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Impact of CRISPR/Cas9 on the expressions of selected immune genes in salmonid cells

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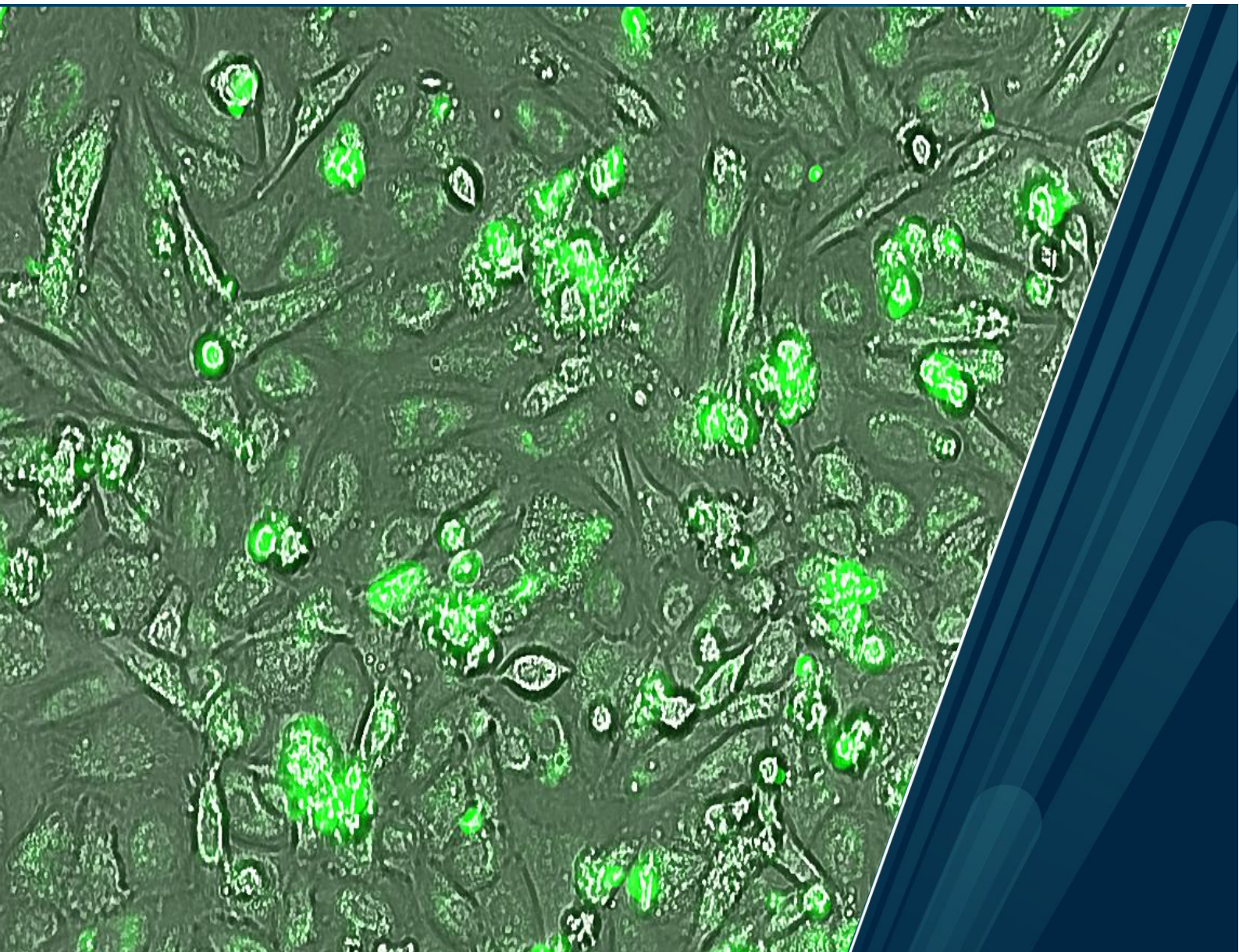

“…, for he who promised is faithful”

Hebrews 10:23

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Abstract

The discovery of CRISPR/Cas9 as a gene editing tool has revolutionized the field of molecular biology due to its huge potential for innovative applications. However, the biosafety concerns on CRISPR/Cas9-mediated gene editing like off-target mutations, triggering of cell death, and induction of cellular stress need to be elucidated. This thesis aims to investigate the impacts of electroporation and the individual components of CRISPR/Cas9, *i.e*. sgRNA, Cas9 and RNP complex on ASK-1 cells, using the gene expression of *hsp90, hsp70, mhc I, igt* and *igm* as indicators of the impacts. Electroporation was used as a transfection method to edit the *cr2* gene and deliver the individual components of CRISPR/Cas9 into the ASK-1 cells. The relative gene expression of the target genes was measured using RT-qPCR and *ef-1α* was used as the reference gene. The results of this study showed that electroporation as a cell transfection method do not affect the expression of *hsp90* and *mhc I* in ASK-1 cells. On the other hand, the *hsp70* was significantly upregulated in Day 2 and Day 7 samples of sham treatment (shocked cells only), however, this might not be due to electroporation since the effect was not seen in other treatments where electroporation was also employed as a mode of transfection. The CRISPR/Cas9 components, sgRNA, Cas9, and RNP complex did not show any effect on the expression of *hsp70* and *mhc I*. Moreover, the expression of *hsp90* was not affected by sgRNA and Cas9 components, however it was significantly upregulated in the RNP complex treatment group, both in Day 2 and Day 7 samples. This effect might be the consequence of *cr2* gene mutation, as many studies have shown that *hsp90* was upregulated during mutations to buffer its lethal effect. The gene expression of *igm* and *igt* genes were too low to detect, thus, their gene expression cannot be calculated. To conclude, this study has shown that the electroporation and the CRISPR/Cas9 components did not cause cellular stress nor affected the expression of the immune genes. The impacts that were seen seem to be not attributed to the CRISPR/Cas9-mediated gene editing procedure and components. However, these findings were limited only for the protocols and conditions used in this study using ASK-1 cell line as a model.

Abbreviations

18s RNA	18s ribosomal RNA
ACTB	β-actin
ASK	Atlantic salmon kidney
bp	Base pair
CP	Crossing point
cr2	Complement receptor 2
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated proteins 9
crRNA	CRISPR RNA
DF	Dilution factor
DNA	Deoxyribonucleic acid
DSB	Double-stranded breaks
EF-1α	Elongation factor-1α
FACS	Fluorescence-activated cell sorting
G6PDH	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOI	Gene of interest
HKG	Housekeeping gene
HSP	Heat shock protein
hsp70	Heat shock protein 70 kilodaltons
hsp90	Heat shaock protein 90 kilodaltons
Ig	Immunoglobulin
kDa	Kilodalton
MGEs	Mobile genetic elements
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
ng	Nanogram
NRT	No reverse transcriptase
NTC	No template control
NUC	nuclease
PAM	protospacer adjacent motif
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
RNA	ribonucleic acid
RNP	Ribonucleoprotein
RNP complex	ribonucleoprotein complex
RPS20	ribosomal protein S20
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
sgRNA	single guide RNA
TALENS	Transcription Activator-like Effector Nucleases
tracrRNA	trans-activating RNA
ZFN	zinc finger nucleases

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1 INTRODUCTION

Over the years, different types of genomes editing tools have been developed to make precise targeted changes to the genome of living cells. They have played a vital role in solving problems in the fields of agriculture, human medicine, veterinary medicine, and aquaculture.

In 2012, a new technology, the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated proteins 9 (CRISPR/Cas9) emerged that revolutionized genome editing (Kozovska et al., 2021). This tool is so powerful that the discoverers, Emmanuelle Charpentier and Jennifer A. Doudna have received a Nobel Prize in Chemistry in 2020. The CRISPR/Cas9 technology has been applied in the fields of agriculture (Kumar et al., 2021), medicine (Liu et al., 2021) and in epigenetics (Kozovska et al., 2021). In aquaculture, the technology was used to modify and introduce favorable genes in fishes to make them more resistant to diseases and enhance their natural immunity (Ferdous et al., 2022). For example, CRISPR/Cas9 was used to edit the genes of channel catfish (Au - Elaswad et al., 2018), grass carp (Ma et al., 2018), and farmed carp (Chakrapani et al., 2016) to make them disease-resistant. CRISPR/Cas9 was also used in editing Atlantic salmon (A. salmon; *Salmo salar*) to produce a sterile fish (Wargelius et al., 2016) and increase the omega-3 production (Datsomor et al., 2019).

Compared to the other gene editing tools such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and meganucleases that can also cause targeted mutations in cells, CRISPR/Cas9 has been widely accepted since its discovery due to its precision, site specificity, relatively low cost, ease of design and simplicity. For biosafety and risk assessment purposes, precise and targeted gene editing tools are paramount because they avoid indiscriminate mutations at non-target genomic sites, thus limiting or completely eliminating unwanted off-target effects. The efficiency of the CRISPR/Cas9 technology notwithstanding the off-target and on-target effects wherein the mutations at sites that differ from the target region (Fu et al., 2013) or induces unwanted pleotropic effects after targeted gene editing; trigger an RNA-sensing innate immune response which results in cell death (Kim et al., 2018); and its long-term effects have still not fully been elucidated. Further, the CRISPR/Cas9 was found to induce cellular stress (Johnston et al., 2020) in certain cell types.

1.1 CRISPR/Cas system

The CRISPR/Cas system is an RNA-guided adaptive immune system of several bacteria and archaea (Newsom et al., 2021). The CRISPR was first observed in *Escherichia coli* in 1987 (Ishino et al., 1987) while the association of Cas genes to CRISPR was discovered much later (Jansen et al., 2002).

The CRISPR is composed of palindromic repeats and spacers. The palindromic repeats are identical short segments of DNA that is 20 to 40 base pairs (bp) in length (Jansen et al., 2002), while the spacers, on the other hand, are unique DNA fragments which were later discovered to be identical to viral or bacteriophage DNA fragments that had previously infected the prokaryotes. This has led to the conclusion that CRISPR is a form of adaptive immune response the bacteria and archaea use to protect themselves against mobile genetic elements (MGEs) such as virus and bacteriophage (Shabbir et al., 2019). The cas, on the other hand, are genes that are involved in the cutting of the target DNA (nucleases), mediating the integration of spacer into the CRISPR array (integrases), or unwinding the DNA (helicases) (Asmamaw & Zawdie, 2021).

When a bacteriophage injects its viral genome inside the bacterial cell, the bacteria use the CRISPR/Cas system to counteract the infection. The Cas enzyme excises a short fragment from the viral DNA and incorporates it as a spacer into the CRISPR repeat-spacer array within the host genome as a new spacer, together with the other fragments taken from previous infections (Jiang & Doudna, 2017). These viral fragments or spacers are inserted in between the repeated palindromic sequences. Through this mechanism, it provides a genetic memory for future invasion of the same virus, thus forming its adaptive immune system. Upon reinfection of the same virus, the CRISPR/Cas system is activated and degrades the viral genome (Bhattacharya et al., 2020).

1.2 CRISPR/Cas9 system as a gene editing tool

The CRISPR/Cas adaptive immune system of prokaryotes has been repurposed into a programmable RNA-guided DNA targeting platform for gene editing known as the CRISPR/Cas9 system.

CRISPR/Cas9 system is a type II CRISPR/Cas system that uses a single Cas protein originally from *Streptococcus pyogenes* (Li et al. (2020). It is composed of Cas9 and single-guide RNA (sgRNA) (**Figure 1**.). Cas9 is a large (152 kDas DNA endonuclease and is sometimes called the "genetic scissor" (Asmamaw & Zawdie, 2021). It consists of two regions, the recognition lobe and the nuclease (NUC) lobe. The recognition lobe are the domains responsible for binding to the guide RNA, while the NUC lobe are the domains responsible for cutting each single-stranded DNA and initiates the binding to the target DNA through the protospacer adjacent motif (PAM) (Asmamaw & Zawdie, 2021). The double-stranded breaks (DSBs) are made at a site 3 bp upstream of the PAM (Jiang & Doudna, 2017). The sgRNA, on the other hand, is a complex containing the CRISPR RNA (crRNA) and trans-activating RNA (tracrRNA). The crRNA is an 18-20 bp that is specifically designed to pair with the target DNA, while the tracrRNA is a loop that provides a binding scaffold for the Cas 9 endonuclease (Asmamaw & Zawdie, 2021). The crRNA provides the specificity and can be designed to target any gene sequence. This is one of the reasons this gene editing tool is powerful and revolutionary.

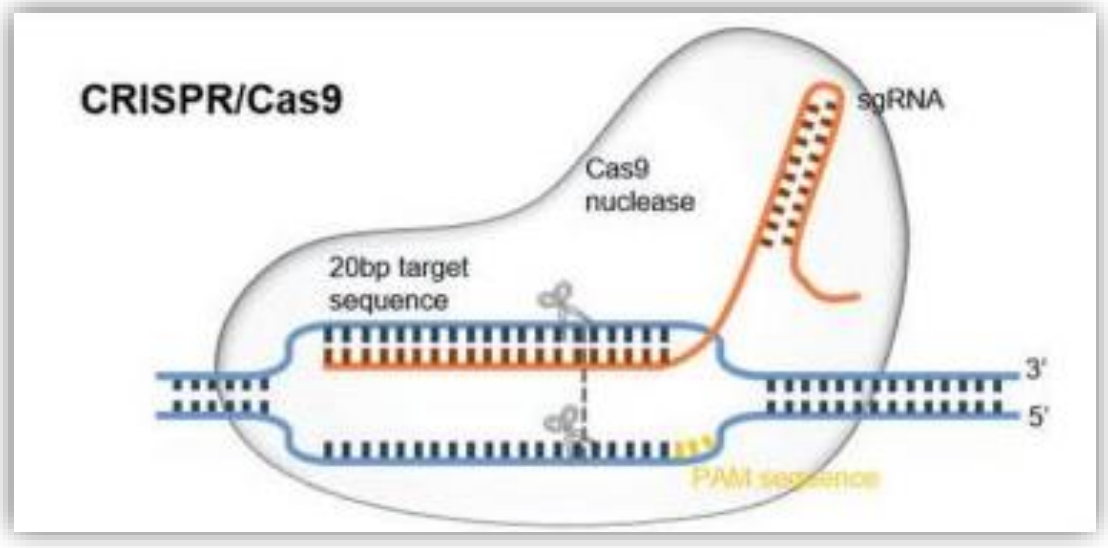

Figure 1. ***CRISPR/Cas9 structure showing the Cas9 and sgRNA components****. Source: Li et al., 2020*

The mechanism of CRISPR/Cas9 gene editing involves three steps, *i.e*. recognition, cleavage, and repair. The sgRNA recognizes the target sequence in the gene of interest through its crRNA

complementary base pair component. It will then direct the Cas9 to make a DSB at a site 3 bp upstream to appropriate PAM that will trigger the local DNA melting followed by RNA-DNA hybrid formation. This will activate the Cas9 for DNA cleavage (Asmamaw & Zawdie, 2021). The gene is edited when the host' cellular machinery repairs the DSBs by deletion and/or insertion (indel) causing mutations (Strømsnes et al., 2022).

1.3 Relationship between cellular stress response and immune gene expression.

Cells are constantly exposed to various stress factors that threaten their functionality. Some examples of stressors are heat, UV, heavy metals, toxin, pathogens, viruses, bacteria, etc. To cope with stress, the cells activate their survival mechanisms (Muralidharan & Mandrekar, 2013), – the cellular stress response. Cellular stress response is a complex process that involves changes in gene expression, protein synthesis, and cellular metabolism that respond to changes in intracellular and extracellular conditions as well as fluctuations that damage the structure and function of macromolecules (Poljšak & Milisav, 2012). Depending on the type and severity of stress, cells can re-establish themselves to homeostasis or adopt an altered state (Poljšak & Milisav, 2012).

Basically, there are four types of cellular stress responses: induction of cell repair mechanism, induction of autophagy, triggering of cell death, or temporary adaptation (adaptive response) (Poljšak & Milisav, 2012). Most of these responses are geared toward cellular homeostasis. The adaptive response, however, is observed during low exposure to stress, *e.g*. low dosage of radiation, low dose of mutagen, low exposure to chemicals, etc. (Poljšak & Milisav, 2012).

1.3.1 Heat shock proteins as cellular stress-related genes

Heat shock proteins (HSPs) are markers for cellular stress. One example is the use of electroporation, a transfection method for introduction of genetic materials into cells, and which is used in CRISPR/Cas9 mediated gene editing. The HSPs are a family of intracellular proteins which are highly conserved in many organisms ranging from bacteria to humans (Smith et al., 1999). They are named according to their molecular weight, *e.g*. hsp30, hsp70, hsp90, etc. which refers to 30, 70 and 90 kilodaltons (kDa) in size, respectively. Under normal condition,

HSPs chaperone proteins from one compartment to the other; help refold damaged proteins; and protect newly synthesized protein to form into functional form (Jurivich & Zhou, 2007).

Under cellular stress, the HSPs protect the cells from damage. One study has shown that the mRNAs of hsp70 and hsp90 were upregulated during thermal stress to provide protection against the cytotoxic consequences of protein denaturation (Smith et al., 1999). Some studies have also reported the expression of HSPs during stressful conditions such as cold temperature (Matz et al., 1995), UV light (Cao et al., 1999), wound healing, tissue remodelling (Laplante et al., 1998), and stressful environmental conditions. Thus upregulation of HSP was described as a part of stress response (Santoro, 2000).

1.3.2 Heat shock proteins and immune response

The HSPs were also seen to be involved in the pathways of innate and adaptive immune system. A study showed that in the MHC I antigen presentation pathway, HSPs role is to transfer peptides from proteasome to MHC I. Failure to bind the peptide to hsp70 and hsp90 resulted to MHC I not being able to present the peptide (Binder et al., 2001).

Immune sentinels have HSP receptors that can detect their presence in the extracellular environment. The abundant presence of HSPs at the extracellular environment indicates a loss of cellular integrity, *i.e*. non-conventional cellular expression on the membrane, pathological cell death, and abnormal active secretory mechanism which can elicit an immunological response against cancer cells or pathogen-infected cells, an inflammatory response, or suppress an on-going immunity (Binder, 2009).

