Neon user manual was followed, using the setting for 1600V, 3 pulse for 10ms. Four replicates were electroporated from each individual tube, including the control tube without RNP, all 24 samples electroporated separately, to avoid cross contamination. The electroporated samples were seeded on a 24 well plate as shown below, with six columns of samples and four rows of replicates for each sample.

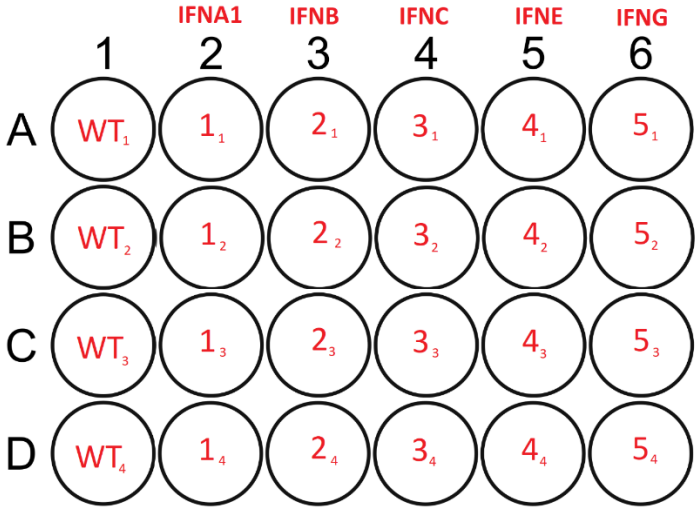

FIGURE 1.2.1.3: VISUAL REPRESENTATION OF THE 24 WELL PLATE SEEDED WITH FOUR REPLICATES OF EACH ELECTROPORATION EXPERIMENT.

The plate was incubated in the same cell growth conditions as mentioned above until confluent. The media in the wells was exchanged with normal, freshly combined growth media with antibiotics about 24 hours post-transfection.

2.2.2 Sequencing

2.2.2.1 DNA extraction

Once the cells were confluent, media was removed from the wells and the wells were washed twice with PBS. After washing, all the remaining PBS was removed with a small pipette. Quick Xtract DNA was added to each well and incubated for 5 minutes at room temperature, according to the manufacturers protocol. Cells were carefully scraped off the bottom of the wells with a pipette tip, collected in individual, labelled tubes and heated to 65°C for 15 minutes, then to 98 °C for 2 minutes using the PCR machine. The DNA concentration of each sample was measured using Nanodrop and summarized in table A II:1 in Appendix II: additional information and raw data.

2.2.2.2 PCR

PCR was done using the Platinum II Hot-Start Green PCR Master Mix kit (Thermofisher, 2018). The suggested PCR-protocol was followed, with 35 cycles of denaturation, annealing and extension.

20µl reactions were prepared for each DNA extract and primer combination (see table A II:2, in Appendix II) using varying amounts of nuclease free water, 10µl Platinum™ II Hot-Start Green PCR Master Mix (2x), 0,4µl 10µM of both forward and reverse primer, 1-6 µl template DNA* and 4µl Platinum™ GC Enhancer.

*Amount of template DNA was roughly estimated, based the Nanodrop readings of concentration, so that total amount of DNA in each sample would not be higher than the recommended amount of <500ng/rxn (Thermofisher, 2018)

2.2.2.3 Gel electrophoresis

30 well gels were made using 1% agarose powder and TAE Buffer (Tris-acetate-EDTA). The gels were loaded with GeneRuler 1kb ladder and samples and ran at 120V until the visible dye was 2/3rd down the gel, around 35min. Once the gels were done, they were imaged using The ChemiDoc XRS+ Gel Imaging System and the Image Lab 6.0 software.

2.2.2.4 Sequencing

PCR products were prepared for sequencing using the instructions from Eurofins Genomics (Eurofins genomics, 2021), with minor alterations.

ExoSAP-IT™ Express PCR Product Cleanup was used, following the protocol given by the distributor (Appliedbiosystems, 2017) with minor modification. 1µL PCR product was mixed with 4µL (1:10 diluted in nuclease free water) ExoSAP-IT™ Express reagent and the samples were incubated in the PCR machine at 37°C for 15 minutes, then at 80°C for 15 minutes and lastly cooled to 4°C and kept on ice.

The cleaned samples were combined with 5µL 5 pmol/µL of their respective forward primers*, labelled with stickers from GATC and delivered at KBM Reception, NMBU, for outsourced sequencing at Eurofin Genomics.

*As we did not test if the M13 primer-tail worked as expected, it was decided upon to use the respective forward primers as sequencing primers.

2.3 Planned onward experiment

This part was not completed due to time issues.

2.3.1 Challenging with ISA virus

The planned experiment included conducting an ISA virus challenge of the electroporated SHK-1 cells as follows:

After electroporation, gene-edited cells from each sample were to be seeded in 96 well plates and infected with ISA virus using the same method as described in 2.1.4, with the virus concentration around the baseline established in earlier titration. Within the next days and weeks, the wells were to be scored by signs of virus infection, and one would evaluate if there were a detectable difference between the wildtype SHK-1 cells and the electroporated cells with (hopefully) knocked out interferons.

2.3.2 Creating overview of modified, closely related genes

After observing the electroporated cells resistance to ISAV, it was planned to chart what genes were modified. For this, one would design specific primers for each of the close relatives to the initial genes of interest, do another PCR of the electroporated DNA with these new primers, and have it sequenced.

3 Results

3.1 Virus titration

The three separate virus harvests were not mixed in the lab, but analyzing the results showed no noticeable variation between the three batches. 96 wall plates were seeded with SHK-1 cells and virus and incubated as described in methods, 2.1.4. The first round of virus titration was discarded, as some wells had dried out by day 7, and some wells contained contaminations. The second round of titration was scored successfully and showed no noticeable variation between the effect of "fresh" virus, straight from cell culture media, and virus from frozen storage.

Wells were scored as (++) if all, or nearly all, cells within the well were dead and floating around the media, or cells were no longer distinguishable, but a shapeless mass in the center and around the edges of the well. Wells with prominent signs of decay, such as visible vacuoles within most live cells, and many dead cells floating around and loss of the typical "flower" growth pattern seen in confluent SHK-1 cell culture, were scored as (+). Wells with no noticeable sign of viral infection was scored as (-). These wells would also have the occasional dead cell floating around the media, but the wells were still confluent, and one could clearly see the typical growth patterns.

The figure below summarizes the scorings of the three 96 well plates used to calculate the $TCID_{50}$. The virus concentrations in each well are as mentioned in methods, 2.1.4, and the plates were scored 9 days after normal cell culture media was changed with virus dilutions. Column 7 and 8 contains control samples, without virus, all scored as (-). This results in 2 columns x 8 samples x 3 plates = 48/48 samples scored as (-). Column 1-6 contains virus, with each row having a different virus concentration. The top row; A, contains media with a virus concentration of $Nx10^{0}$, the same concentration of virus as was in the media immediately after harvesting. 17/18 wells on row A is scored as (++), and the last well is scored as (+). In the next row, B, the virus concentration is $Nx10^{-1}$; original harvesting concentration diluted by 10. For this row two wells were scored as (++), one well was scored as (-) and the remaining 15/18 was scored as (+). In row C the virus concentration is $Nx10^{-2}$; original harvesting concentration diluted by 100. Here, all 18/18 wells have been scored as (+). For the next row, D, the concentration is $Nx10^{-3}$, so the same original harvesting concentration diluted by 1 000. Here 14/18 wells have been scored as (+) and the remaining four wells has been scored as (-). In row E the virus concentration is $Nx10^{-4}$, original harvesting concentration diluted by 10 000. 12/18 wells were scored as (+) and the remaining 6/18 wells were scored as (-). In row F the virus concentration was $Nx10^{-5}$, diluted by 100 000. Here 13/18 wells were scored as (-) and the remaining 5/18 were scored as (+). In row G the virus concentration was $Nx10^{-6}$, corresponding to original harvest concentration diluted by 1 000 000. 17/18 wells were scored as (-) and one single well was scored as (+). In row H the final virus concentration was $Nx10^{-7}$, original harvest concentration diluted by 10 000 000. Here, all but one well was scored as (-), and one was scored as (+).