There is some suggestion that autoimmunity can be triggered by the sustained release of HSPs in the extracellular environment. During homeostasis, the HSPs are inaccessible to their receptors. However, when there is a continued release of HSPs outside the cell, proinflammatory conditions are elicited, and when these conditions are sustained for a long time it can result to autoimmunity (Binder, 2014).

1.4 Gene expression quantification

1.4.1 Housekeeping genes

Housekeeping genes are genes that are constitutively expressed in a cell because they are required for basic cellular functions and cell maintenance. This is also the reason why they are frequently used to normalize mRNA levels between different samples. However, since their expression also varies, depending on the type of cells or cellular conditions, it is critical to use the right housekeeping gene in gene expression studies. The criteria that can be used in selecting the appropriate housekeeping genes (or reference gene) are good PCR efficiency and stable expression. Some examples of housekeeping genes are elongation factor-1α (EF-1α), β-actin (ACTB), 18s ribosomal RNA (18s RNA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PDH), and ribosomal protein S20 (RPS20). These genes have been profiled and validated in immune relevant tissues and cells of A. salmon (Ingerslev, 2006; Julin, 2009). The reference gene is important in calculating the the relative gene expression of the selected immune genes.

1.4.2 Immune genes

1.4.2.1 Immunoglobulin

Immunoglobulins (Ig) or antibodies are Y-shaped glycoproteins produced by B lymphocytes that play an important role in humoral immune response. They can be membrane-bound where they function as antigen receptors on B cells to initiate B cell activation, or as secreted antibodies that neutralize and opsonize microbes and toxins (Abbas et al., 2020).

There are three major classes of immunoglobulin isotypes identified in teleosts, *i.e*. IgM, the predominant surface Ig isotype, IgD and IgT/IgZ (Peñaranda et al., 2019). In A. salmon, the amount of μ transcripts was 200 times more than δ, and the τ transcripts is 20 times more than δ (Hordvik, 2015).

IgM is the first antibody that is secreted when an antigen is detected by the body. Due to its pentameric conformation, it has higher avidity, however it tends to have a lower affinity during the primary response (Abbas et al., 2020). IgT/IgZ in salmonid fish has four constant domains and is specialized to mucosal immune response (Hordvik, 2015).

1.4.2.2 Major Histocompatibility Complex I

The genes of Major Histocompatibility Complex (MHC) are involved in the adaptive immune system. In general, foreign peptides as a result of degradation of intracellular pathogens are presented by the MHC class I molecules to cytotoxic T cells (Abbas et al., 2020).

The genotypes of MHC class I in A. salmon have been found to provide resistance to infectious diseases such as *Aeromonas salmonicida*, infectious salmon anemia virus (ISAV), and infectious haematopoietic necrosis virus (IHNV) (Kjøglum et al., 2006).

1.5 Relative quantification of gene expression

The quantitative Reverse Transcription Polymerase Reaction (qRT-PCR) is a molecular biotechnology technique that is used to amplify, detect and quantify nucleic acids for various applications. This method has high sensitivity, good reproducibility and has a wide quantitative range (Bustin et al., 2005).

There are two methods to quantify the gene expression. One is by absolute quantification and the other is by relative quantification. Absolute quantification determines the gene expression by relating the PCR signal to a standard curve (Svec et al., 2015). The standard curve can be prepared using diluted PCR products, sample from a treatment group, etc. The relative quantification, on the other hand, relate the PCR signal of the target gene to a reference. A reference can be an endogenous control, exogenous control, a reference gene index or a target gene index (Pfaffl, 2006). Relative quantification does not require a standard curve to determine the level of expression. The gene expression is calculated by taking the differences between the ΔCt (threshold cycle) or ΔCP (crossing point) measured (Pfaffl, 2006).

There are two ways to perform the relative quantification. One is by using the double delta Ct (ΔΔCt) of Livak method and the other one is by Pffafl method. The difference between the two is that the Livak method assumes that the PCR efficiency of the target and the reference gene is around 2 (100%) and the PCR efficiency difference between them is within 5%. The Pfaffl method does not have this assumption, thus it uses the calculated PCR efficiency of the target gene and the reference gene.

The PCR Efficiency describes how a target gene is amplifying. The ideal PCR efficiency is when the number of molecules double per amplification cycle, which corresponds to PCR Efficiency of 2 or 100% PCR Efficiency. However, efficiency is still considered acceptable or "good" when it lies between 1.9 and 2.1 which corresponds to 90% - 110% (Svec et al., 2015).

In order to achieve the best optimal relative expression results, normalization of expression is important. The relative expression data have to be normalized by using the same amount of template and/or by using a stable reference gene. It is very important that the reference gene is stable because this will be used to normalize the expression of the target gene as well as the kinetics of the PCR (Pfaffl, 2006).

1.6 Complement receptor 2

In mammals, the complement receptor 2 (CR2 or CD21) is part of the regulators of complement activation system that plays an important role in humoral immune response (Boackle, 2018). They are expressed by B lymphocytes and function as a receptor to C3d (Boshra et al., 2006). The involvement of CR2 enhances the antigen-dependent activation responses of B cells (Abbas et al., 2020). In teleost, little is known about their role and structure, however, it has been shown in carp that the C3d can bind to peripheral lymphocytes (Boshra et al., 2006).

1.7 Atlantic Salmon Kidney (ASK) cells

Atlantic salmon kindey (ASK) is an epithelial cell line isolated from the kidney of *Salmo salar* (**Figure 2**). In euryhaline teleost, the kidney's main function is ion transport and osmoregulation. In salmon, the kidney, together with the gills and intestine are the primary organs that is involved in osmoregulation (Klykken et al., 2022).

Kidney is one of the major lymphoid tissues in teleost fishes (Press & Evensen, 1999). The ASK cell line was used to improve understanding on host immune response against pathogens. One study has shown that ASK cells were an effective model to characterize the interferon and IFN-induced gene expression upon salmonid alphavirus infection (Munir et al., 2020). Several studies also used the ASK cells to investigate A. salmon's response upon ISAV infection (Andresen et al., 2020); (Svingerud et al., 2013); (Rolland et al., 2005)

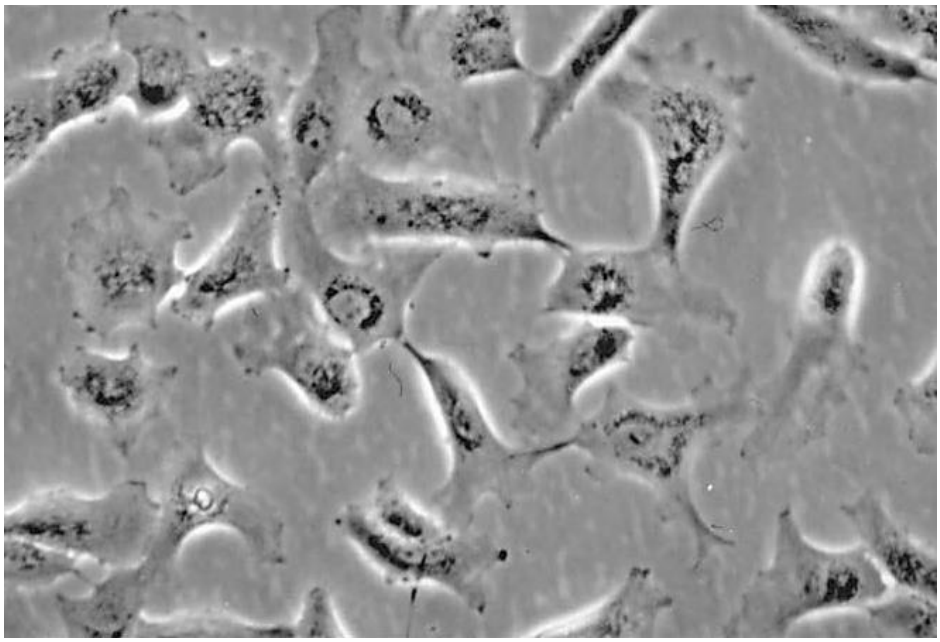

*Figure 2.**Atlantic Salmon Kidney cells of Atlantic salmon (Salmo salar)**. Image taken at 200X magnification. Source:* Wergeland & Jakobsen, 2001

1.8 Main objective

Most studies on electroporation as a method of transfecting cells were focused on its efficiency as a gene editing procedure. Studies on the impact of the procedure itself on the cells is lacking. Furthermore, there are no studies available that look at the impacts of the individual components of CRISPR/Cas9, namely, sgRNA and Cas9, while very few studies are available investigating the impacts of the RNP complex on the cells. However, this is understandable as this gene editing technology is still new. The principal components of the editing system were only discovered in less than a decade (Gostimskaya, 2022). In this study, the focus will be to investigate these areas and hope that it can provide additional information that can address these gaps and lack of information.

This study aims to evaluate the impact of the CRISPR/Cas9 gene editing tool on the expression of selected immune genes when the Atlantic Salmon Kidney (ASK-1) cell line is subjected to gene editing.

Specific objectives:

1. To separately determine the impacts of Cas9, sgRNA, and Cas9-sgRNA complex (ribonucleoprotein -RNP) on the expression of stress marker genes, *hsp70* and *hsp90*, as well as immune genes *mhc I*, *igt* and *igm* in ASK-1 cell;
2. To determine the impacts of the electroporation transfection method on the gene expression of stress marker genes, *hsp70* and *hsp90*, as well as immune genes *mhc I*, *igt* and *igm* ASK-1 cell;

1.9 Justification for the selection of the target genes

This study has chosen the genes, *hsp90, hsp70, igm, igt* and *mhc I* to investigate the impacts of CRISPR/Cas9 gene editing procedure and its components on their expression. The immune genes were chosen because of their main role in the adaptive immune system and their expression in kidney cells.

1.10 *cr2* as the gene target for CRISPR/Cas9-mediated gene editing

The complement receptor 2 (*cr2*) gene was the CRISPR target in this study. The choice of the *cr2* gene was arbitrary and was chosen from a list of genes previously optimized for CRISPR/Cas9 gene editing in the project group at NORCE (Strømsnes et al., 2022).

The sgRNA sequence is UGCGUGUGUGGAUAGGACAA and the product size is 289 bp (Strømsnes et al., 2022).

1.11 Justification for the selecting ASK-1 cell as a model

The main reason the ASK-1 cell was chosen as a model in this study is because the kidney is one of the major lymphoid tissues in teleost fishes (Press & Evensen, 1999). Moreover, the ASK cells were also used to improve understanding on host immune response against pathogens. Several studies used the ASK cells to investigate A. salmon's response upon ISAV infection (Andresen et al., 2020); (Svingerud et al., 2013); (Rolland et al., 2005)

2 MATERIALS AND METHODS

2.1 Biological material

This study has used Atlantic salmon kidney 1 (ASK-1) cells as a model to measure the mRNA expression of the target genes. These were obtained from the Federal Research Institute for Animal Health, Germany.

2.2 Chemicals, kits and primers

The chemical and kits that is used in this thesis is shown in **Table 1**.

Table 1. ***List of important chemicals and kits used during the conduct of this thesis including the product number and where was it used.***

Purpose	Chemicals/Kits	Product number	Source
Cell culture	Gibco™ Leibovitz L-15 with 4 mM L-glutamine and L-amino acids (1x)	11415-049	Thermofisher Scientific, Netherlands
Cell culture	Trypsin-EDTA solution (0.25%)	T 4049	Sigma-Aldrich, USA
Cell culture	Dulbecco's Phosphate Buffered Saline	D 8537	Sigma-Aldrich, USA
Cell culture	Fetal Bovine Serum	S00K910331	Biowest (Biowest, Brazil)
sgRNA synthesis	EnGen ® sgRNA synthesis kit, *S. pyogenes*	NEB E3322V/S	New England Biolabs, UK
sgRNA synthesis	Monarch RNA Cleanup kit	NEB T2040	New England Biolabs, UK
sgRNA synthesis, qPCR	RNAse-free water	J71786-XCR	Thermofisher Scientific
Gel electrophoresis	DNA loading dye (6X)	R0611	Thermo Scientific
Gel electrophoresis	RNA loading dye (2X)	00867048	Invitrogen (Thermo Scientific)
Gel electrophoresis	SeaKrem® Le Agarose	0000645702	Bio Nordika, US
Gel electrophoresis	PAGE Gel red Nucleic Acid	19P1230	Biotium, US
Gel electrophoresis	Gene ruler 1 kb +	SM 1333	Thermo Scientific

Cell density determination	Trypan blue stain (0.4%)	EBT-001	NanoEntek
Electroporation	CAS9GFPPRO	0000139756	Sigma-Aldrich, Israel
Electroporation	Gibco Opti-MeM (1X) Reduced serum medium	31985-047	Thermofisher Scientific, UK
	Neon Transfection System 10 µl	MPK10096	Invitrogen,US
RNA isolation	RNAqueous™ total RNA isolation kit	AM 1914	Invitrogen, Lithuania
cDNA synthesis	Quantitech Reverse Transcription Kit	205311	Qiagen, Germany
Endpoint PCR	DreamTaq PCR Master Mix (2x)	00762885	Thermo Scientific, Lithuania
qPCR	Power track SYBR Green Master mix (2X)	01104866	Applied Biosystems
qPCR	Yellow buffer (40X)	0196 6885	Applied Biosystems
DNA Isolation	GenElute Mammalian Genome DNA Miniprep kit	SLCF0735	Sigma Aldrich, US

Table 2. ***Housekeeping genes evaluated in this study as potential reference gene candidates for relative quantification of target genes***

Housekeeping gene	Gene name	Sequence (5'-3')	GeneBank Accession number	Tm (˚C)	Amplicon size	Reference
Elongation factor 1α	EF-1α	Fwd: GCTGTGCGTGACATGAGG Rev: ACTTTGTGACCTTGCCGC	AF321836	64.6 64.0	88	(Ingerslev et al. 2006)
18S ribosomal RNA	18S rRNA	Fwd: CCTTAGATGTCCGGGGCT Rev: CTCGGCGAAGGGTAGACA	AJ427629	64.0 64.1	67	(Ingerslev et al. 2006)
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Fwd: AAGTGAAGCAGGAGGGTGGAA Rev: CAGCCTCACCCCATTTGATG	BU693999	67.1 67.7	96	(Julin, Johansen, and Sommer 2009)
β-actin	ACTB	Fwd: AAGATGAAATCGCCGCAC Rev: ATGGAGGGGAAGACAGCC	AF012125	63.8 64.2	97	(Ingerslev et al. 2006)
Glucose-6-phosphate dehydrogenase	G6PDH	Fwd: TGGTGCAGAACCTCATGGTCCTCA Rev: ATCCCGGATGATTCCAAAGTCGTC	CB498878	73.9 71.6	155	(Julin, Johansen, and Sommer 2009; Jorgensen et al. 2006)
Ribosomal Protein S20	RP S20	Fwd: AGCCGCAACGTCAAGTCT Rev: GTCTTGGTGGGCATACGG	AY953432.1	63.8 64.1		(Ingerslev et al. 2006)

Table 3. ***An overview of the target gene primers used in this study***

Immune gene	Gene name	Sequence (5'-3')	GeneBank Accession number	Tm (˚C)	Amplicon size	Reference
Heat shock protein 90	hsp 90	Fwd: TTGCGTGGAACTAAGGTGA Rev: CCAATGAACTGAGAGTGCT	NM_001146473.1	61.9 57.9	104	(Gadan et al. 2012)
Heat shock protein 70	hsp 70	Fwd: TGACGTGTCCATCCTGACCAT Rev: CCAGCCGTGGCCTTCAC	AJ632154	67.9 68.3	57	(Gadan et al. 2012)
Major Hiscompatibility Complex I	mhc I	Fwd: GAAGAGCACTCTGATGAGGACAG Rev: CACCATGACTCCACTGGGGTAG	ABX44766.1	64.4 67.7	112	(Chang et al. 2015)
Immunoglobulin M	IgM	Fwd: TGAGGAGAACTGTGGGCTACACT Rev: TGTTAATGACCACTGAATGTGCAT	Y12457.1	66.1 65.1	69	(Chang et al. 2015)
Immunoglobulin T	IgT	Fwd: CAACACTGACTGGAACAACAAGGT Rev: CGTCAGCGGTTCTGTTTTGGA	ACX50290	66.2 69.4		(Chang et al. 2015)

2.3 Flow of the project

The main objective of this thesis is to study the impacts of electroporation and CRISPR/Cas 9 components in ASK-1 cells, by using the expression of *igm*, *igt*, *mhc I*, *hsp90* and *hsp70* as indicators. The workflow and the different methods that were used in this study is presented in Figure 3.