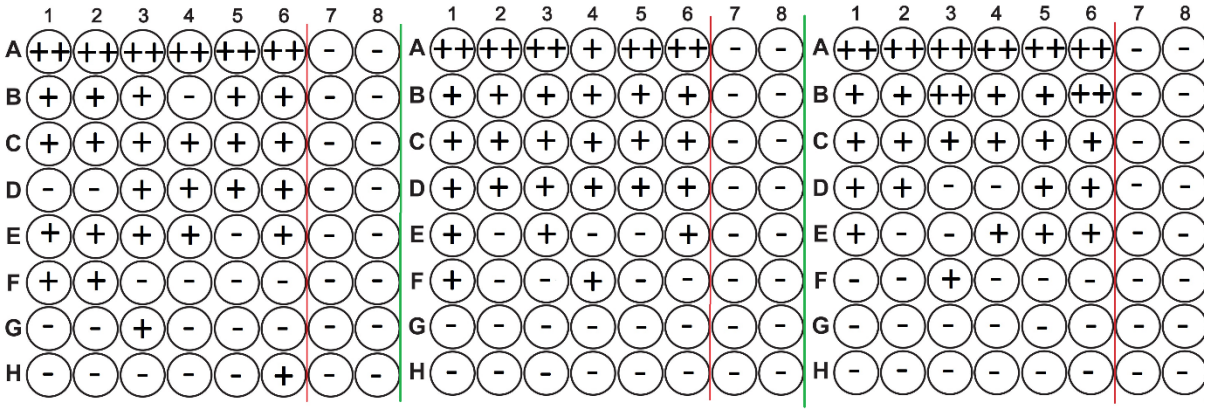

FIGURE 2.1: VISUAL OVERVIEW OF SCORED VIRUS TITRATION, USING 64X3 WELLS OF THREE 96 WELL PLATES. THE GENERAL TREND IS THAT THE WELLS CONTAINING HIGHER VIRUS CONCENTRATION, HAS A HIGHER DEGREE OF CELL DEATH AND DECAY.

The scorings were plotted into the $TCID_{50}$ calculator, resulting in a $TCID_{50\ /ml}$ of 1,67E+06 (+sd 9,94E+05, -sd 6,23E+05).

3.2 Bioinformatics

The interferon of interest was, as mentioned, located on salmobase.org, and the phylogenetic trees were analyzed for related genes in the Atlantic salmon genome. The figure below shows one example of the trees with the relevant genes underlined in red, this one showing ifnb and its related genes. All additional phylogenetic trees utilized are in Appendix II: Additional information and raw data.

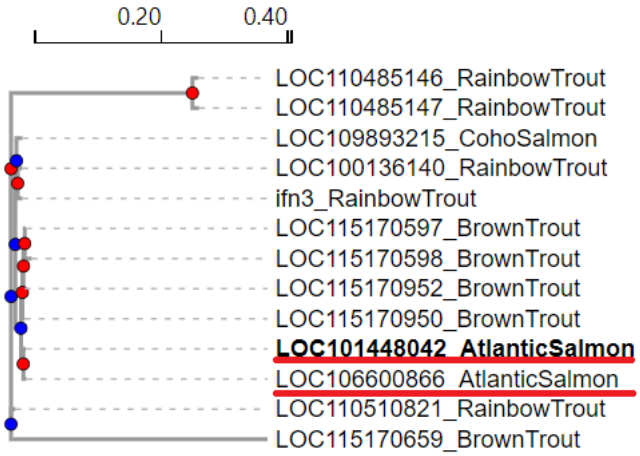

FIGURE 3.2.1: ILLUSTRATIVE FIGURE MODIFIED FROM SALMOBASE.ORG, ILLUSTRATING IFNB AND ITS CLOSELY RELATED GENES.

The phylogenetic findings are summarized in the table below. There are some blank spaces in the column containing gene IDs from NCBI.com, as the genes in question were not available in the database.

TABLE 3.2.1: THE INITIAL GENES OF INTEREST (HIGHLIGHTED IN YELLOW), THEIR CLOSELY RELATED GENES AND THEIR RESPECTIVE GENE IDS (RESPONDING TO THE NCBI DATABASE) AS WELL AS THE CHOSEN NAME FOR EACH GROUP.

Group	Initial gene of interest	Related genes	Gene ID from NCBI
A	Ifna1	ifna1	100137019
		LOC101448041	101448041
		ifna2	100136436
		LOC106596334	
		LOC106590949	106590949
		LOC106600865	106600865
		LOC106600963	106600963
		LOC106607463	106607463
		LOC106600783	106600783
		LOC106600964	106600964
		LOC106600969	106600969
B	ifnb	LOC101448042	101448042
		LOC106600866	106600866
C	ifnc	LOC101448043	101448043
		LOC106600965	106600965
		LOC106600970	106600970
		LOC106607525	106607525
		LOC106594533	106594533
		LOC106597742	
		LOC106597870	106597870
		LOC106597883	
		LOC106594534	106594534
		LOC106607529	106607529
		LOC106595256	
		LOC106607528	106607528
E	ifne	ifne	106600961
		LOC106607408	106607408
G	ifng	ifng	100136413

3.3 Gel electrophoresis

Gel electrophoresis was conducted and imaged as mentioned in methods. The gel images are edited to highlight the results as follows:

Figure 3.3.1 shows the gelphoto from the run containing PCR-product of DNA from the cells edited for Ifna1 and Ifnb and wildtype DNA, as well as three wells filled with 1Kb ladder and a negative and positive control from the PCR run.

From left to right, the gel-image shows:

A ladder in the first well, followed by PCR-product from replicates 1, 2, 3 and 4 (respectively) of the Ifna1-edited DNA combined with the A1 primer-pair. The sixth well from the left shows the PCR-product of wildtype DNA combined with the same A1 primer pair, followed by another 1Kb ladder in the seventh well. The eighth to twelfth wells contain PCR-product of Ifna1-edited DNA replicates 1, 2, 3 and 4, and

wildtype DNA, all combined the A2 primer-pair. The next wells contain a third 1Kb ladder, followed by PCR-product of Ifnb-edited DNA replicates 1, 2, 3 and 4, as well as wildtype DNA, all combined with the B primer pair. The right-most two wells contain a negative and a positive, respectively.

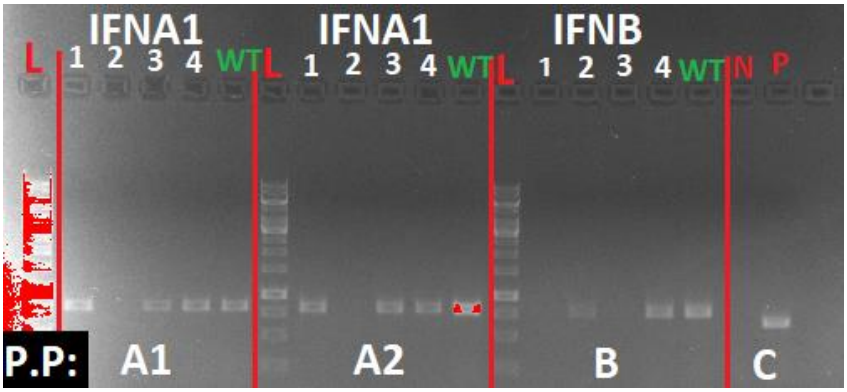

FIGURE 3.3.1: GEL-PHOTO FROM RUN CONTAINING DNA-SAMPLES EDITED FOR IFNA1 AND IFNB, THREE 1KB LADDERS AND NEGATIVE AND POSITIVE CONTROL. EDITS HIGHLIGHT THE DNA-EDITS IN QUESTION (IFNA1 AND IFNB IN WHITE), ELECTROPORATION REPLICATION NUMBER (WHITE NUMBERS FROM 1 TO 4), WILDTYPE DNA (GREEN WT), NEGATIVE- AND POSITIVE CONTROL AND LADDERS (RED N, P AND L) AND PRIMER PAIRS IN WHITE ON THE BOTTOM OF THE IMAGE (WHITE A1, A2, B AND C FOR CONTROL).