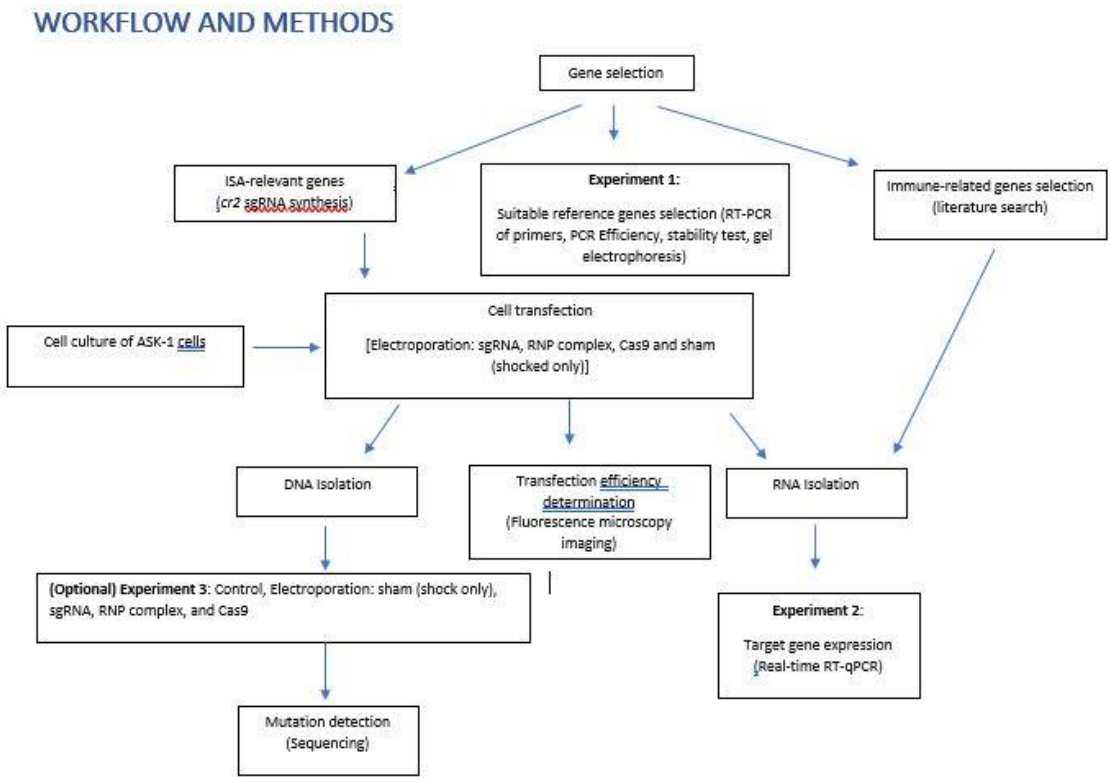

Figure 3. ***The workflow and the methods that were conducted in this project are presented here****.*

2.4 Cell culture

The ASK-1 cells were cultured in Falcon T75 and T175 flasks containing Gibco™ Leibovitz L-15 medium (1x) supplemented with 4 mM L-glutamine and L-amino acids (Thermofisher Scientific, Netherlands) and 10% Fetal Bovine Serum (Biowest, Brazil). The cells were routinely passaged at 1:2 when it reached ~70% confluency using modified Dulbecco's Phosphate Buffered Saline (PBS) without calcium chloride and magnesium chloride (Sigma-Aldrich, USA) and Trypsin-EDTA solution (0.25%) (Sigma-Aldrich, USA). They were kept in the dark while being incubated at 22°C.

2.5 Cell transfection

2.5.1 Experimental set-up in well plates

There were two experimental groups used in this study, the two-day post-electroporation (2D) and 7-day post-electroporation (7D). The nucleic acids were isolated 2 days after electroporation in 2D and after 7 days in 7D group.

Each experimental group has four treatments, *i.e*. RNP-complex (cells transfected with 9:1 sgRNA:Cas9), Cas9 (cells transfected only with Cas9), sgRNA (cells transfected only with sgRNA) and sham (shocked only cells).

The RNP-complex treatment group was cultured in 6-well plates so the cells have enough space to grow, since this group will be transfected with 60 µl of the complex per well. This will avoid passaging of the cells during the experiment period (Plate # 1). This plate contained 5 ml of filtered L-15 conditioned media. A conditioned media is a 1:1 volume of fresh media and old spent media with 20% FBS. The old spent media was filtered using 20 µm cellulose acetate membrane filter (VWR, Puerto Rico) before mixing with 20% FBS. The rest of the treatment groups were cultured in a 12-well-plate with 2 ml filtered L-15 conditioned media (Plate # 2). This plate contained the control, sgRNA, Cas9 and sham treatment groups (**Figure 4**).

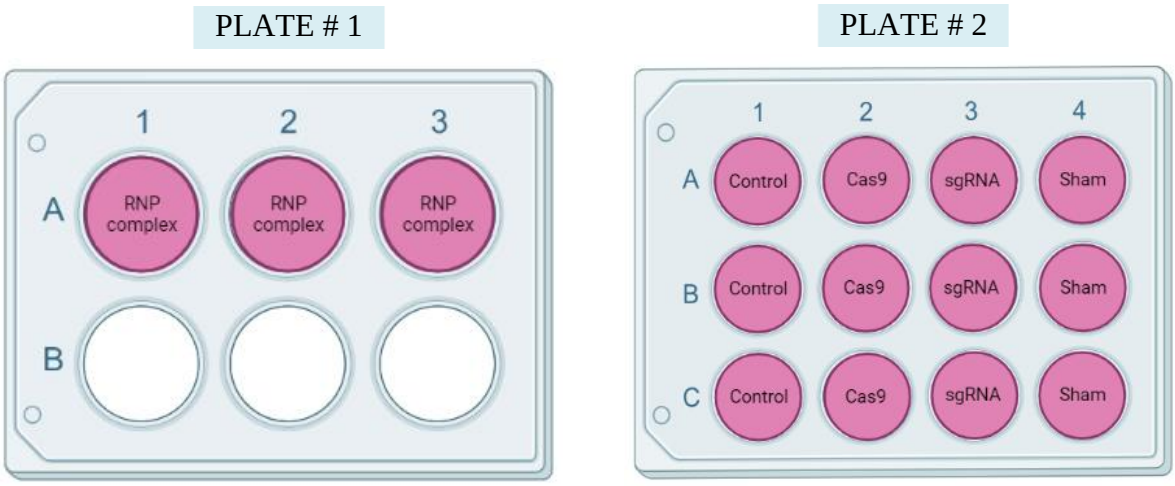

Figure 4. ***Experimental set up for electroporation****. Plate # 1 contains the RNP complex treatment group and the Plate # 2 contains the sgRNA, Cas9, sham (shocked only cells) and the control groups. All groups were set-up in triplicate. Each well contains filtered L-15 conditioned media. The plates were kept in the dark at 22˚C. The Images were created in BioRender.com*

The experimental set-up for single-cell clonal isolation of cells transfected with RNP complex is a 6-well plate containing 5 ml of filtered (20 µm cellulose acetate membrane filter, VWR, Puerto Rico) L-15 conditioned media (**Figure 5**). This plate contains the RNP complex with 3 replicates, Cas9 (positive control) and sham (negative control). There was only one sample prepared for these groups because they will only be used in setting the gating during Fluorescence-activated cell sorting (FACS).

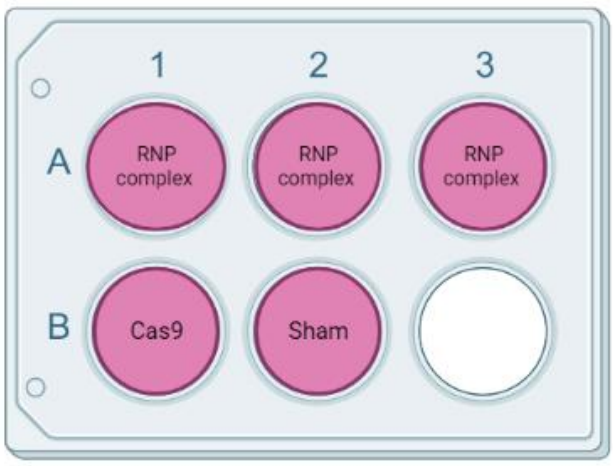

Figure 5. ***Experimental set-up for single-cell cloning of RNP complex treatment group.*** *Each well contains filtered L-15 conditioned media. The plates were kept in the dark at 22˚C Images were created in BioRender.com*

2.5.2 Synthesis of sgRNA

The single guide RNA (sgRNA) oligos targeting the *cr2* genomic site in A. salmon genome was designed and synthesized using the EnGen ® sgRNA synthesis kit, *S. pyogenes* (New England Biolabs, UK). The synthesis was performed following the manufacturer's protocol, except for the incubation step which was modified to 2 hours at 30˚C (instead of 30 min). The synthesized sgRNA was purified using the Monarch RNA Cleanup kit following the manufacturer's protocol (New England Biolabs, UK) and eluted with 25 µl RNAse-free water (Thermo Scientific). A 1 µl purified sample was used to evaluate the purity as well as the concentration of the synthesized sgRNA using Nanodrop 2000c Spectrophotometer (Thermo Scientific).

The quality and size of the synthesized sgRNA was visualized in gel electrophoresis. Prior to running in the gel, the purified samples were denatured to avoid the formation of RNA secondary structures. A 10 µl RNA loading dye (2X) (Invitrogen (Thermo Scientific) was added to 10 µl sample and incubated at 61˚C for 1 hour. The agarose gel was prepared using 2% SeaKrem® Le Agarose (Bio Nordika, US), 70 ml TBE buffer (TRIS, Boric acid, EDTA), and 0.7 µl PAGE Gel red Nucleic Acid (Biotium, US) and run at 90V for 1 hour.

2.5.3 Transfection of cells

2.5.3.1 Preparation of adherent cells

The culture media was aspirated from the flask and the cells were washed at least twice with Dulbecco's PBS without calcium chloride and magnesium chloride (Sigma-Aldrich, USA). The cells were detached using 2 ml Trypsin-EDTA solution (Sigma-Aldrich, USA) and resuspended in 8 ml Gibco^{TM} Leibovitz L-15 medium (1x) supplemented with 4 mM L-glutamine and L-amino acids (Thermofisher Scientific, Netherlands) and 10% FBA. It was centrifuged at 300 g for 5 min at room temperature. The media was aspirated and the cells were washed twice with PBS and resuspended in 750 µl Opti-Mem (1x) reduced serum medium (Thermo Scientific, UK).

2.5.3.2 Cell density determination

From the resuspended cells, 15 µl aliquot was mixed with 15 µl trypan blue stain (0.4%) (NanoEntek), and a 10 µl of this mixture was loaded to each side of the counting slide. The

cell density was determined using EVE automated cell counter (NanoEntek Eveplus, Korea). The target cell density for electroporation was 10^6 per ml which was based on the previously optimized protocol (Strømsnes et al., 2022).

2.5.3.3 RNP complex formation

The RNP complex was prepared at sgRNA:Cas9 (CAS9GFPPRO, Sigma-Aldrich) by mixing the sgRNA and Cas9 (9:1) and incubating for 15 min in room temperature. The formed RNP complex was placed on ice until used.

2.5.3.4 Preparation of CRISPR/Cas 9 components

Each treatment group contains the cells, Opti-MeM and a component of CRISPR/Cas9. The mixture contains cells:CRISPR/Cas9 component in 1:1 ratio. The calculation of each mixture and the volume of each component were shown in **Table 4**.

Table 4. ***The mixture for each CRISPR/Cas9 components used in the experiment is presented here***

Groups	**CRISPR/Cas9 component**				**Cells total volume** (µl)
	sgRNA (µl)	**Cas 9** (µl)	**Opti-MEM** (µl)	**Total volume** (µl)	
RNP complex	X	1	Y	30	30
Cas9	-	1	29	30	30
sgRNA	X	-	Y	30	30
Sham shock control	-	-	30	30	30
Negative control	-	-	30	30	30

X: volume of the sgRNA is dependent on the concentrations of the synthesized sgRNA; Y: volume of Opti-Mem; (-) nothing was added

The volume of sgRNA (X) depends on the concentration of sgRNA synthesized (Z). See **Equation 1** for the calculation. The volume of Opti-Mem (Y) per treatment group is the difference between 30 µl and total volume of CRISPR/Cas9 component(s).

$$\text{Equation 1.} Vol\ of\ sgRNA\ (X) = \frac{7.8\ ug}{Z\ (ug/ul)}$$

2.5.3.5 Electroporation protocol

Cell transfection was done by electroporation. Electroporation of the cells was performed using Neon Transfection System (Invitrogen, US). The previously optimized parameters: 1600 V, 10 ms, 3 pulses were applied using 10 µl Neon tip. One electroporation shock has a total volume of 10 µl, *i.e*. 5 µl cell sample from a group and 5 µl CRISPR/Cas9 component(s); see **Table 5** .

*Table 5.**The number of electroporation shock per well and the total volume per well used were described below**. Each electroporation shock has a volume of 10 µl, which is composed of 5 µl cell sample from a group and 5 µl CRISPR/Cas9 component.*

Group	No. of shocks per well	Total volume per well (µl)
RNP complex	6	60
Cas9	3	30
sgRNA	2	20
Sham	2	20
Control	0	30

The electroporated cells were dispensed in 6-well plate (RNP complex treatment group) and 12-well plate (the rest of the treatment groups and control) containing filtered conditioned media according to the setup in (**Figure 6),** and incubated in the dark at 22^{o}C. This experiment was repeated twice, one for 2-day post-electroporation and one for 7-day post-electroporation set-up.

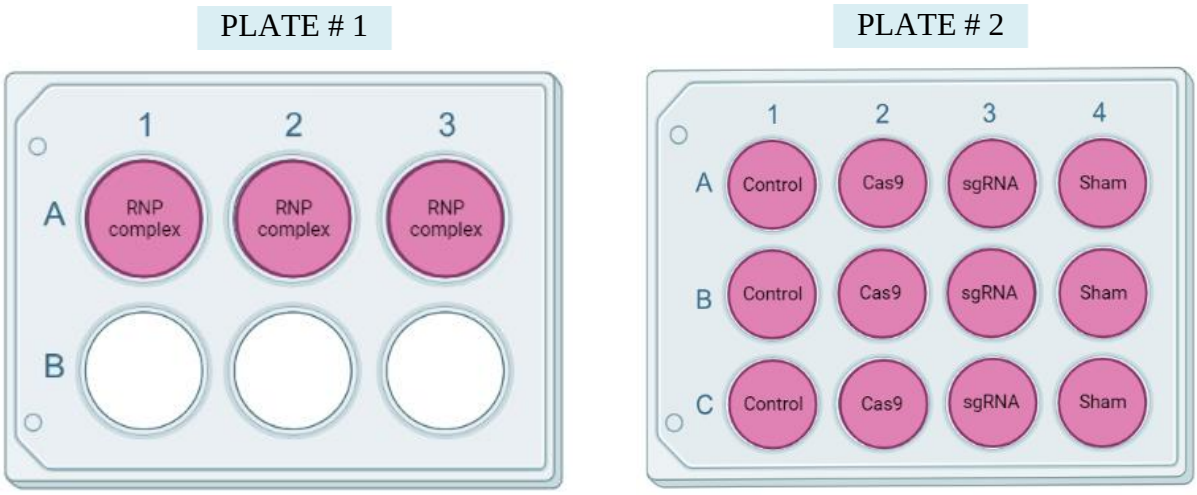

Figure 6. ***Electroporation set-up containing the cell sample and the CRISPR/Cas9 component per well****. Each group has three technical replicates. The plates were incubated in the dark and kept at 22˚C. Images were created in BioRender.com*

2.6 Fluorescence Imaging

Cell images were taken using Zeiss Axio imager using 200X magnification and Axiocam 202 mono prior to isolation of nucleic acids and flow cytometry-assisted cell sorting (FACS) to determine the transfection efficiency. To avoid bias in capturing the images, all images were taken after 10s, both for the light channel and fluorescent channel. Images were processed using the ImageJ program. The transfection efficiency (%) was computed per replicate using **Equation 2.**

$$\text{Equation 2. } Transfection\ efficiency\ (\%) = \frac{number\ of\ fluorescent\ cells}{total\ number\ of\ cells}\ (100)$$

2.7 Total RNA Isolation

The cell culture media were aspirated and the cells were washed twice with PBS. The total RNA was extracted using RNAqueous^{TM} total RNA isolation kit (Invitrogen, Lithuania) following the manufacturer's protocol, and eluted in 50 µl elution buffer. The yield and purity

were measured using the Nanodrop 2000c Spectrophotometer (Thermo Scientific). As a quality control measure, synthesis of cDNA was performed immediately after RNA isolation.

2.8 cDNA synthesis

Residual gDNA was removed from the isolated total RNA prior to cDNA synthesis by adding 2 µl gDNA Wipeout Buffer (7x) to 10 µl RNA template and incubated at 42˚C for 2 min. The cDNA was synthesized using Quantitech Reverse Transcription Kit (Qiagen, Germany). A master mix was prepared by mixing 1 µl Quantitech Reverse Transcriptase, 4 µl Quantiscript RT buffer (5x) and 1 µl RT primer mix. The master mix was added to the RNA template and incubated at 42˚C for 15 min and at 95˚C for 3 min to inactivate the Quantitech Reverse Transcriptase. All samples were stored at -20˚C until further use.

2.9 Primer Integrity Test

2.9.1 Endpoint PCR

The cDNA template was amplified using DreamTaq PCR Master Mix (2x) (Thermo Scientific, Lithuania). The mixture contains 25 µl DreamTaq PCR Master Mix (2x), 1 µl Forward primer (10 µM), 1 µl Reverse primer (10 µM), 5 µl cDNA template (5 ng/µl), and 18 µl nuclease-free water in a 50 µl reaction volume. The endpoint PCR was carried out with the following setting: 95˚C (1 min), followed by 35 cycles of 95˚C (30 s), 58˚C (30s), 72˚C (1 min), and 72˚C (10 min), 4˚C ∞.

The list of primers used in this study is listed in **Table 2** and **Table 3**.

2.9.2 Gel electrophoresis

Gel electrophoresis was performed to visualize the endpoint PCR products. A 1% agarose was prepared using SeaKrem® Le Agarose (Bio Nordika, US), 70 ml TBE buffer (TRIS, Boric acid, EDTA), and 0.7 µl PAGE Gel red Nucleic Acid (Biotium, US) and run at 90V for 1.5 hours. The amplicons were added with 6x DNA loading dye (New England Biolabs, UK) prior to

running in the gel. A 1 kb + gene ruler (Thermo Scientific) was used as a ladder to determine the size of the amplified products.

2.10 Selection of a suitable reference gene

In order to determine the most suitable reference gene for the relative quantification of the target genes under the conditions applied in this study, six housekeeping genes, namely, *ef-1α, actb, 18s RNA, g6pdh, gapdh,* and *rp s20* were evaluated. The criteria used for the evaluation were primer integrity, good PCR Efficiency % (90-110%) and stable expression of the gene under the conditions applied in this study.

2.10.1 Determination of primer integrity

A cDNA template which was prepared from ASK-1 cell from control group was amplified in endpoint PCR using different housekeeping gene primers, *i.e. ef-1α, actb, 18s RNA, g6pdh, gapdh,* and *rp s20*. A master mix for each primer was prepared using 25 µl DreamTaq Master mix, 1 µl forward primer (10 M), 1 µl reverse primer (10 M) and 18 µl nuclease-free water. A 5 µl cDNA template (25 ng) was added to the master mix to give a total volume of 50 µl per reaction. The PCR settings used were as follows: initial denaturation at 95˚C for 1 min, 1 cycle; 35 cycles of denaturation at 95˚C for 30 s, annealing at 58˚C for 30 s, extension at 72˚C for 1 min; and final extension at 72˚C for 10 min. The PCR was performed using S1000 Thermal Cycler (Bio-rad).