The next gel-image shows a run containing PCR-product from the electroporation of Ifne and Ifng, with four 1Kb ladders and negative and positive control.

From left to right, the wells contain:

The first ladder is placed in the left-most well, followed by an empty well. The next five wells contain PCR-products of replicates 1, 2, 3 and 4 of Ifne-edited DNA, followed by wildtype DNA, all combined with primer-pair E1. The next well contains a ladder. The next five samples contain the PCR-product of the E2 primer pair with replicate 1, 2, 3 and 4 of the Ifne-edited DNA and the wildtype DNA, in that order. Then follows a ladder, and then the PCR-product of Infg-edited DNA replicate 1, 2, 3, 4 and wildtype DNA combined with the G1 primer pair. The last three wells contain ladder, negative- and positive control respectively.

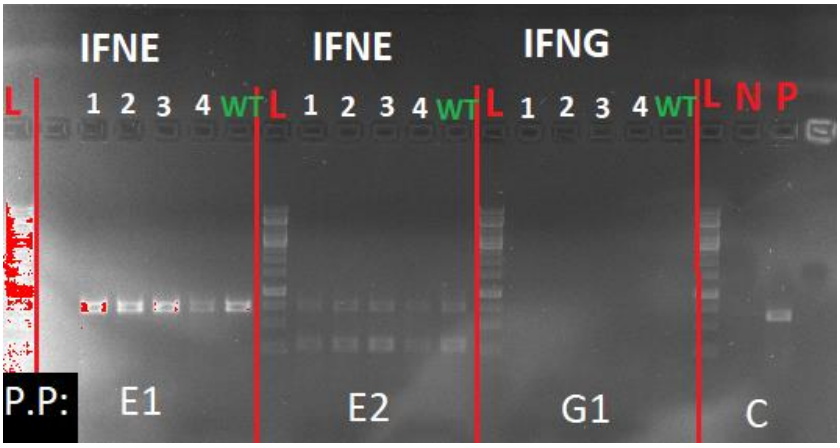

FIGURE 3.3.2: EDITED GEL-PHOTO OF RUN CONTAINING PCR-PRODUCT OF INFE- AND IFNG-EDITED SAMPLES, 1KB LADDER AND NEGATIVE- AND POSITIVE CONTROL. FOR FULL FIGURE EDIT EXPLANATION, SEE FIGURE TEXT OF THE PREVIOUS GEL-PHOTO; FIGURE 3.3.1

The final gel-image is a highlighted gel-photo of the remaining PCR-products of the Ifnc-edited DNA and a PCR re-run of the Ifng-edited samples shown in the last figure, as well as PCR-products from wildtype DNA with the same primers, and two 1Kb ladders.

From left to right, the wells contain:

The first well in the image was filled with a 1Kb ladder, but due to sensitivity issues with the imaging software it has been partly cropped from the figure. The next four wells are filled with the respective PCR re-run products of the Ifng-edited DNA samples with the G primer pair. The sixth well in the image has wildtype PCR-product produced with that same G primer pair. The next well contains a fully visible 1Kb ladder. Then follows the four replicates of the Ifnc-edited DNA, and the wildtype DNA, with the C1 primer pair. Next comes another 1Kb ladder. The rightmost five wells contain PCR-product of replicate 1, 2, 3, and 4 of Ifnc-edited DNA, and wildtype DNA, combined with the C2 primer-pair. This gel-run did not have positive- and negative control, as they were run together with earlier PCR samples whos' controls are run on the previous gel, that was already imaged by the time this gel was run. The ladders in the gels also serve as positive control for the gel.

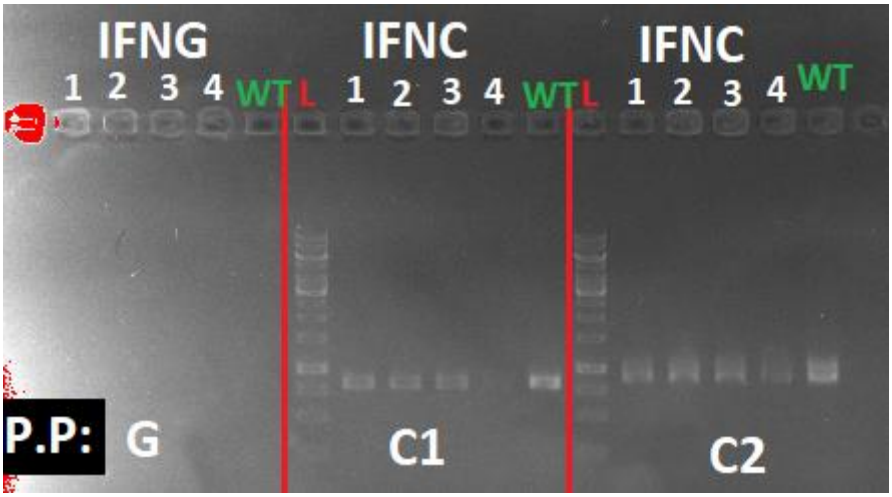

FIGURE 3.3.3: THE FIGURE SHOWS EDITED GEL-IMAGE FROM THE LAST GEL-RUN, CONTAINING SAMPLES OF IFNC- AND IFNG-EDITED DNA, AS WELL AS CPR-PRODUCT OF WILDTYPE DNA USING THE CORRESPONDING PRIMERS. FOR FULL DETAILS ON EDITED DETAILS, SEE FIGURE TEXT OF THE FIRST GEL-IMAGE; FIGURE X.X.

From the gel-images above one can see that most PCR-product produced bands in the expected areas, just around the ladders 1000bp mark, with some exceptions. The ifng-edited CPR-product shows no bands in either of its runs. The wells containing negative controls with no primers in the PCR-run also shows no bands, as is expected. The other samples that don't produce any clear bands includes both 2nd replicates of the ifna1-edited DNA, the 1st and 3rd replicate of the ifnb-edited DNA and the 4th replicate of the ifnc-edited DNA combined with the C1 primer pair. The ifne-edited DNA does however produce two distinct rows of bands, one at the expected 1000bp mark and another closer to the end of the gel, corresponding to a shorter DNA piece.

3.4 Post-sequencing data analysis

For the complete list of samples sent for sequencing and their content, see table A II:2 in Appendix II: Additional information and raw data.

The data received from external sequencing was analyzed using BioEdit Sequence Alignment Editor and ice.syntego.com. First, all AB1 files were opened in BioEdit, and files containing reads too short to be useful were discarded. Second, sequenced data was aligned with their respective datasets from the NCBI database to conclude whether the reads covered the areas of interest. Screen photos from the process are placed below.

3.4.1 Ifna1 data analysis

Sequencing data was aligned in BioEdit as shown in the figure below. Here, only samples 320, 316, 313, 314 and 317 contained sequencing data for long enough stretches to analyze them.