The PCR products were visualized in the gel electrophoresis. The 1% agarose gel was prepared using SeaKrem® Le Agarose (Bio Nordika, US), 70 ml TBE buffer (TRIS, Boric acid, EDTA), and 0.7 µl PAGE Gel red Nucleic Acid (Biotium, US). The samples were added with 6x DNA loading dye (New England Biolabs, UK) and the gel electrophoresis was performed using 90V for 1 hour. A 1 kb + gene ruler was used as a ladder.

2.10.2 Determination of PCR Efficiency

The next criteria is PCR Efficiency. The PCR Efficiency was calculated using qPCR. A master mix for each primer which contains 0.8 µl forward primer (10 µM), 0.8 µl reverse primer (10 µM), 0.5 µl yellow sample buffer, 10 µl Power track SYBR green master mix (2x) and 6.9 µl nuclease-free water was prepared. A 1 µl of cDNA template (5 ng) was added to the master mix to give a total volume of 20 µl per reaction. A stock solution was prepared and diluted in RNAse-free water to give a final concentration of 5 ng/ul (1:1). A serial dilution with a dilution factor of 10 was prepared to make 4 standards, *i.e*. 1:1, 1:10, 1:100 and 1:1000.

The qPCR was performed using Light Cycler 480 ® (Roche) with the amplification program settings: pre-incubation at 95˚C for 2 minutes, amplification at 95˚C for 15 seconds, 40 cycles of annealing at 60˚C (60 s), and 1 cycle of extension at 72˚C for 10 minutes.

Data analyses were performed using the LightCycler software version 1.5. The crossing point (CP) was calculated by using the maximum second derivative function on the LightCycler software. The PCR Efficiency was determined using an external standard. The PCR Efficiency was calculated based on Pfaffl, et al. (2001). The slope (a) was taken from the linear regression model (

Equation *3*) wherein the model was fitted by plotting the CP values of the standard curve (y) against the log transformed concentration of the standard curve (x).

Equation 3. Linear regression formula $y = \alpha x + b$

The PCR Efficiency and PCR Efficiency % for each housekeeping genes were calculated using **Equation 4** and **Equation 5**, respectively.

Equation 4. PCR Efficiency: $E = 10^{-1/slope}$

Equation 5. $PCR\ \% = (E - 1) \cdot 100$

2.10.3 Stable expression evaluation

The next criteria in choosing the suitable reference gene is stable expression. Only the housekeeping genes that exhibited primer integrity and good PCR Efficiency were chosen to evaluate their stability. The impact of electroporation on the stability of expression of the qualified housekeeping genes was determined by comparing the expression of these genes in the control group and electroporated group.

The cells were electroporated (shocked only) using Neon Transfection System (Invitrogen, US). The previously optimized parameters: 1600 V, 10 ms, 3 pulses were applied using 10 µl Neon tip. One electroporation shock has a total volume of 10 µl, *i.e.* 10 µl cell sample.

The control and electroporated cells were cultured in 12-well plates with 2 ml Gibco™ Leibovitz L-15 medium (1x) supplemented with 4 mM L-glutamine and L-amino acids (Thermofisher Scientific, Netherlands) and 10% Fetal Bovine Serum (Biowest, Brazil). Additional media was added during the experiment to prevent the wells from drying up.

Seven groups were compared in this experiment, *i.e.* Day 1, Day 2, Day 3, Day 4 (Plate # 1), Day 5, Day 6 and Day 7 (Plate # 2). The different days refer to the day the total RNAs were isolated. There were 3 replicates per group. The cells in Plate # 1 and Plate # 2 were electroporated, while the cells in Plate # 3 is the control group (**Figure 7**).

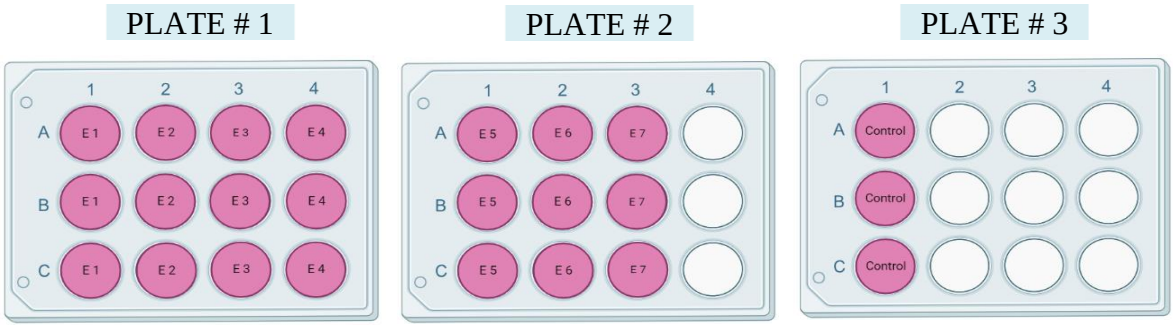

Figure 7. ***Experimental set-up comparing the gene expression of 6 different housekeeping genes in electroporated and control groups****. Plate #1 and Plate #2 contained samples that were electroporated while Plate #3 contained the control group. Plate #1 has Day 1 to Day 4 samples while Plate #2 has Day 5 to Day 7 samples. The RNAs were isolated every day for 7 days and each day has 3 replicates. The day number corresponds on the day the RNA was isolated. Images were created in BioRender.com*

Using quantitative absolute quantification, the expression of each housekeeping gene was calculated using the maximum second derivative function on the LightCycler software. Their stability was evaluated using BestKeeper-1 software (www.gene-quantification.de), an excel-based tool that was developed by Pfaffl et al. (2004). The factors that were looked at to determine the stability of expression was the variation, *i.e.* standard deviation (SD) and coefficient of variance (CV) of the calculated gene expression. A housekeeping gene that has a SD of more than 1 will be considered unstable (Pfaffl et al., 2004). All data processing was based on the crossing points (CPs).

2.11 Relative quantification of target genes expression

2.11.1 qPCR analysis

The qPCR 96-well set-up is consisted of samples from control group, treatment group, negative controls (no template control and no reverse transcriptase control), positive control (sample in 1:20 dilution) and standard curve. The control group had 3 replicates, *e.g.* 2D Control 1 (A1-A3), 2D Control 2 (A4-A6) and 2D Control 3 (A7-A9), and each replicate has 3 technical repeats, *e.g.* 2D Control 1 (A1, A2, and A3). Similarly, the treatment groups consisted of 3 replicates and 3 technical repeats. (**Figure 8**).

A total of 40 assays were analysed, *i.e.* 2 experimental set-up (Day 2 and Day 7), 4 treatment groups (sham, sgRNA, Cas9, RNP complex), and 5 target genes (*hsp90*, *hsp70*, *igm*, *igt* and *mhc I*).

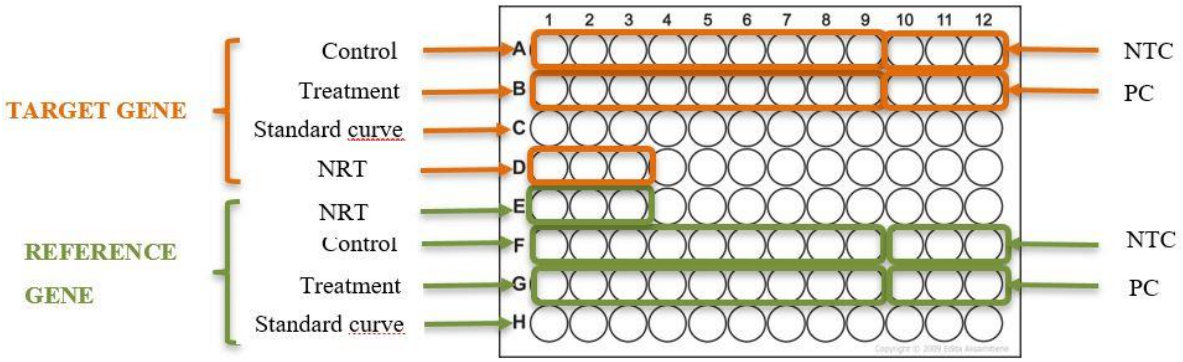

Figure 8. ***qPCR set-up on a 96-well plate****. Each well contains the master mix and sample, except the NTC wells where the samples were replaced with RNAse-free water. The A to D wells contain the target gene the E to H wells contain the reference gene (ef-1α). NTC: No template control; PC: Positive control; NRT: No reverse transcriptase control.*

Each well contains 1 µl of cDNA template with a stock concentration of 5 ng/µl for *hsp90, igt* and *mhc I*, and 20 ng/ul stock concentration for *hsp70* and *igm*. The master mix contained 0.8 µl forward primer (10 µM), 0.8 µl reverse primer (10 µM), 0.5 µl yellow sample buffer, 10 µl Power track SYBR green master mix (2x) and 6.9 µl nuclease-free water. The total PCR mix volume is 20 µl.

The following is the describes the content of each well:

Control wells: The well contains 19 µl master mix and 1 µl cDNA template from control group.

Treatment wells: The well contains 19 µl master mix and 1 µl cDNA template from treatment group.

No template control: The well contains 19 µl master mix and 1 µl RNAse-free water.

No reverse transcriptase: The well contains 19 µl master mix and 1 µl NRT sample. The NRT sample was prepared by mixing 4 µl Quantiscript RT buffer (5x), 1 µl RT primer mix, 10 µl RNA template from a treatment group and 1 µl RNAse-free water. The sample was incubated at 42˚C for 15 min and at 95˚C for 3 min.

Positive control: The well contains 19 µl master mix and 1 µl cDNA template from PC samples. The PC samples were prepared by mixing 4 µl Quantiscript RT buffer (5x), 1 µl RT

primer mix, 10 µl RNA template from a treatment group and 1 µl Quantitech Reverse Transcriptase. The sample was incubated at 42˚C for 15 min and at 95˚C for 3 min. The RNA template is composed of 3 biological triplicate samples of a treatment group diluted in 1:20 RNAse-free water.

Standard curve: The standard curve for *hsp90, igt* and *mhc I* contained 15 µl master mix and 5 µl cDNA template per well, while the *hsp70* and *igm* contained 7.9 µl cDNA template and 12.1 µl master mix.

2.11.2 Standard curve preparation

***hsp90, igt* and *mhc I* standard curve preparation:**

A stock solution containing cDNA template from 7D RNP complex was prepared and diluted in RNAse-free water to give a final concentration of 5 ng/ul (1:1). A serial dilution with a dilution factor of 10 was prepared to make 4 standards, *i.e.* 1:1, 1:10, 1:100 and 1:1000.

***hsp70* and *igm* standard curve preparation:**

A stock solution containing cDNA template from 7D RNP complex was prepared and diluted in RNAse-free water to give a final concentration of 20 ng/ul (1:1). A serial dilution with a dilution factor of 10 was prepared to make 4 standards, *i.e.* 1:1, 1:10, 1:100 and 1:1000.

2.11.2.1 PCR settings

The qPCR was performed with the Light Cycler 480 ® (Roche) using the program settings: pre-incubation at 95˚C for 2 minutes, amplification at 95˚C for 15 seconds, 40 cycles of annealing at 60˚C (60 s), and 1 cycle of extension at 72˚C for 10 minutes.

2.11.3 Relative quantification of gene expression

The mRNA gene expression was quantified based on the mathematical model of Pfaffl, which states that the relative expression ratio (*R*) of a target gene is calculated based on the PCR Efficiency (E) and the CP deviation of an unknown sample versus a control, and expressed in

comparison to a reference gene (Pfaffl, 2001). In this model, the target gene expression is normalized by the reference gene expression (**Equation 6)**.

$$\text{Equation 6. } ratio\ (R) = \frac{(E\ target)^{\Delta CP\ target(control - sample)}}{(E\ reference)^{\Delta CP\ reference\ (control - sample)}}.$$

The CP or "crossing point" is the point where the fluorescence is registered above the background fluorescence (Pfaffl, 2001). E_{target} refers to PCR Efficiency of the target gene while the $E_{\text{reference}}$ to the PCR Efficiency of the reference gene. The $\Delta CP_{\text{target}}$ is the deviation of control group minus the sample of the target gene, while the $\Delta CP_{\text{reference}}$ is the deviation of control – sample of the reference gene (Pfaffl, 2001). The ΔCP was calculated by taking the average CP of the control group and deduct it to the CP of each sample. The ΔCP is calculated for each sample.

The calculation of PCR Efficiency was discussed in detail in Section 2.11.2

The data analyses were performed using the LightCycler software version 1.5. The CP was calculated by using the maximum second derivative function on the LightCycler software. Gene expression was normalized to a reference gene and presented as expression change relative to the controls (See Appendix III: Target gene relative expression calculation

An example of the calculation of the relative gene expression ratio is shown in Appendix IV: Target gene relative expression ratio calculation.

2.12 Fluorescence Activated Cell Sorting (FACS)

The Day 7-post electroporation ASK-1 cells that were transfected with RNP complex was resuspended in 1.5 ml L-15 conditioned media. It has a concentration of ~2.4 x 10^6 cells/ml. A conditioned media is a 1:1 volume of fresh media and old spent media with 20% FBS. The old spent media was filtered using 20 µm cellulose acetate membrane filter (VWR, Puerto Rico) before mixing with 20% FBS. The cells were sorted through a 130 µm nozzle using BD FACS

Aria III cell sorter (BD, NJ, USA). Single-cell events were gated using cells transfected with Cas9 as a positive control and sham cells as a negative control. The cells that contained the RNP complex-EGFP were separated from the cells that did not have the fluorescent protein using 488 nm laser. The GFP-positive cells were collected into a 96-well plate containing 150 µl of filtered conditioned media for single-colony culture. It was incubated in the dark at 22˚C. The cells were checked once to two times per week and refill with conditioned media every 2 weeks.

2.13 DNA isolation

The culture media was aspirated from the well-plate and the adherent cells were washed with PBS twice. A 20 µl proteinase K solution followed by 200 µl of lysis solution C were added to lyse the cells and were scraped from the well-plate using a cell scrapper. The genomic DNA was isolated using GenElute Mammalian Genome DNA Miniprep kit (Sigma Aldrich, US) following the manufacturer's protocol. The DNA was eluted in 25 µl elution buffer included in the kit. The yield and purity were measured using the Nanodrop 2000c Spectrophotometer (Thermo Scientific). All samples were stored in - 20˚C until further use.

2.14 Statistical analysis

All statistical analyses were performed using R software version 4.2.3. The normality of data distribution was checked using Shapiro-Wilk test. Linear regression model was used to model the mRNA expression of the target genes in the different treatments and control group. Kruskal-wallis was used to validate and/or clarify the result of the model. Dunn test was used for post-hoc analysis.

3 RESULT

The use of CRISPR/Cas9 system as a genome editing tool has revolutionized the field of molecular biology since its discovery. Since this is a relatively new technology, there are still few studies conducted on its side effects and risks. This study has investigated the impact of electroporation, as a cell transfection method, and the impacts of the components of CRISPR/Cas9 on ASK-1 cells, using gene expression of *hsp90*, *hsp70*, *mhc I*, *igt*, and *igm* as indicators. The relative gene expression of the target genes was measured using RT-qPCR and normalized using EF-1α as the reference gene.

3.1 Cell culture of ASK-1 cells

The image of untreated ASK-1 cells is shown in Figure 9. The cells were cultured in Falcon T75 and T175 flasks with L-15 media, kept in the dark and incubated at of 22˚C.

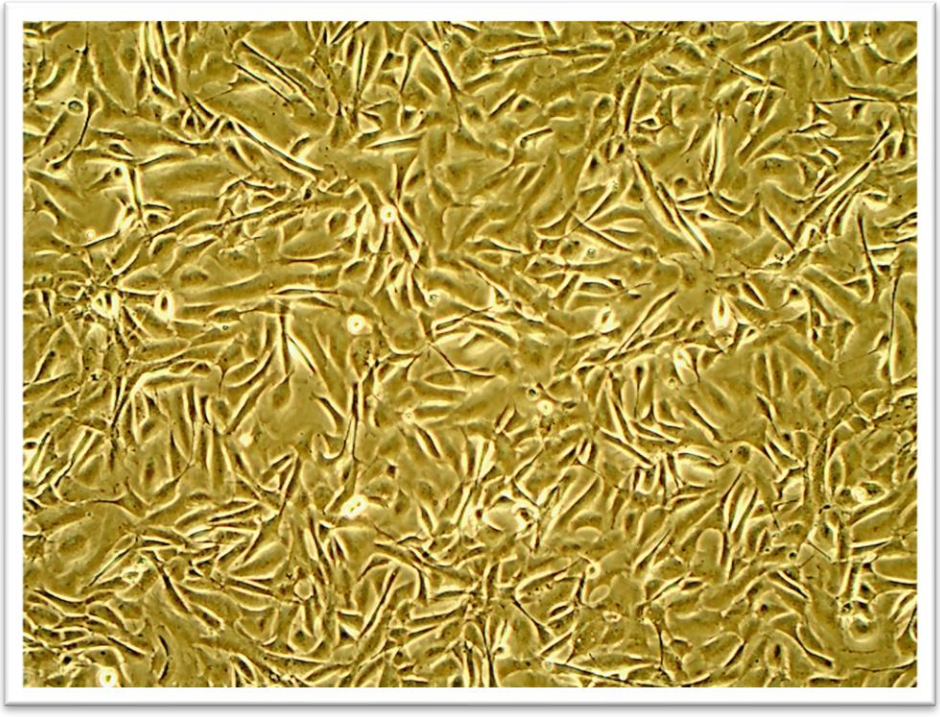

Figure 9. ***Cell culture of Atlantic Salmon Kidney cells of Salmo salar****. This is the image of untreated ASK-1 cells taken at 100x magnification. Source: J. Palerud*

3.2 Evaluation of the best reference gene candidate

3.2.1 Primer integrity

For the calculation of the relative expression of the target genes, six housekeeping genes, *i.e. gapdh*, ef-*1α, g6pdh, rp s20, actb, and 18s rRNA* were evaluated in this study to select the best candidate for a reference gene. These housekeeping genes were chosen because they were already tested in A. salmon based on published papers.

The integrity of the primers for the housekeeping genes was assessed by evaluating the band size, presence or absence of non-specific bands of PCR amplicons on (1%) agarose gel. All the tested primers showed single clear bands except for RP S20. Among the 6 primers, the ACTB, EF-1α and 18S rRNA have stronger bands than G6PDH and GAPDH, while the RP S20 did not show any band. The ACTB, EF-1α and 18S rRNA have the correct band size but not the G6PDH and GAPDH. The amplicon size of each housekeeping gene is presented in Table 2. The sizes of bands were estimated using the GeneRuler 1 kb plus DNA ladder (Figure 10).

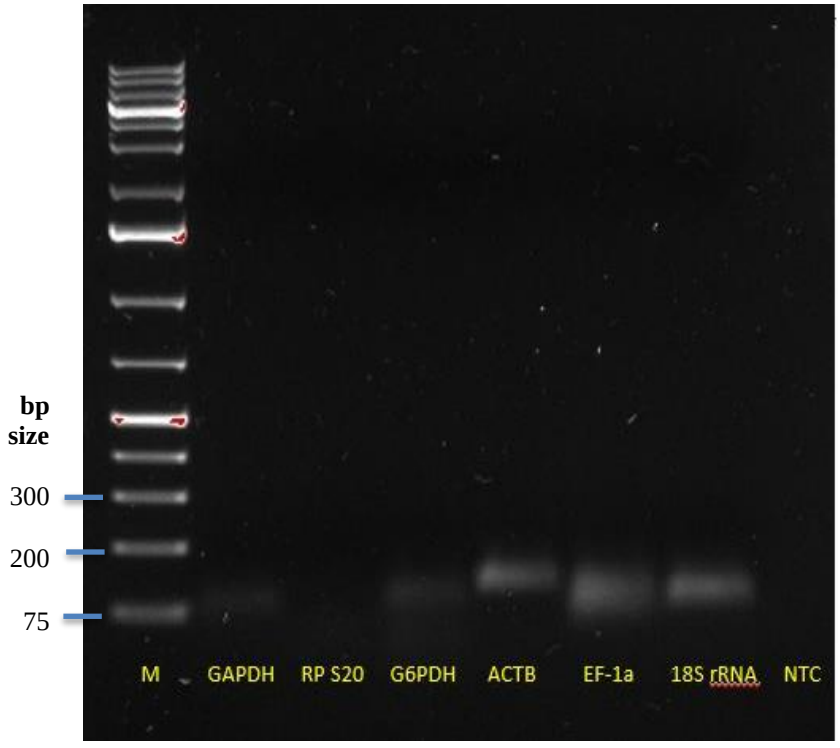

Figure 10. ***Agarose gel (1%) of PCR products of 6 housekeeping genes with cDNA from ASK-1 cells as template.*** *Primers used in the PCR reactions are selected based on literature (Table 2). Each housekeeping genes are indicated in the picture. Negative control (NTC) contains water and no template. M: 1 kb plus DNA ladder.*

3.2.2 PCR Efficiency

The second criterion that was evaluated in selecting a reference gene is good PCR Efficiency. Among the housekeeping genes, the *ef-1α, actb, and 18s rRNA* showed PCR efficiencies within the acceptable range of 94%, 91% and 105%, respectively, and with a coefficient of determination (R^2) of 0.99, 0.98 and 0.99, respectively. The PCR efficiency of *g6pdh* was greater than the acceptable value. The PCR Efficiencies of *rp s20* and *gapdh* could not be calculated because the gene expression was lower than Cp 35, the lowest detectable limit of the RT-PCR instrument. All the PCR efficiencies are shown in Table 6.

Table 6. ***PCR Efficiencies of housekeeping genes.*** *The PCR efficiencies were calculated using a standard curve with a dilution factor of 10. The acceptable range of PCR Efficiency is between 1.9 and 2.1 (90-110%). The EF-1α, ACTB, and 18s fulfilled the criteria to be used as reference genes under the conditions of the study presented in this thesis.*

Housekeeping Gene	R^2	PCR Efficiency	PCR Efficiency %
ef-1α	0.99	1.94	94
actb	0.98	1.91	91
18s rRNA	0.99	1.96	105
rp s20	n.a.	n.a.	n.a.
g6pdh	0.72	2.9	190
gapdh	n.a.	n.a.	n.a.

R^2 (coefficient of determination); n.a. (not available)

3.2.3 Stability of expression

Based on the primer integrity and PCR Efficiencies results, only the *actb*, *18s rRNA* and *ef-1α* were evaluated for stability of expression. Stability of expression of the reference gene, under the conditions of this study is important for internal standardisation of target gene expression data. Using the BestKeeper, a computing software that analyses the CPs statistically, the *ef-1α* showed the most stable expression compared to *actb and 18s rRNA* since its standard deviation is 0.95, and its coefficient of variance (%CP) is 3.54 (**Table 7**). The expression of a gene should have a standard deviation of < 1.0 and low CP variation to be considered stable (Pfaffl et al., 2004).

Table 7. ***Descriptive statistics of crossing point (CPs) values of the three housekeeping gene candidates.*** *The values presented here were analysed using BestKeeper, a computing software. The most stable housekeeping gene was highlighted.*

Factor	*ef-1α*	*actb*	*18s rRNA*
n	84	82	84
Geometric mean (CP)	26.76	27.04	22.53
Arithmetic mean (CP)	26.78	27.12	27.30
Min (CP)	24.87	23.55	19.27
Max (CP)	29.54	35	28.58
Std dev (±CP)	**0.95**	1.64	1.46
CV (% CP)	**3.54**	6.06	6.44

n: number of samples, Min (CP) and Max (CP): extreme values of CP, std dev (standard deviation), CV: coefficient of variance.

Figure 11 is the graphical representation of the descriptive statistics of each housekeeping gene presented in **Table 7**. It shows the spread of data points of *ef-1α, actb and 18s rRNA.* Each point represents the CP value of a sample. In this graph, *ef-1α* has the lowest variation in expression when compared with *actb* and *18s rRNA*. The *ef-1α* has CP values between 24 and 30, while *18s rRNA* shows the bigger variation in expression, having CP values between 19 and 35.

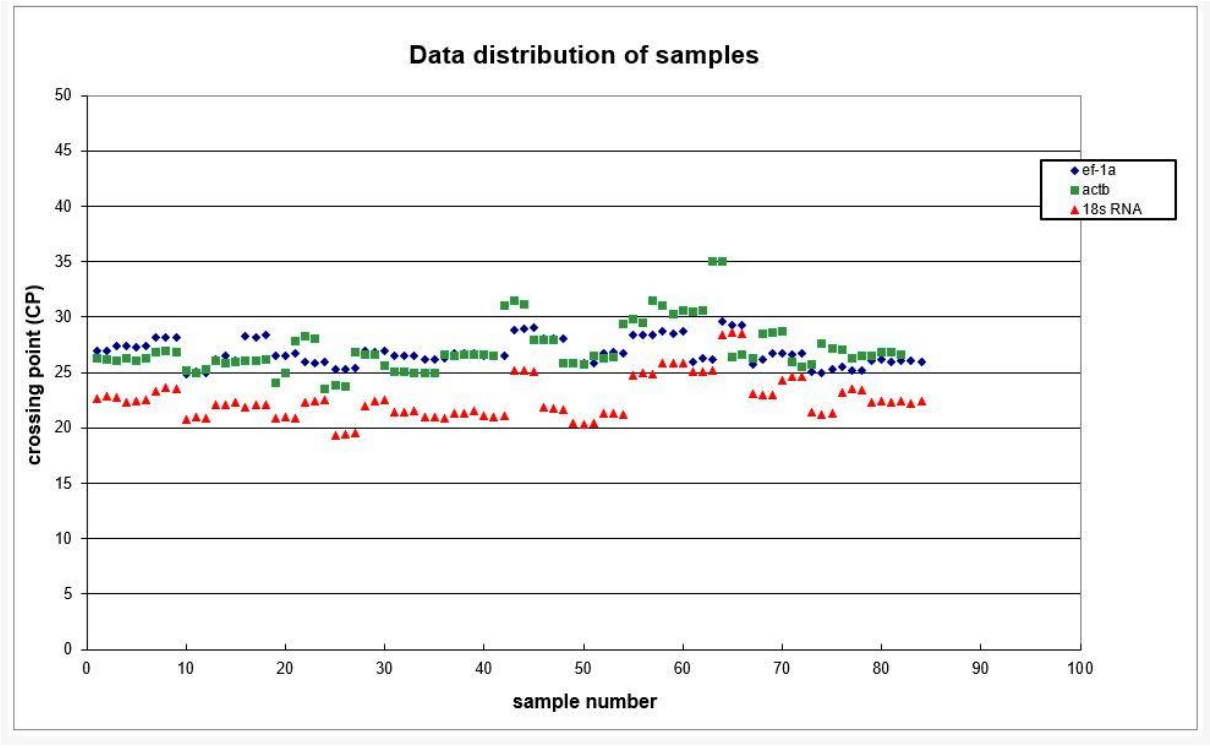

Figure 11. ***Data distribution of 80 different cDNA samples of electroporated or untreated ASK-1 cells.*** *The CP values were obtained following RT-PCR using 3 primers representing the housekeeping genes, ef-1α, 18s rRNA and actb. Calculations were done using BestKeeper. Each data point represents one CP value (crossing points) of a sample. The ef-1α has the lowest variation while the 18s rRNA showed the highest coefficient of variation among the three housekeeping genes.*

3.3 Fluorescence Microscopy

The Day 2 and Day 7 cells did not have any observable difference in terms of integrity and viability. Significant mortalities were also absent in all the groups. The images presented in **Figure 12** were the images that best represent the condition of the cells in their group.

Fluorescence imaging was used to analyse the transfection efficiency. Transfection efficiency is the ratio of cells that fluorescent over the total number of cells. The transfection efficiency for Cas9 and RNP complex in Day 2 and Day 7 samples is *ca*. > 95%.

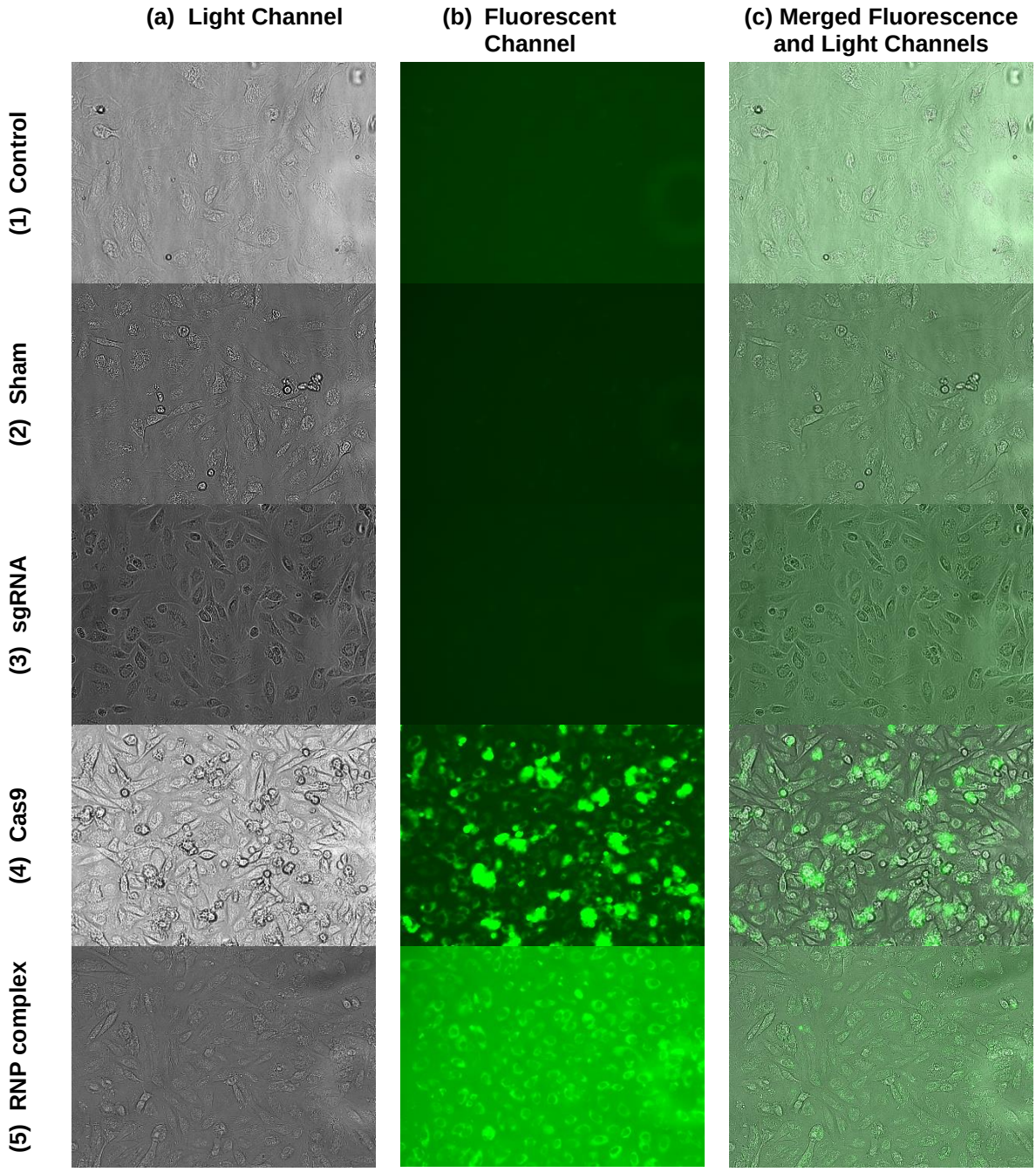

Figure 12. ***Fluorescence imaging of transfected and control ASK-1 cells.*** *The vertical panel shows the images of cells taken with (a) light, (b) fluorescence, and (c) overlay of light and fluorescent channels. The horizontal panel are images of cells in the (1) control, (2) sham, (3) sgRNA, (4) Cas9 and (5) RNP complex group. The images were taken using 200X magnification for both the light and fluorescence Channels. All images were processed using ImageJ program.*

3.4 Relative quantification of target gene mRNA expression

The relative mRNA expression of the target genes was calculated based on the CP values of the target genes which was normalized to the CP values of *ef*-1α and presented as an expression change relative to the control samples.

3.4.1 Quality of qPCR analyses

The negative reverse transcriptase controls (nRTs) for *hsp90, hsp70, mhc 1, igt, and igm genes*, did not show any amplification. Similarly, there was no amplification in all the no template controls (NTC). The positive control, which is a diluted sample (1:20) did not show any presence of PCR inhibitors in all the samples.

3.4.2 mRNA expression of *hsp90*

The linear model showed that in Day 2 samples, the relative expression of *hsp90* was significantly upregulated in cells transfected with RNP complex (r^2=0.97; $p < 0.001$). The treatment groups Cas9 and sgRNA have no effect on the expression of *hsp90*. Additionally, the Sham group, where cells were subjected only to electroporation without Cas9 or sgRNA in the electroporation milieu, *i.e*. neither Cas9 nor sgRNA were incorporated into the cells during electroporation did not show a significant effect on the expression of *hsp90* (**Figure 13**).

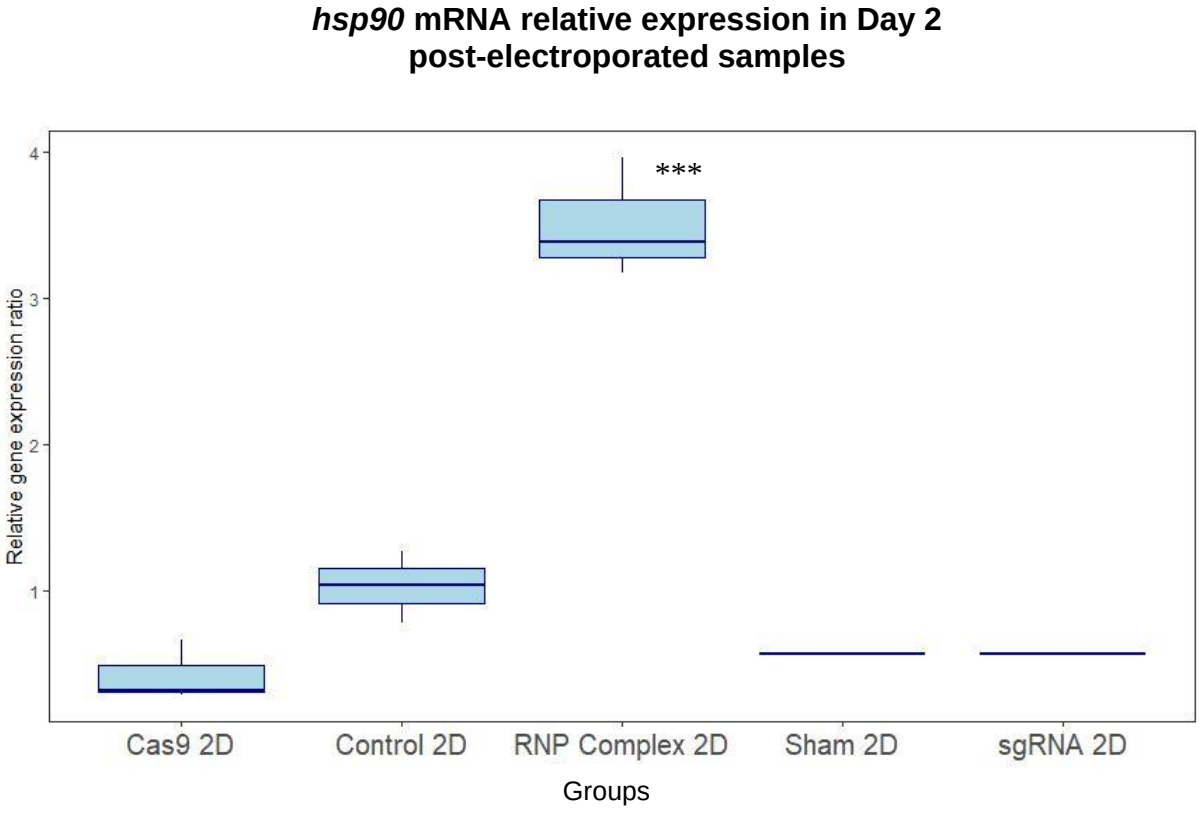

Figure 13. ***The relative gene expression of hsp90 in the control and treatment groups in Day 2 post-electroporated samples.*** *The gene expression data was normalized to EF-1α. The RNP complex group has significantly higher relative expression of hsp90 than the rest of the groups. Control and treatment groups have n=9. The data were analysed using a linear regression model. Statistical significance (***) denotes p < 0.001. r^2=0.97.*

Similar to Day 2, the linear model showed that the relative expression of *hsp90* in Day 7 samples was significantly elevated in cells transfected with RNP complex (r^2=0.72; $p < 0.01$), while the rest of the groups did not show any significant effect on the expression of *hsp90* (**Figure 14**).