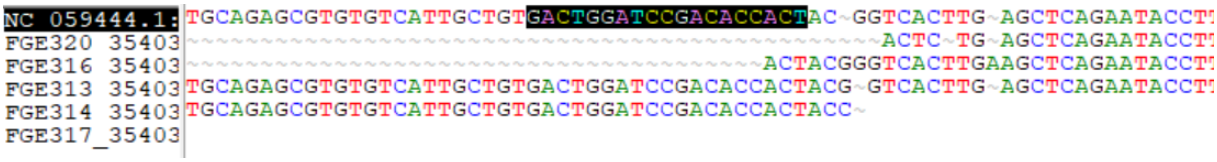

FIGURE 3.4.1.1: SEQUENCE ALIGNMENT OF IFNA1 GENE FROM THE NCBI DATABASE (TOP ROW) AND THE CORRESPONDING SEQUENCED PCR-PRODUCTS. THE SEQUENCE AREA WITH BLACKED OUT BACKGROUND (ON THE TOP ROW) IS THE FIRST GRNA IN THE GENE.

The wildtype PCR-product with the A1 primer pair, GATC sample ID FGE309, did not produce useable sequencing data, so the samples with the same primer hair has not been analyzed using ICE. The sample with GATC sample ID FGE320 was the only replicate of ifna1-edited DNA and the A2 primer pair that was useable, and was analyzed in ICE. The result output from ICE is shown in the figure below.

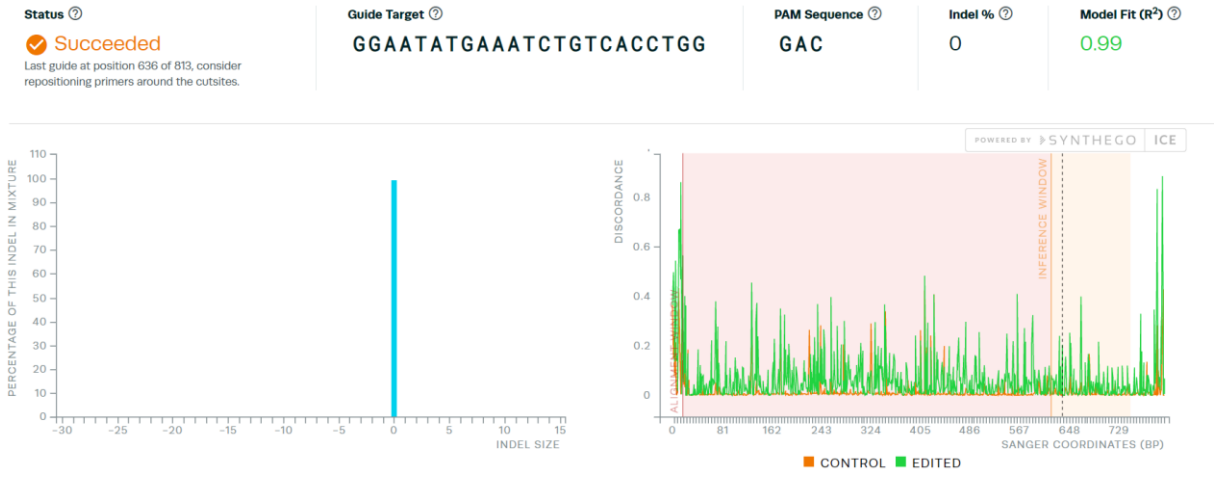

FIGURE 3.4.1.2: THE IMAGE SHOWS THE SUCCESSFUL ICE ANALYSIS OF THE UNSUCCESSFUL GENE EDITING.

As seen in the figure above, the indel% of this sample was 0%. In other words, the ifna1 has not been successfully edited.

3.4.2 Ifnb data analysis

For infb, only two of the five samples sent for sequencing gave long enough data stretches to analyze in a meaningful manner, sample FGE307 and FGE311. None of them contain wildtype DNA.

```
NC_059444.1:  ~~~~~~~~~~~~ATGGCTGTATTGAAATGGTTGAGCATTTGCCTGACTCTGTTCTGCCAAGGCACAGTA
FGE307_35403  ATAAAAGAAACATGGCTGTATTGAAATGGTTGAGCATTTGCCTGACTCTGTTCTGCCAAGGCACAGCA
FGE311_35403  ATAAAAGAAACATGGCTGTATTGAAATGGTTGAGCATTTGCCTGACTCTGTTCTGCCAAGGCACAGCA
```

FIGURE 3.4.2.1: FIGURE OF ALIGNMENT OF USEFUL IFNB SEQUENCING DATA AND THE CORRESPONDING NCBI DATA FILE (TOP ROW)

The samples were analyzed in ICE, out of pure curiosity. The figure below summarizes the ICE result output, with FGE307 submitted as wildtype control sample and FGE311 submitted as edited sample.

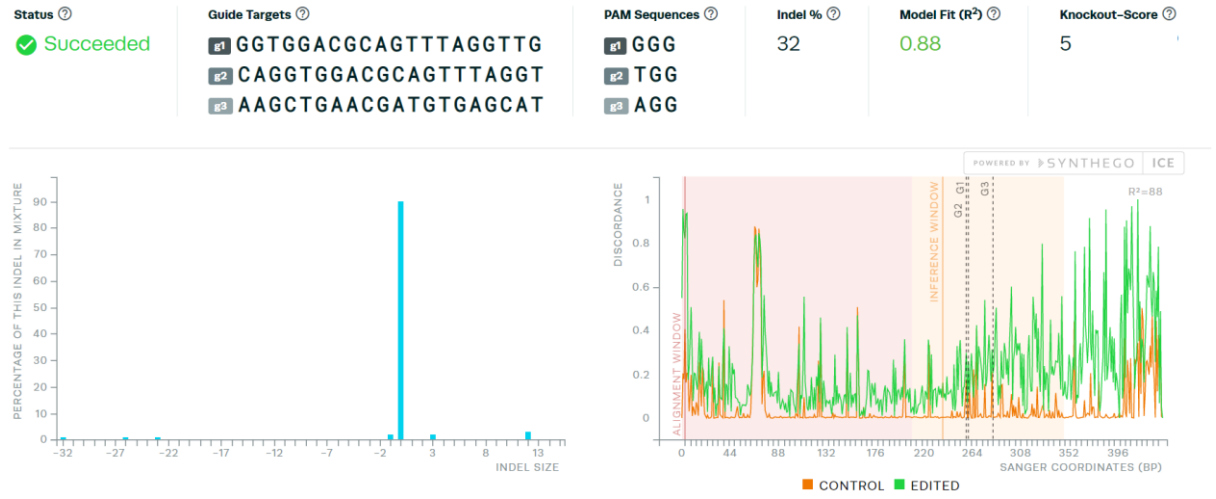

FIGURE 3.4.2.2: THE FIGURE SHOWS THE ICE RESULT OUTPUT FOR IFNB, WITH FGE307 AS CONTROL INPUT (ORANGE) AND FGE311 AS EDITED SAMPLE INPUT (GREEN).

The figure above shows quite some sequence variation in both the FGE307 sample and the FGE311 sample, after the gRNA attachment area. On the top of the figure, it displays an indel% of 32%.

3.4.3 Ifnc data analysis

Of the ifnc-edited DNA samples, only GATC ID FGE 297 was unsuited for ICE analysis. It was however long enough to align with the others in BioEdit, as shown in the figure below.

```
NC_059444.1:  GTATTATCCACCTATGTATAACTGAATAGCTCAACGTGAATGTTGTGTTTTCTCTTTCTTTCAGGGGGGTAATTTTCCTCTGGAGTGTCTGCAGGAGAACGT
FGE301_35403  GTATTATCCACCTATGTTTAACTGAATAGCTCAACGTGAATGTTGTGTTTCCTCTTTCTTTCAGGGGGGTAATTTTCCTCTGGACTGTCTTCAGGAGAACGT
FGE303_35403  GTATTATCCACCTATGTTTAACTGAATAGCTCAACGTGAATGTTGTGTTTCCTCTTTCTTTCAGGGGGGTAATTTTCCTCTGGAGTGTCTTCAGGAGAACGT
FGE304_35403  GTATTATCCACCTATGTTTAACTGAATAGCTCAACGTGAATGTTGTGTTTCCTCTTTCTTTCAGGGGGGTAATTTTCCTCTGGAGTGTCTTCAGGAGAACGT
FGE305_35403  GTATTATCCACCTATGTTTAACTGAATAGCTCAACGTGAATGTTGTGTTTCCTCTTTCTTTCAGGGGGGTAATTTTCCTCTGGAGTGTCTTCAGGAGAACGT
FGE300_35403
FGE297_35402  ~~~~~~~~~~~~~~~~~~~~~~~~AAAAGCTCAACGTGAATGTTGTGTTTCCTCTTTCTTTCAGGGGGGTAATTTTCCTCTGGAGTGTCTTCAGGAGAACGT
FGE302_35403  GTATTATCCACCTATGTTTAACTGAATAGCTCAACGTGAATGTTGTGTTTTCTCTTTCTTTCAGGGGGGTAATTTTCCTCTGGAGTGTCTTCAGGAGAACGT
FGE275_35402  GTATTATCCACCTATGTTTAACTGAATAGCTCAACGTGAATGTTGTGTTTTCTCTTTCTTTCAGGGGGGTAATTTTCCTCTGGAGTGTCTGCAGGAGAACGT
FGE299_35402  ~~~~~~~~~~~~~~~~~~TAACTTGAAATAGCTCAGCGTGAATGTTGTGTTTTCTCTTTCTTTCAGGGGGGTAATTTTCCTCTGGAGTGTCTGCAGGAGAACGT
FGE298_35402  GTATTATCCACCTATGTTTAACTGAATAGCTCAACGTGAATGTTGTGTTTTCTCTTTCTTTCAGGGGGGTAATTTTCCTCTGGAGTGTCTTCAGGAGAACGT
```

FIGURE 3.4.3.1: FIGURE OF ALIGNMENT BETWEEN USEFUL IFNC SEQUENCING DATA AND THE CORRESPONDING NCBI DATA FILE (TOP ROW)

The remaining samples were successfully analyzed in ICE. The sequenced data using the C2 primer pair did not show any edits, so those results are left out of the following part of the report. The samples sequenced with the C1 primer, however, did show quite some variation in the targeted sites. The figure below shows the indel efficiency of each of the four samples, as shown on the ICE results page, with

sample FGE300 showing the highest indel% of over 80% and the other samples showing a significantly lower indel% of under 20%.

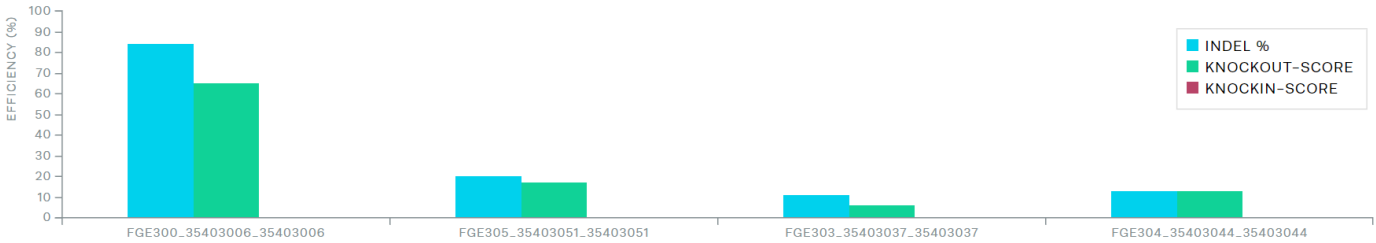

FIGURE 3.4.3.2: DIAGRAM OF INDEL EFFICIENCY OF THE IFNC-EDIT, SEQUENCED WITH THE C1 PRIMER. THIS IMAGE IS CREATED ON ICE.SYNTEGO.COM, USING SEQUENCE DATA RECEIVED FROM GATC.

FIGURE 3.4.3.3: ICE ANALYSIS RESULT FOR THE FGE300 SAMPLE.

The figure above shows the ICE analysis results for the FGE300 sample, with an indel% of 84.

3.4.4 Ifne data analysis

Most of sequenced infe-samples contained long, useful results. Only the GATC ID FGE261 was short, and therefore excluded from further analysis. The sequence data from the samples using the E1 primer pair was easily aligned using BioEdit, as shown in the figure below. There seems to be a single nucleotide polymorphism (SNP) between the NCBI data-file and the SHK-1 cells sequenced in this experiment, located in the middle of the first gRNA aimed at the gene.

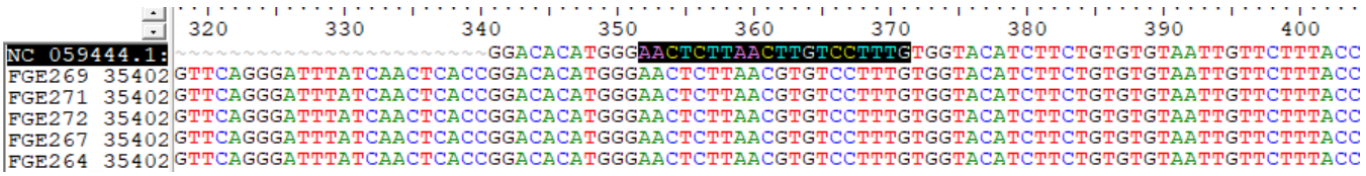

FIGURE 3.4.4: ALIGNMENT OF USEFUL IFNE SEQUENCE DATA, WITH E1 PRIMER PAIR, AND THE NCBI DATA FILE ON THE CORRESPONDING GENE. THE BLACK HIGHLIGHTED STRETCH ON THE TOP ROW SHOWS THE FIRST GRNA AIMED AT THE IFNE GENE.

4 Discussion

Even though parts of the experiment have been cut short due to time issues, parts of the experiment was conducted successfully, and its results show great promise for further analysis, optimization and onward studies.

4.1 Results

4.1.1 Virus titration

The virus titration results show, as mentioned in the results section, the expected trend that the more virus introduced, the more death and decay in the cell cultures. There was no noticeable difference between “fresh” virus used directly after harvesting and virus that had been frozen in the filtrated medium solution for a few weeks. From the result one can see that a virus concentration of around $Nx10^{-4}$ is a decent concentration for further use, as some, but not all wells showed clear signs of infection. This would be a good concentration to compare genetically edited and wildtype SHK-1 cells’ resistance to the ISAV infection, as one could easily score whether more or fewer wells of edited cells get visibly infected by the virus concentration.

However, the number of days between infection and scoring is of great importance, so the concentration should only be used if the experiment designed uses the same timespan for infection. It is also important to note that even though our virus samples, harvested on three separate occasions, showed the same trend of infection degree over time, these results are not necessarily transferable to other studies as these are biological samples.

Even if this is a widely used method of virus infection scoring, it is in no way a very accurate method. The wells in question contain thousands of cells, tiny living organisms, that are almost never all dead or all alive. In practice there is no real (+) or (-), but more of a gradient line with varying degrees of visibly infected cells, combined with a myriad of reasons for the cells’ health conditions, that has nothing to do with the virus infection. The scientist observing the cells is another such biological component and is probably unknowingly biased to what wells to score as (+) and (-).

The results from the top row of wells, with undiluted virus in media directly from virus production, raises some questions as the amount of virus, and nothing else for that matter, is measured before titration. This media has, in our production setup, been used by the SHK-1 cell line producing the virus for several weeks without any refill of nutrients. This could result in the top row dying from lack of nourishment rather than virus infection, or a combination of the two. This concern grows smaller, as the dilutions do, further down the 96 well plate, as higher and higher percentage of the media is freshly made, and not reused. When evaluating the results, one sees that it isn’t an issue either way, as the desired virus concentration is far below the original one and the overall trend stays the same even with the top row excluded.