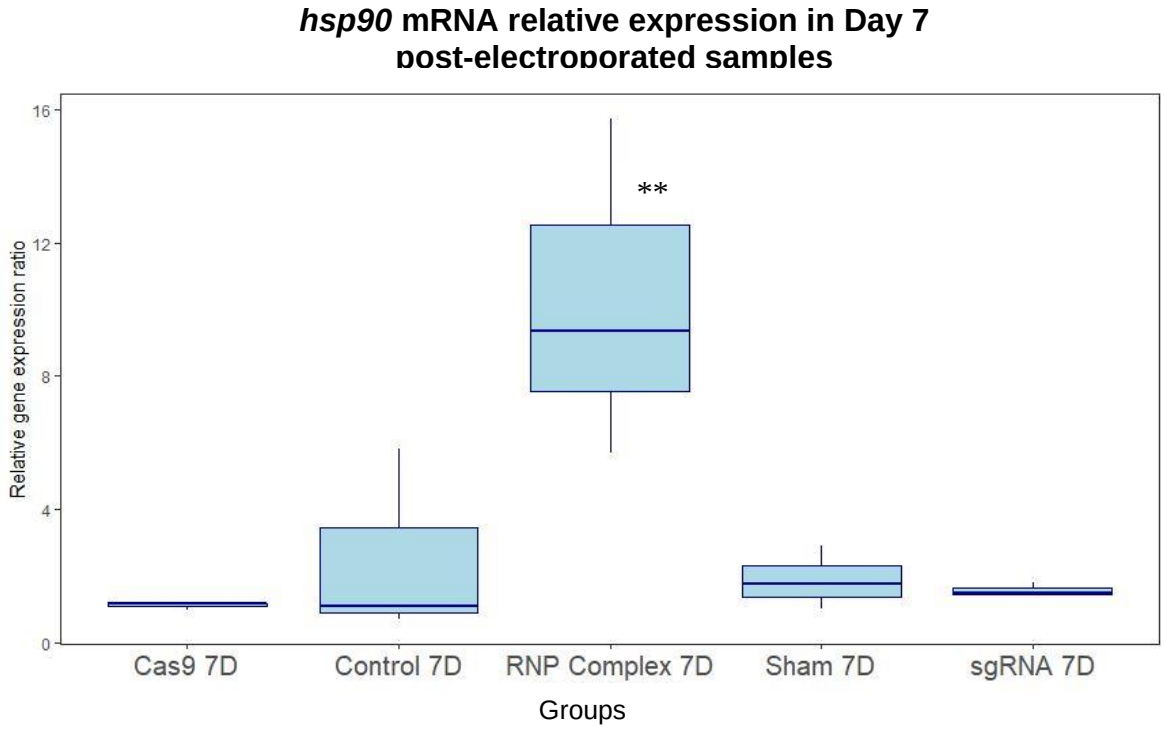

Figure 14. ***Relative gene expression of hsp90 in the control and treatment groups in Day 7 post-electroporated samples****. The gene expression data was normalized to EF-1α. The RNP complex group has significantly higher The relative expression of hsp90 than the rest of the groups. Control and treatment groups have n=9. The data were analysed using a linear regression model. Statistical significance (**) denotes p < 0.01. r^2=0.72.*

When Day 2 and Day 7 samples were compared, the Day 7 samples of the RNP complex group showed the highest expression of *hsp90* (r2=0.77; p <0.001), while the rest of the groups showed no significant effect to *hsp90* (**Figure 15**). The duration of incubation following electroporation had no impact on *hsp90* expression as there was no significant difference in the expression of the gene between Day 2 and Day 7 except for 7D RNP complex .

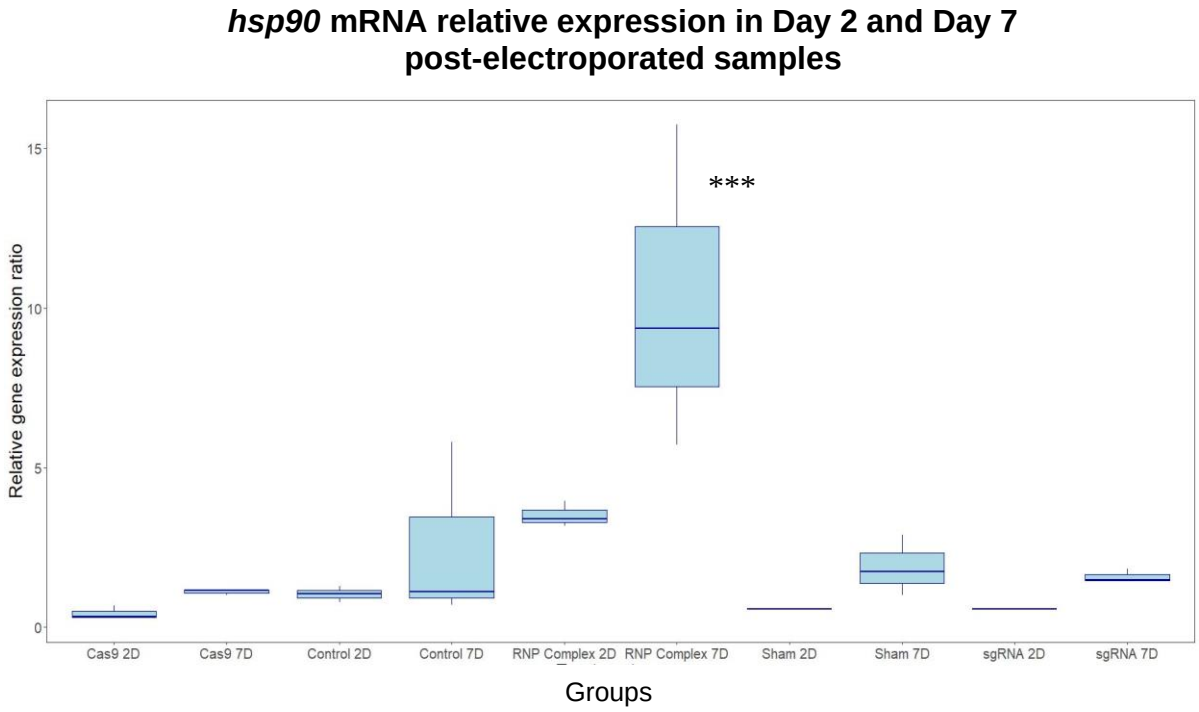

Figure 15. ***Comparative analysis of the relative gene expression ratio of hsp90 in Day 2 and Day 7 post-electroporated samples****. The gene expression data was normalized to EF-1α. The Day 7 samples transfected with RNP complex has significantly higher relative expression of hsp90 than the rest of the groups. The expression of hsp90 between the Day 2 and Day 7 samples among the same treatment and control group was not statistically different. Control and treatment groups have n=9. The data were analysed using a linear regression model. Statistical significance (***) denotes p < 0.001. r2=0.77.*

3.4.3 mRNA expression of *hsp70*

The mRNA expression of *hsp70* was significantly elevated in the cells from the Sham treatment group but not in the rest of the groups (r^2= 0.81; p < 0.001) (**Figure 16**). The cells in the sgRNA group showed a wide variation in the expression, however this is not significant.

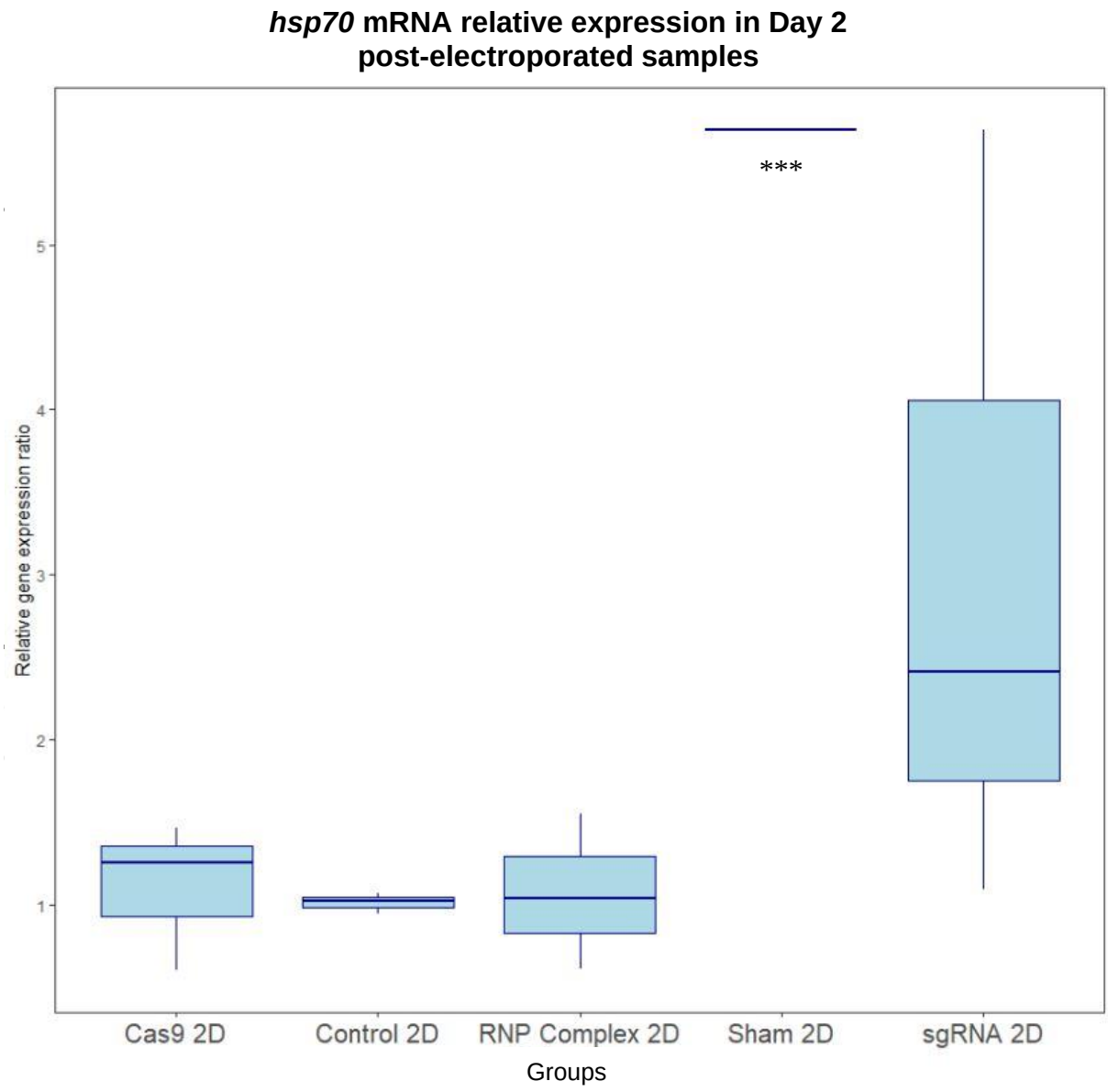

Figure 16. ***The relative gene expression of hsp70 in the control and treatment groups in Day 2 post-electroporated samples.*** *The gene expression data was normalized to EF-1α. The Sham group has significantly higher relative expression of hsp70 compared to the rest of the groups. Control and treatment groups have n=9. The data were analysed using a linear regression model. Statistical significance (***) denotes p < 0.001. r^2= 0.81*

In modelling the relationship of the different groups with the relative gene expression of *hsp70* in Day 7 samples, *hsp70* was significantly elevated in the cells from the Sham treatment group which was also seen in Day 2 samples (r^2= 0.83; p < 0.001). The rest of the groups did not show any effect on the expression of hsp70. The cells transfected with the RNP complex seems suppressed, however, this expression was not significant (**Figure 17**).

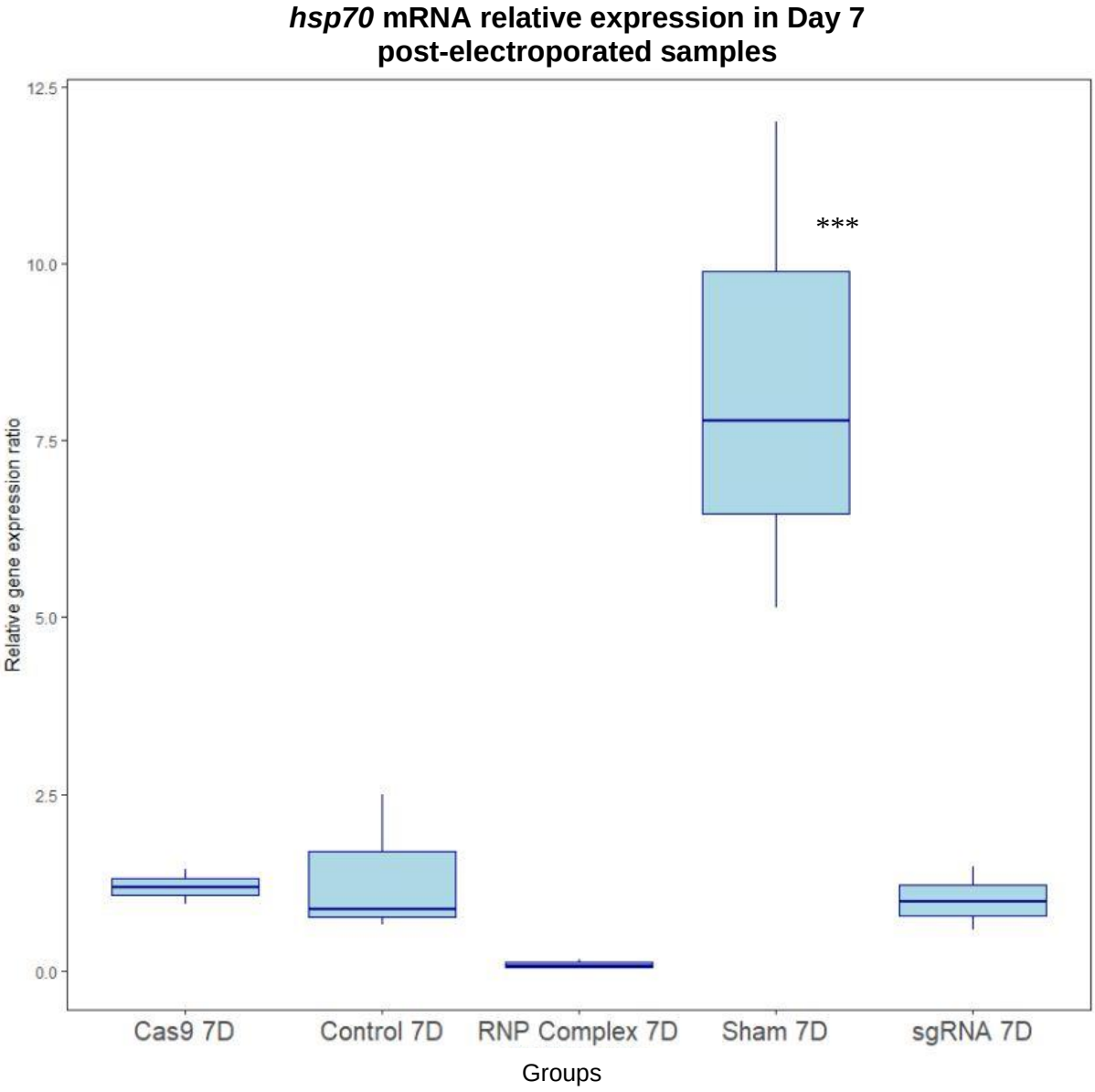

Figure 17. ***Relative gene expression ratio of hsp70 in the control and treatment groups in Day 7 samples****. The gene expression data was normalized to EF-1α. The cells in the Sham group have significantly higher relative expression of hsp70 than the rest of the groups. Control and treatment groups have n=9. The data were analysed using a linear regression model. Statistical significance (***) denotes p < 0.001. r^2= 0.83*

When the *hsp70* expression of Day 2 and Day 7 samples were modelled, the cells in Sham group from both days have shown a significantly higher relative expression of hsp70 among the groups (r^2=0.67; $p < 0.01$) (**Figure 18**). Then Dunn test was used to determine the effect of incubation days on the expression of *hsp70*. The results show that there is no significant difference between Day 2 and Day 7 samples.