With that, I would like to conclude the titration as useful as an indication, nothing less or more, and evaluate other options for measure under 4.2: revised methods.

4.1.2 Gel electrophoresis

This was the first step in the process allowing us to evaluate the primer choice. As mentioned in the results part, most PCR-products produces the expected bands around 1000bp.

The exceptions include PCR-product of both 2nd replicates of the ifna1-edited DNA. As shown in table x.x in Appendix II: Additional information and raw data, the nanodrop measured very low amounts in this sample, and as the PCR is done with separate pairs of primers, one can assume that this result shows that the sample in question simply does not have enough DNA available in solution to produce the needed PCR-product volume.

The 1st and 3rd replicate of the ifnb-edited samples also read very low DNA concentration, making the same conclusion logical, seeing as the 2nd and 4th replication with the same primer pair produced the expected bands, with higher DNA concentration in their solutions.

The 4th replicate of the ifnc-edited sample also shows low DNA concentration, though not as low as the other DNA samples that didn't produce any bands. If one looks closely, it looks like there is faint band in the expected area for the C1 primer-pair, but the lack of DNA in the sample does not explain the difference in intensity between the sample prepared from the same DNA sample replicate with the other primer-pair. With this in mind, it seems plausible that something else is off with the sample. Other variations could probably have caused it, for example something wrong with the pipetting technique, causing less volume of DNA sample or primer to enter the PCR tube. Other reasons could be insufficient mixing or vortexing, resulting in unevenly distributed sample in the tube, or the PCR-mixture not getting to the right temperatures.

All samples with the E2 primer pair showed the same band corresponding to a smaller DNA segment. This could be due to contamination of the samples, but it seems unlikely since none of the other samples have bands in that size range. A logical explanation could however be that the primer pair produces an additional, smaller PCR-product from the salmon genome.

None of the samples using infg-edited DNA-samples produced any bands, even though the nanodrop got quite high DNA concentrations from the readings. As only a single primer pair was tried out on these samples, it would make sense if that were where the problem lies. This could include the primers not attaching for some reason, or the PCR temperatures could be wrong for the specific primer pair.

4.1.3 Post-sequencing data analysis

The overall quality of the sequenced samples was low, and there were many samples that had be cut short due to their lack of information. Amongst those of low quality were a few of the wildtype samples, without whom the further ICE analysis was not completable.

The sequence data with longer reads did however provide some information. Most of them were easily aligned with their respective NCBI data files, proving that the primers had adhered to the intended sites, and provided the decent PCR-products of the targeted genes.

The sequence data from samples using the E2 primer pair were also easily aligned, just not with the intended gene. This indicates it was a poor primer choice for the intended gene, although it has received consistent long useful reads. This supports the suspicion from the gel run, that the primers adhere to another gene in the Atlantic salmon genome, and that the PCR therefore produces another, unintended gene transcript.

Of the sequencing data analyzed in ICE, only a few gave meaningful results, and even fewer showed significant gene editing. The ifna1 sample was successfully analyzed with ICE, but proved unaffected by the editing attempt, with 0% editing efficiency. The ifnb gene did not provide any control sample sequence, but the two samples that had decent sequencing data output were paired up as a silly little project, only driven by my own curiosity for the ICE analysis. Although this provided an indel efficiency of 32%, the result provides no actual, usable information due to it being produced by two individually edited samples and does not contain a negative control that the software needs to function as intended. Ifnc proved to be the overall most efficiently edited gene, with up to 80% efficiency in one of the samples. This high efficiency is however not present in the three remaining replicates of the sample, who all remain under 20%, so there is no reason to conclude that this was the result of particularly efficient gRNAs.

In conclusion, there is much more optimizing, trial and error ahead before being able to draw any revolutionary conclusions from these results.

4.2 Revised methods

The Benchling website has proved to not be a very good alignment tool. Upon realizing this, it was already too late to re-align the genes used, design new gRNAs, order and receive them and re-do the electroporation experiment. In hindsight, a better alignment software should have been used, probably resulting in more precise alignments, followed by better gRNAs and higher knockout efficiency in several genes. However, as the lack of time prevented us from creating primers for the closely related genes, they were never sequenced. The bad alignments did not affect the gRNA creation itself, as it was conducted in chopchop, with only the initial genes of interest as input. They did however affect the *choice* of gRNA for each gene group, resulting in some of the less optimal gRNAs getting priority based on their position on the gene alignment. For later reference, a better alignment tool should be used to re-align the related genes and get a better understanding of what domains are conserved between them.

In retrospect, I also question my seemingly blind trust in information sources. Other databases than salmobase.org should have been included in grouping closely related genes. But once more, it does not affect any other part of the experiment than what genes were put together in a table, as primer-pairs were not designed for the related genes, and they were not sequenced. The same goes for off-target effects, whom were taken into consideration in advance of gRNA selection, but excluded from the entire thesis as they were not primed and sequenced, resulting in no data on the matter.

It is impossible to replicate biological samples and experiments in a perfect manner due the myriad of tiny variations, most of which we are completely unaware of and unable to control. Therefore, it would be valuable to include more accurate, tangible, quantitative statistical methods, for example by utilizing a kit for assessing cell culture health parameters, determining a baseline for healthy cell culture metabolism and cross-check ISAV-infected cell lines with this. Even though the manual scoring of death by virus infection by visual observation of confluency in cell culture is a commonly used method, I have doubts about it. I, personally, feel like it is more of a nonchalant guesstimation than an accurate scientific method of measure.

4.3 Downstream experiments

As the intended experiment was not completed, and should be completed, replicated and optimized before further studies are prompted. This includes re-sequencing all samples to provide a better idea of what gRNAs actually worked, and at what efficiency. There should be designed new primer pairs for the latter two gRNAs aimed at ifne, and the gRNAs aimed at ifng.

Then, primers specific for each individual closely related gene should be designed, they should be sequenced, and the knockout efficiency of each gene should be examined. The gRNAs knockout efficiency on the genes sequenced in this study might not be representative for its efficiency on the genes with similar sequence.

This can be done in parallel with the planned ISAV challenge experiment. If that yields any results, the field of interesting genes will be narrowed down to only those differing in ISA virus resistance from the wildtype. If the virus challenge does not show any difference in resistance, one could move on to evaluating whether the genotypic edit has resulted in a phenotypical change.

Further study is also needed to verify that the genes are actually knocked out, as the sanger sequencing only provide information on the change in genomic DNA sequence. Analysis of transcription and translation of the genes is needed to verify the absence of functional protein. Alternative splicing, alternative start codons and insertion/deletion of a multiple of three base pairs can result in the continued production of functional gene products, even if the genomic DNA is successfully altered.

If any specific interferons or interferon-like genes prove to play an important role in ISA virus resistance it would be relevant for further studies. This could include studying its form and exact function, and its biological pathways in salmon immune response. It would also be useful to conduct further knockout studies in other cell types, then organs, then entire organisms, as the interferons are known to function differently is different tissues. Knowing its differences from similar interferons and what is causing it to be particularly effective in ISA virus resistance, would shed light on the mysteries of salmon immune response and possibly provide a deeper understanding of interferons in general.

Such an understanding of the subtle differences in interferons could possibly be used in selective breeding and genetic modification, creating designer immune responses that could protect aquaculture (and possibly humanity) from future virus infections and epidemics.

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Appendix I: Reagents, equipment, and software

Reagents

Product	Catalogue number	Manufacturer
2-Mercaptoethanol	31350010	Gibco
50X TAE Electrophoresis buffer	B49	Thermo Scientific
Agarose	A9539	Sigma
Cell Counting Kit, 30 dual-chambered slides, 60 counts, with trypan blue	1450003	Bio Rad
ECACC General Cell Collection: SHK-1	97111106	European Collection of Authenticated Cell Cultures (ECACC)
ExoSAP-IT™ Express PCR Product Cleanup Reagent	75001.1.EA	Applied Biosystems™ ThermoFisher Scientific
Fetal Bovine Serum	26400044	Gibco
GeneRuler 1 kb DNA Ladder	SM0312	Thermo Scientific
Leibovitz's L-15 media	11415064	Gibco
NEON™ Transfection Kit	MPK1096K	Invitrogen
Nucleic Acid Staining Solution	250-67A532	Nordic BioSite
Opti-MEM™ Reduced Serum Medium	31985047	Gibco
Penicillin-streptomycin	15140122	Gibco
Phosphate buffered saline	20012027	Gibco
Platinum™ II Hot-Start Green PCR Master Mix (2X)	14001013	Invitrogen ThermoFisher Scientific
QuickExtract™ DNA Extraction Solution	QE09050	Lucigen
Trypsin-EDTA solution	25200056	Gibco

All gRNAs were ordered from IDT (Integrated DNA Technologies) at idtdna.com

All primers were ordered from Invitrogen at Thermofisher.com

Equipment

Product	Catalog number	Manufacturer
NanoDrop 8000 Spectrophotometer	ND-8000-GL	Thermofisher Scientific
Neon Transfection System	MPK5000	Invitrogen
TC-20 Automated cell counter	1450102	Bio Rad
The ChemiDoc XRS+ Gel Imaging System		Bio Rad

Software

Software
BioEdit Sequence Alignment Editor
Image lab 6.0

Appendix II: Additional information and raw data.

Bioinformatics

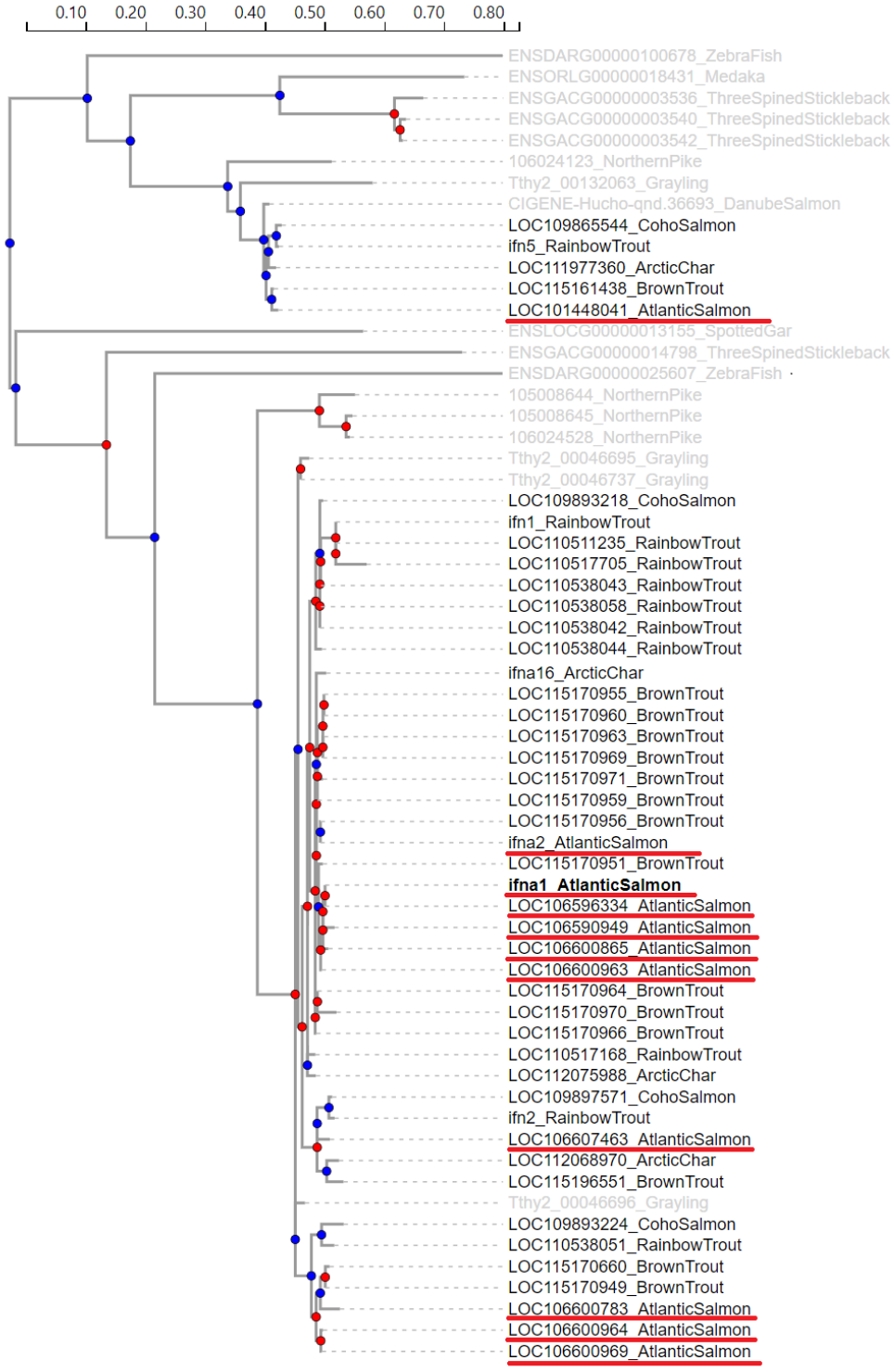

FIGURE A II:1: RAW DATA OF INFA1S CLOSELY RELATED GENES. PHYLOGENETIC TREE COLLECTED FROM SALMOBASE.ORG, WITH MODIFICATIONS UNDERLINING THE GENES IN THE ATLANTIC SALMON GENOME.