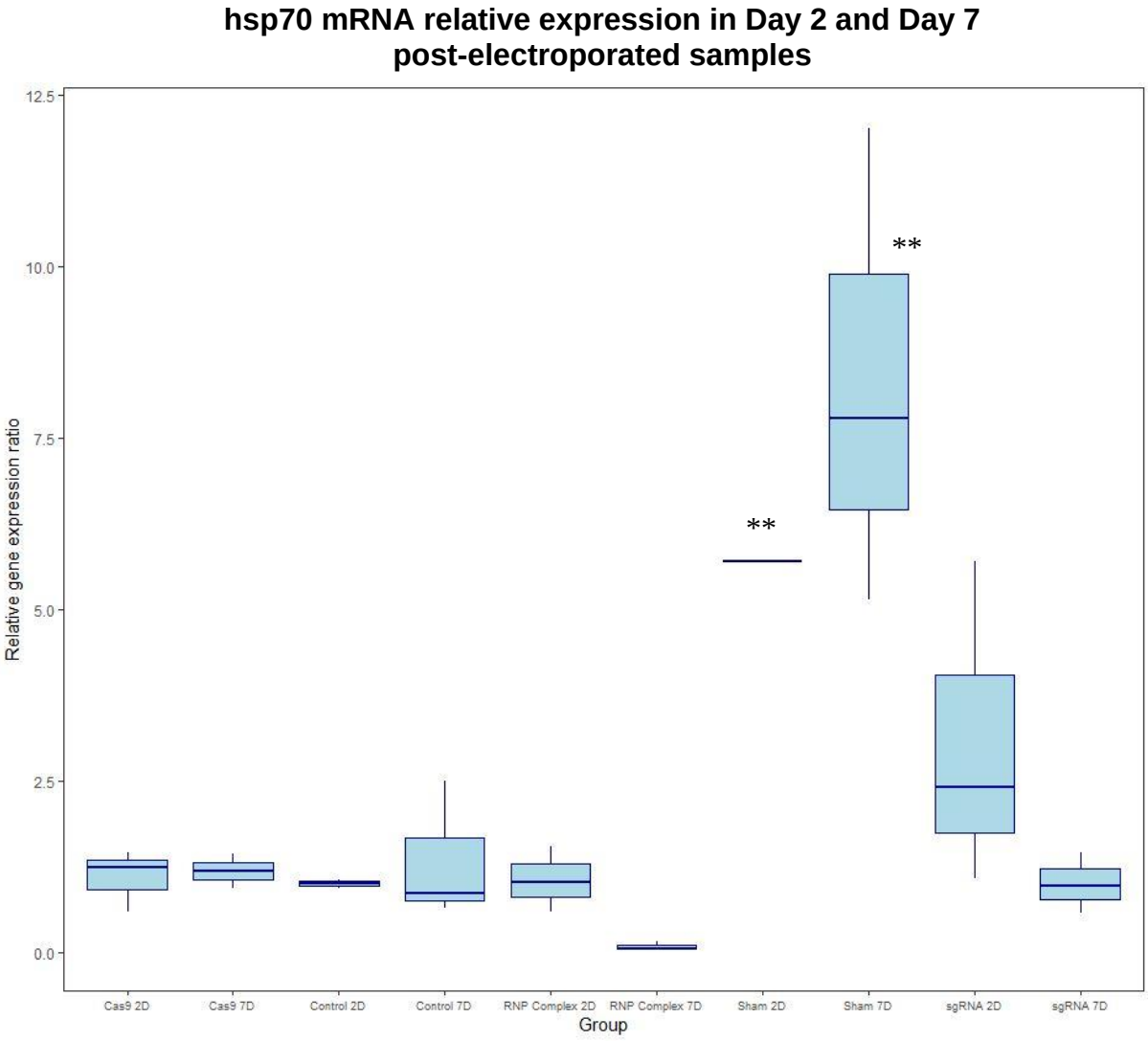

Figure 18. ***Comparative analysis of the relative gene expression ratio of hsp70 in Day 2 and Day 7 post-electroporated samples****. The gene expression data was normalized to EF-1α. Among the groups, the cells from Sham group shown a significantly higher expression than the rest of the groups. Control and treatment groups have n=9. The data were analysed using a linear regression model Statistical significance (**) denotes $p < 0.01$. r^2=0.67*

3.4.4 mRNA expression of *mhc I*

In Day 2 samples, all the groups did not have a significant effect on the expression of mh*c I* (*H(4)=8.17, P = 0.08*) (**Figure 19**).

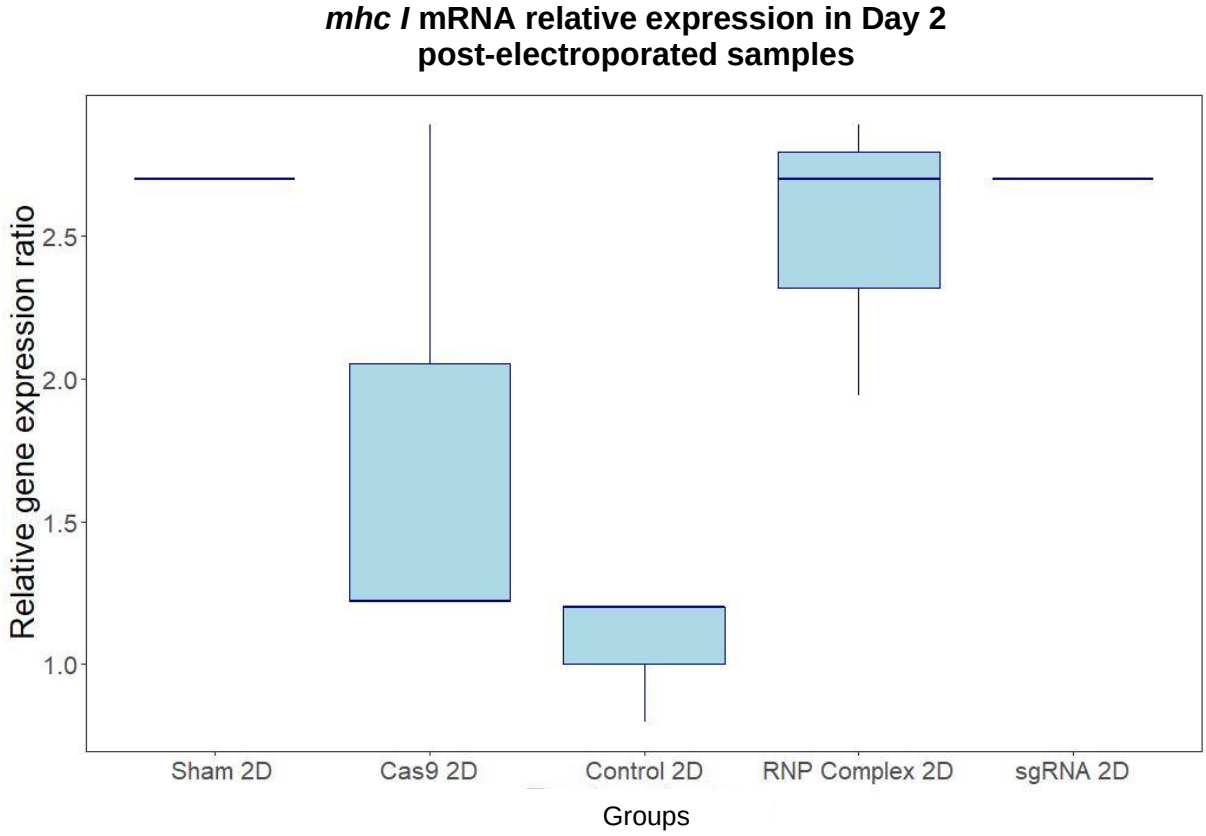

Figure 19. ***Relative gene expression ratio of mhc I in the control and treatment groups in Day 2 samples****. The gene expression data was normalized to EF-1α. There was no significant difference on the expression of mhc I among the groups. Control and treatment groups have n=9. Kruskal wallis sum test was used to analyse the data: H(4)=8.17, P = 0.08 .*

In Day 7 samples, all the groups did not show any significant effect on the relative expression of *mhc I* (*H (4) = 8.78, P = 0.07*) (**Figure 20**). This result is also similar to result of the Day 2 samples.

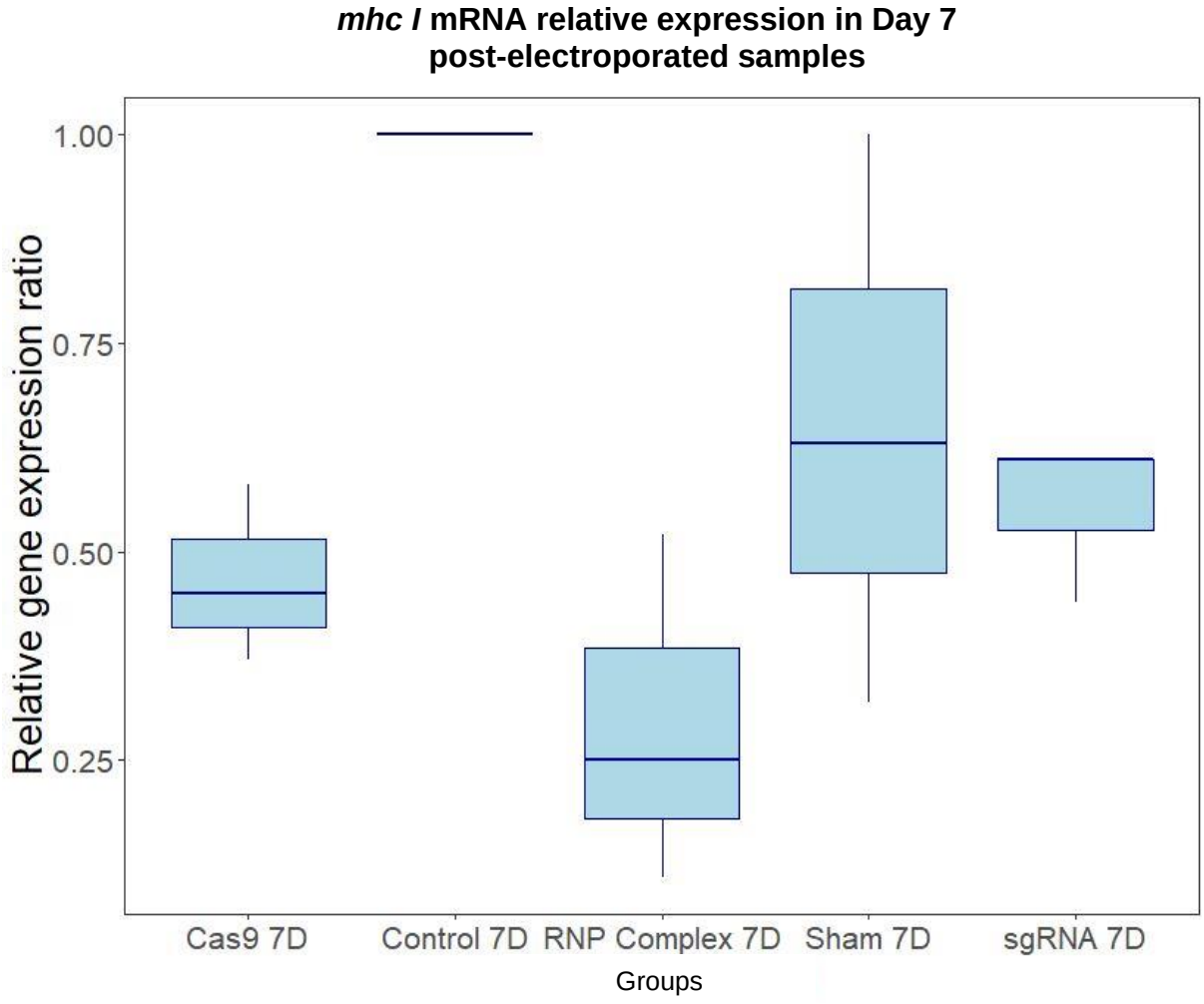

Figure 20. ***Relative gene expression ratio of mhc I in the control and treatment groups in Day 7 samples****. The gene expression data was normalized to EF-1α. There was no significant difference on the expression of mhc I among the groups. Control and treatment groups have n=9. Kruskal wallis sum test was used to analyse the data: H(4)=8.78, P = 0.07.*

When the samples from Day 2 and Day 7 were compared in the same group, a significantly higher expression of mhc I were seen in the cells of Day 2 samples (H (9) = 25.92, P = 0.002) (**Figure 21**). The result from the Dunn test showed that the incubation period has an effect on the mhc I. These *are the* p values of the following treatment groups: Cas9 ($p < 0.05$), sgRNA ($p < 0.05$), Sham ($p < 0.05$) and RNP complex ($p < 0.01$). The cells from the Day 2 control group did not significantly differ with the cells from Day 7 *control*. However, the Dunn test showed that when these gene expressions were compared to the level of expression in the control group, they were not significant.

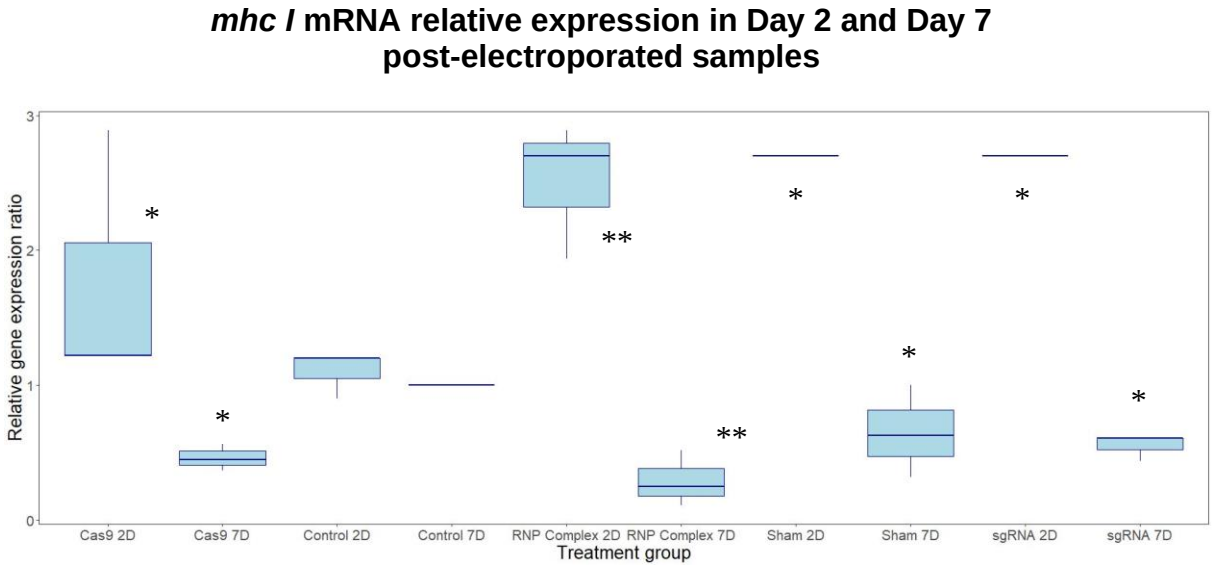

Figure 21. ***Comparative analysis of the relative gene expression ratio of mhc I in Day 2 and Day 7 samples****. The gene expression data was normalized to EF-1α. All treatment groups in Day 2 have significantly higher expression of mhc I than in Day 7 samples. In the control group, the level of expression in Day 2 did not differ with the Day 7 samples. Control and treatment groups have n=9. Kruskal wallis sum test was used to analyse the data: H (9) = 25.92, P = 0.002. The p value of each group was taken from the Dunn test results. Significance (* and **) denotes p < 0.05 and p < 0.01, respectively.*

3.4.5 mRNA expression of *igm* and *igt*

The *igm* and *igt* were not expressed in all the samples, *i.e*. control, treatment groups and standard curve. Except for 3 samples, all the CP values were either 0 or 35. These values mean that the signals were below the detection limit of the machine. Consequently, the relative gene expression of these genes cannot be calculated as the PCR Efficiency cannot be derived from standard curve values. The CP values of the reference genes on the other hand, were as expected. All the measurements taken from the target genes were shown in **Table 9.**

Table 9. ***Average gene expression of igt and igm genes in ASK-1 cells of Day 2 and Day 7 samples.*** *Except for 3 samples, CP values from Day 2 and Day were either 0 or 35. These CP values denote signals that are too low for the machine to detect, thus there are considered with high uncertainty.*

Group	IgM CP value (mean)	IgT CP value (mean)
2D Control 1	35	0
2D Control 2	35	0
2D Control 3	35	0
2D Sham 1	0	0
2D Sham 2	0	0
2D Sham 3	0	0
2D sgRNA 1	0	0
2D sgRNA 2	0	0
2D sgRNA 3	0	0
2D Cas9 1	0	0
2D Cas9 2	0	0
2D Cas9 3	0	0
2D RNP complex 1	0	0
2D RNP complex 2	0	0
2D RNP complex 3	35	0
Standard 1:1	33.5	0
Standard 1:10	35	0
Standard 1:100	0	0
Standard 1:1000	0	0
7D Control 1	35	0
7D Control 2	35	0
7D Control 3	35	0
7D Sham 1	35	0
7D Sham 2	35	0
7D Sham 3	35	0
7D sgRNA 1	35	0
7D sgRNA 2	35	0
7D sgRNA 3	35	0
7D Cas9 1	35	0
7D Cas9 2	35	0
7D Cas9 3	35	0
7D RNP complex 1	35	0
7D RNP complex 2	0	0
7D RNP complex 3	33.7	0
Standard 1:1	34.6	0
Standard 1:10	35	0
Standard 1:100	0	0
Standard 1:1000	0	0

3.5 Single cell cloning

The cloned single-cell with transfected RNP complex was cultured on a 96-well plate. This image was taken 7 weeks after FACSing, and the cells show that they have started to adhere, proliferate, and spread out (Figure 22). The confluency in this well is *ca*. > 50%. The rest of the wells of the 96-well plate have confluency between 0 and 30%, and 15% being the average. The 0% means that it is either the cell has died or the confluency is too few to see under the 100X magnification.