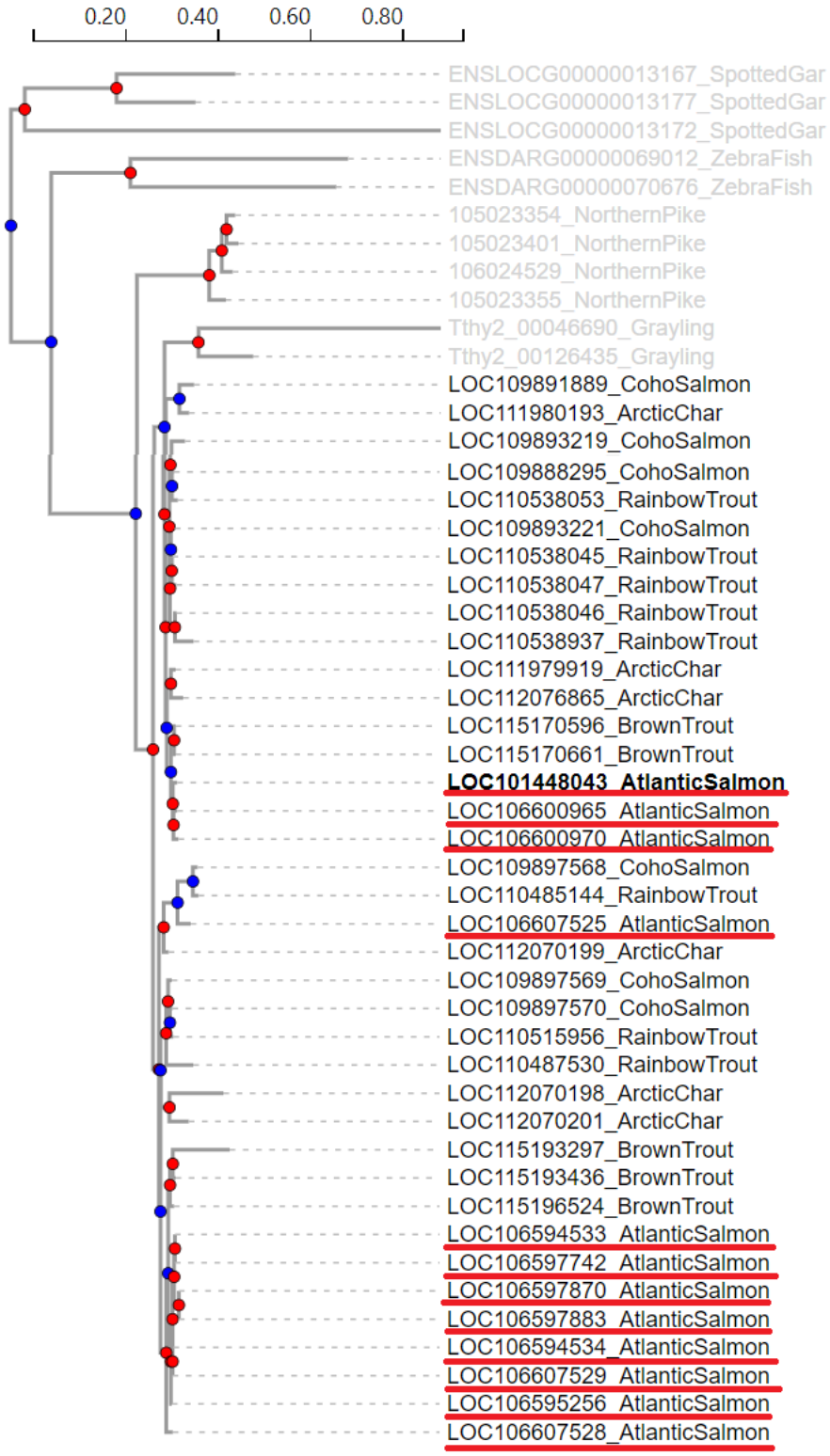

FIGURE A II:2 : PHYLOGENETIC TREE OF IFNC AND ITS CLOSELY RELATED GENES MODIFYED FROM SALMOBASE.ORG. GENES OF ATLANTIC SALMON IS UNDERLINED WITH RED.

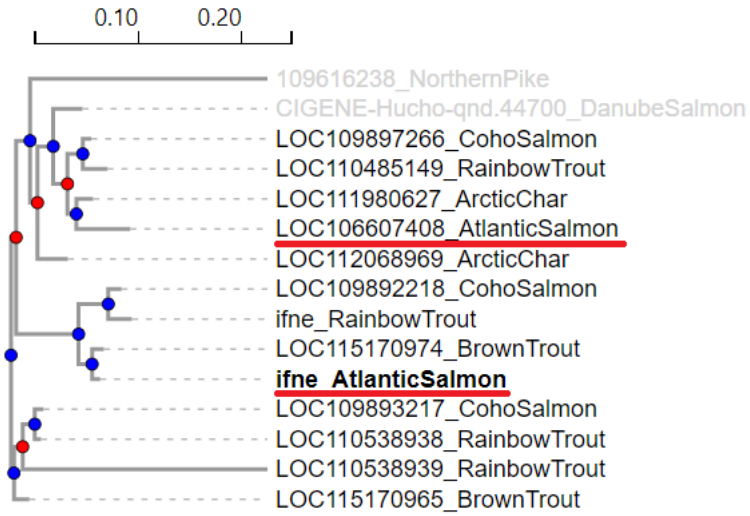

FIGURE A II:3 : PHYLOGENETIC TREE OF IFNE AND ITS RELATED GENES MODIFIED FROM SALMOBASE.ORG, UNDERLINING THE GENES IN ATLANTIC SALMON WITH RED.

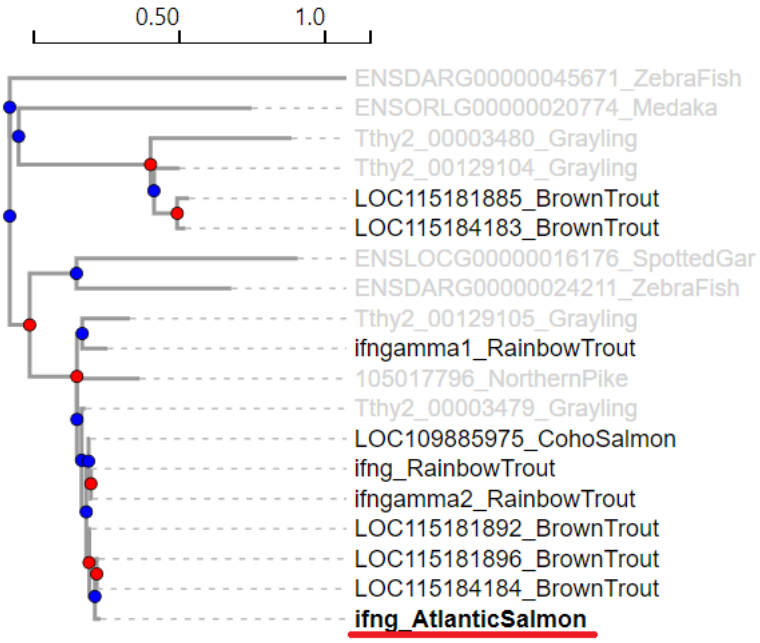

FIGURE A II:4 : PHYLOGENETIC TREE MODIFIED FROM SALMOBASE.ORG, UNDERLINING SOLELY IFNG, AS IT IS THE ONLY GENE ON THIS TREE FOUND IN THE ATLANTIC SALMON GENOME.

Lab raw data

TABLE A II: 1 : DNA CONCENTRATION MEASURED BY NANODROP

Gene	Duplicate nr.	DNA concentration (ng/µl)
Ifna	1	112,3
	2	26,13
	3	97,16
	4	129,0
ifnb	1	32,91
	2	81,22
	3	31,69
	4	131,3
ifnc	1	76,99
	2	83,11
	3	54,41
	4	47,67
ifne	1	171,6
	2	141,7
	3	210,7
	4	95,19
ifng	1	203
	2	350
	3	353
	4	150
WT	1	258,7
	2	235,8
	3	336,3

	4	228,2

TABLE A II:2 : LIST OF GATC ID SEQUENCES, AND THE CONTENTS OF THE RESPECTIVE SAMPLES. USEFUL SEQUENCING DATAFILES ARE MARKED WITH AN X.

GATC ID seq.	Useful seq. data (X)	Template DNA	Electroporation Replication number	Primer pair
317	X	Infa1-edited	1	A1
312			2	
313	X		3	
314	X		4	
309		Wildtype	1	
318		Infa1-edited	1	A2
319			2	
320	X		3	
315			4	
316	X	Wildtype	1	
310		Infb-edited	1	B
311	X		2	
306			3	
307	X		4	
308		Wildtype	2	
303	X	Infc-edited	1	C1
304	X		2	
305	X		3	
300	X		4	
301	X	Wildtype	3	
302	X	Infc-edited	1	C2
297	X		2	
298	X		3	
299	X		4	
275	X	Wildtype	3	
271	X	Infe-edited	1	E1
272	X		2	
267	X		3	
269	X		4	
264	X	Wildtype	4	
265	X	Infe-edited	1	E2
266	X		2	
261			3	
262	X		4	
263	X	Wildtype	4	

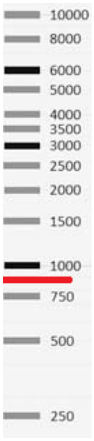

FIGURE A II: 5 : 1KB LADDER, ADAPTED FROM THERMOFISHER.COM. THE AREA OF EXPECTED PCR PRODUCT SIZE IS HIGHLIGHTED IN RED.

Appendix III: Genomic data

FASTA files of all utilized genes are put in attached PDF file.

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