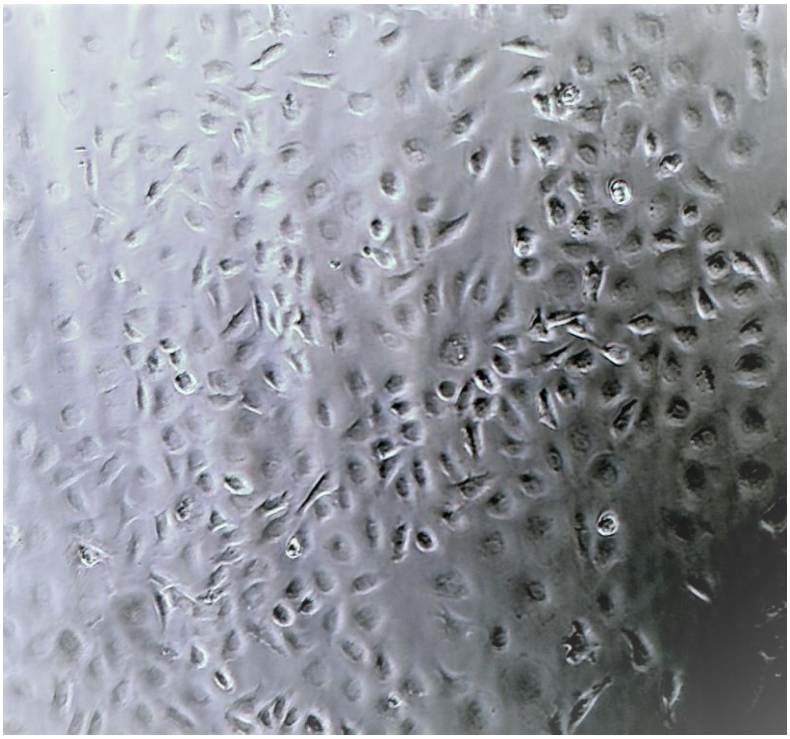

*Figure 22.**Cloned single cell transfected with RNP complex.** Using Fluorescent-assisted cell sorting(FACS), ASK-1 cells transfected with the RNP complex was sorted and cloned. One cell was placed per well on a 96-well plate. This image was taken 7 weeks after FACSing using 100X magnification. The confluency is ~ >50%.*

4 DISCUSSION

The discovery of CRISPR/Cas9 system as a genome editing tool has revolutionized the field of molecular biology. However, since technology is relatively new, there are few published studies on its biosafety. In this, study, electroporation was used to transfect the cells with the components of CRISPR/Cas9. One of the aims was to determine the impacts of electroporation as a gene editing procedure on ASK-1 cells using the expressions of *hsp90, hsp70, igt, igm and mhc I* genes as indicators.

4.1 Electroporation as a gene editing procedure

Electroporation sends out electric field pulses that cause some structural rearrangement of the cell membrane which creates "pores" that are transient. Besides pore formation, the electrical field also provides a local driving force that transports ions and molecules inside the cell (Weaver, 1995). When the cells fail to reseal the initial pores, permanent membrane damage, oxidation stress, and leakage of intracellular molecules will result to loss of cell viability and cell death, which are the common effects of electroporation (Jakstys et al., 2020).

In terms of biosafety, this study did not observe any negative impact of electroporation as shown by the high viability of cells in all treatment groups following electroporation. (Figure 12). Further, this study did not observe any evidence that electroporation can cause cellular stress (Figure 15 and Figure 18). The *hsp90* is upregulated only in cells transfected with RNP complex, but upregulation was absent in the other treatment groups, although these cells were also exposed to electric shocks. In the same manner, *hsp70* was upregulated only in cells belonging to the Sham group, but not in the other treatment groups which were also exposed to electric shocks.

The HSP90 and HSP70 are markers of stress response. They are upregulated during stress, as their main role in proteostasis is to chaperone the newly synthesized proteins to fold correctly and to refold the denatured proteins (Vabulas et al., 2010). Zarate et al (2006) showed that the mRNA expression of *hsp90* and *hsp70* in kidney cells increased by two-fold and three-fold, respectively above the control group levels when it was exposed to thermal stress for 15 min. In another study, the mRNA expression of *hsp70* peaked after 2 hours and continued up to 6 hours when A. salmon was exposed to thermal shock (Smith et al., 1999). Electroporation is a

stressor, so it is expected to cause an upregulation of these genes in ASK-1 cells. However, this was not observed in this study, and one possible reason can be attributed to the timepoint of measurement of the mRNA expressions, *i.e.*, 2 days and 7 days post-electroporation, while in the aforementioned studies, the measurements were done a few hours post-electroporation. The effect electroporation as a stressor, can be transient. It can be assumed that the increased expressions of these genes in ASK-1 in response to electroporation are not long-term or permanent, thus the upregulation of these genes were not captured when the mRNA expression were measured. However, the data from this study is not enough to support this hypothesis and since there is lack of published studies on A. salmon or salmonids cells in relation to impact of electroporation.

Another possible explanation was that the protocols used in electroporation was optimal for the cells. This hypothesis is supported by the viability of the cells after electroporation was done. The Day 2 and Day 7 cells remain to be viable and there was no significant mortalities observed among all the groups.

This study has shown that electroporation does not affect the *mhc I* gene expression in ASK-1 cells. This was evident by the similar level of expression of these genes in all the treatments with the control group. Based on these results, it can be said that electroporation as a gene editing procedure does not disrupt the immune pathways involving MHC I in ASK-1 cells.

Genome editing of salmonid cell lines is still in early stages (Gratacap, 2020) and the result of this study have shown the potential of using electroporation as a gene editing procedure to deliver the CRISPR/Cas9 components without causing any significant stress to the cells. This study has also achieved a high transfection efficiency (> 95%) of RNP complex and Cas9 in salmonid cells, which was similar to what has been reported by Strømsnes et al. (2022) and Gratacap et al. (2020).

It is a fact that electroporation can cause cell injury and cell death (Batista Napotnik, 2021). However, the cells die only when the damaged caused by electroporation were beyond repair. When the electric pulses used were too many and the amplitude was too high, the damage caused will be irreversible and will eventually lead to cell death. This study has shown that when the parameters used are optimized, electroporation can be safe to use in ASK-1 cell lines and can even deliver high transfection efficiency.

4.2 CRISPR/Cas9 components' impact on target gene expression

The second aim of this study was to investigate the impact of each CRISPR/Cas9 components, namely RNP complex, Cas9 and sgRNA to ASK-1 cells in terms of cellular stress and/or toxicity by looking at the expression of stress marker genes hsp90 and hsp70 and immune genes, *mhc I*, *igm*, and *igt*.

4.2.1 sgRNA component

The sgRNA is one of the essential components of CRISPR/Cas9. It is made up of two parts: the base pairs that complement the target sequence and a binding scaffold for the Cas9 nuclease (Asmamaw, 2021). Its main function is to recognize and bind to the target sequence in the gene of interest and guide the Cas9 nuclease where to cut (Asmamaw, 2021).

In this study, the expression of *hsp90, hsp70 and mhc I,* in the sgRNA group did not have any significant difference from the control group (Figure 15, Figure 18, and Figure 21). This was seen for both Day 2 and Day 7 samples. These results show that the sgRNA component of CRISPR/Cas9 does not have an impact nor can influence the expression of these genes in ASK-1 cells.

The expression of *hsp90* and *hsp70* genes did not show any significant difference with the control. This shows that the sgRNA component of the CRISPR/Cas9 did not cause any stress to the cell.

Although the *mhc I* expression was not different from the control group, the cells from Day 2 samples has a significantly higher expression than Day 7 samples (Figure 21). However, the Dunn test showed that when these gene expressions were compared to the level of expression in the control group, they were not significant.

These results are expected since the sgRNA only contains the complimentary sequence and a scaffold that binds the Cas9 nuclease. These components do not have the mechanism to trigger the DNA melting, thus can't cause any mutation that will stimulate the expression of stress marker genes. However, these observations and interpretations were only limited to the target genes used in this study. However, given the structure of sgRNA, it is unlikely that it can effect change in the DNA.

As far as this study is concerned, the interpretations of the results here are limited to ASK-1 cells only. Since this study is the first to do an analysis on the impacts of sgRNA component to the cell and to the expression of the selected immune genes, comparison with other studies was not possible.

4.2.2 Cas9 component

Cas9 is a DNA nuclease whose main function is to cleave to the DNA and form a double-stranded break (Asmamaw & Zawdie, 2021). It works like a genetic scissor in the CRISPR/Cas9 system. In one of its regions, it contains the PAM interacting domains which is responsible for initiating the binding to the target DNA (Asmamaw & Zawdie, 2021). This is an important feature of the CRISPR/Cas9 system because the PAM interacting domain is specific, meaning that the sgRNA can only bind to the complementary DNA once the correct PAM was found by Cas9.

In this study, the Cas9 component did not show any influence on the gene expression of *mhc I* (Figure 21). The expressions of these genes were not significantly different from the control. Although the expression of *mhc I* in Day 2 cells is significantly higher than the Day 7 cells, the Dunn test showed that this level of expression were not significantly different from the control.

There was also no evidence of cellular stress, as the *hsp90* and *hsp70* were not upregulated nor downregulated (Figure 15, Figure 18). The expressions were similar to the control group.

These results were expected because in the absence of sgRNA, Cas9 remains inactive (Asmamaw & Zawdie, 2021).

As with the sgRNA, the interpretations of the results here are limited to ASK-1 cells only. Since there is no previous studies looking at the effects of Cas9 component alone, comparison with other studies was not possible.

4.2.3 RNP complex component

One method that is used to directly deliver the CRISPR/Cas9 system into the cell is by using ribonucleic (RNP) complex. This method has been widely accepted because the genome editing

is transient, it reduces the off-target effects (Zhang et al., 2021), has lower toxicity and immune response, rapid action and has high gene-editing efficiency (van Hees et al., 2022). This study has investigated the impact of RNP complex, a CRISPR/Cas9 component on ASK-1 cells by using the expressions of *hsp90, hsp70, igt, igm and mhc I* genes as indicators.

This study has used the RNP complex to deliver the CRISPR/Cas9 components into the cell. The sgRNA contains the sequence that will target the *cr2* gene. The results show that the mRNA expressions of *hsp70, and mhc I* genes were not affected by the RNP complex. Their expressions were not significantly different from the control group, both for Day 2 and Day 7 samples.

The *hsp90*, on the other hand was significantly upregulated, both in Day 2 and Day 7 samples, wherein the cells in Day 7 have significantly higher gene expression than the Day 2 cells. This means that after the introduction of RNP complex to the cell, the *hsp90* has continued to increase in level until Day 7.

The hsp90 are highly conserved molecular chaperones that are upregulated during stress. Their role is to protect the proteins from denaturation (Vabulas et al., 2010). In fish, the expression of *hsp90* has been related to cytoprotection, cell survival and immune response (Celi et al., 2012). The high level of expression of *hsp90* in the cells transfected by RNP complex might be caused by the stress during the gene editing of *cr2* gene. This mutation was confirmed in study of Strømsnes et al., (2002), wherein the protocols used in RNP complex delivery and sgRNA design and synthesis were also used in this study. During mutation, a protein's ability to fold is disrupted, thus the *hsp9*0 is upregulated to buffer the lethal effect of mutations (Maisnier-Patin et al., 2005), since the role of hsp90 is to protect the mutated and destabilized proteins from degradation (Cowen & Lindquist, 2005).

This is the first study that has investigated the impact of RNP complex in CRISPR/Cas9-mediated gene editing. The results of this study have shown that it causes stress to ASK-1 cells for as long as 7 days. Many studies have shown that stress can impair the immune response and increase the organism's susceptibility to different diseases (Gadan et al., 2012), (Celi et al., 2012), (Vazzana et al., 2002). In application, although the use of RNP complex ensures high gene editing efficiency and lower off-target effects, it should be considered that it is stressful to the cells and might impair their immune responses, especially if the gene editing procedure induces chronic stress.

4.2.4 Igm and igt mRNA expression

The IgM and IgT are part of the adaptive immune system and they play an important role in the humoral immune response. Some of the properties of the adaptive immune system are the following: it takes time for them to elicit a response, need to encounter an antigen before they are activated and proliferate, and the levels go down when the antigen is neutralized (Abbas, 2020). Based on these properties, it will be expected that level of IgM and IgT will not be highly expressed at all times. It is probable that these genes are expressed but in very little amount in the absence of antigen.

When the *igm* and *igt* were measured, the expression was very low to be detected by the machine. These results are expected since these genes are seen to be upregulated in the presence of antigens (Bakke et al., 2020); (Teige et al., 2019); (Jørgensen et al., 2008). The electric shock from the electroporation did not elicit a response from these genes, although it is also a type of stressor, nor the presence of "foreign" nucleic acids like Cas9-eGFP, sgRNA, and the RNP complex. It may be because the receptors of these immunoglobulins are specific to polysaccharides and lipids (Abbas, 2020).

5 CONCLUSION

The main objective in doing this study is to evaluate whether electroporation and the individual components of CRISPR/Cas9 system have an impact on the gene expression of selected immune genes of ASK-1 cell when it is subjected to gene editing. This study showed that using electroporation as a method of delivering CRISPR/Cas9 components into the cell did not cause a negative impact on the cells in terms of viability, nor did it influence the expression of *hsp90*, *hsp70*, and *mhc I*. Therefore, electroporation is ideal to use as a gene editing procedure when studying the abovementioned genes of ASK-1 cells because of its high efficiency and safety, provided that the parameters and conditions that will be used were the same as the ones that have been used in this study.

This study also showed that the CRISPR/Cas9 components, namely sgRNA and Cas9 did not show any impact on the expression of *hsp90*, *hsp70*, and *mhc I* when editing ASK-1 cells. These components, when used, do not cause additional cellular stress to the cells during gene editing. These components also do not affect the expression of *mhc I* genes. Lastly, the RNP complex did not also show any effect on the expression of *hsp70*, and *mhc I*. It showed an effect on the *hsp90* expression which may be due to the mutation of the *cr2* gene.

The gene expression of *igm* and *igt* genes were too low to be detected, thus, their gene expression cannot be calculated. Consequently, the impacts of electroporation and the CRISPR/Cas9 components on their expression cannot be investigated.

Overall, this study was successful in elucidating the impacts of electroporation and CRISPR/Cas9 components in the cell and target gene expression. Electroporation and the CRISPR/Cas9 components, except for the RNP complex did not cause cellular stress nor affected the expression of the immune genes. However, the findings of this study were only applicable for the protocols and conditions used in this study using ASK-1 cell line as a model.

6 LITERATURE CITED

Abbas, A., Lichtman, A., Pillai, S. 2020. Basic Immunology. Functions and disorders of the immune system. 6th edition.

Andresen, A. M. S., Boudinot, P., & Gjøen, T. (2020). Kinetics of transcriptional response against poly (I:C) and infectious salmon anemia virus (ISAV) in Atlantic salmon kidney (ASK) cell line. *Developmental & Comparative Immunology*, *110*, 103716. https://doi.org/https://doi.org/10.1016/j.dci.2020.103716

Asmamaw, M., & Zawdie, B. (2021). Mechanism and Applications of CRISPR/Cas-9-Mediated Genome Editing. *Biologics*, *15*, 353-361. https://doi.org/10.2147/btt.S326422

Bakke, A. F., Bjørgen, H., Koppang, E. O., Frost, P., Afanasyev, S., Boysen, P., Krasnov, A., & Lund, H. (2020). IgM+ and IgT+ B Cell Traffic to the Heart during SAV Infection in Atlantic Salmon. *Vaccines (Basel)*, *8*(3). https://doi.org/10.3390/vaccines8030493

Binder, R. J. (2009). Hsp receptors: the cases of identity and mistaken identity. *Curr Opin Mol Ther*, *11*(1), 62-71.

Binder, R. J. (2014). Functions of Heat Shock Proteins in Pathways of the Innate and Adaptive Immune System. *The Journal of Immunology*, *193*(12), 5765-5771. https://doi.org/10.4049/jimmunol.1401417

Binder, R. J., Blachere, N. E., & Srivastava, P. K. (2001). Heat shock protein-chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histocompatibility complex I molecules. *J Biol Chem*, *276*(20), 17163-17171. https://doi.org/10.1074/jbc.M011547200

Boackle, S. A. (2018). Chapter 40 - CR2. In S. Barnum & T. Schein (Eds.), *The Complement FactsBook (Second Edition)* (pp. 423-434). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-810420-0.00040-7

Boshra, H., Li, J., & Sunyer, J. O. (2006). Recent advances on the complement system of teleost fish. *Fish & Shellfish Immunology*, *20*(2), 239-262. https://doi.org/https://doi.org/10.1016/j.fsi.2005.04.004

Bustin, S. A., Benes, V., Nolan, T., & Pfaffl, M. W. (2005). Quantitative real-time RT-PCR--a perspective. *J Mol Endocrinol*, *34*(3), 597-601. https://doi.org/10.1677/jme.1.01755

Cao, Y., Ohwatari, N., Matsumoto, T., Kosaka, M., Ohtsuru, A., & Yamashita, S. (1999). TGF-β1 mediates 70-kDa heat shock protein induction due to ultraviolet irradiation in human skin fibroblasts. *Pflügers Archiv*, *438*(3), 239-244. https://doi.org/10.1007/s004240050905

Celi, M., Vazzana, M., Sanfratello, M. A., & Parrinello, N. (2012). Elevated cortisol modulates Hsp70 and Hsp90 gene expression and protein in sea bass head kidney and isolated leukocytes. *General and Comparative Endocrinology*, *175*(3), 424-431. https://doi.org/https://doi.org/10.1016/j.ygcen.2011.11.037

Cowen, L. E., & Lindquist, S. (2005). Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. *Science*, *309*(5744), 2185-2189. https://doi.org/10.1126/science.1118370

Gadan, K., Marjara, I. S., Sundh, H., Sundell, K., & Evensen, Ø. (2012). Slow release cortisol implants result in impaired innate immune responses and higher infection prevalence following experimental challenge with infectious pancreatic necrosis virus in Atlantic salmon (Salmo salar) parr. *Fish Shellfish Immunol*, *32*(5), 637-644. https://doi.org/10.1016/j.fsi.2012.01.004

Gostimskaya, I. (2022). CRISPR-Cas9: A History of Its Discovery and Ethical Considerations of Its Use in Genome Editing. *Biochemistry (Mosc)*, *87*(8), 777-788. https://doi.org/10.1134/s0006297922080090

Hordvik, I. (2015). Immunoglobulin isotypes in Atlantic salmon, Salmo salar. *Biomolecules*, *5*(1), 166-177. https://doi.org/10.3390/biom5010166

Jakstys, B., Jakutaviciute, M., Uzdavinyte, D., Satkauskiene, I., & Satkauskas, S. (2020). Correlation between the loss of intracellular molecules and cell viability after cell electroporation. *Bioelectrochemistry*, *135*, 107550. https://doi.org/https://doi.org/10.1016/j.bioelechem.2020.107550

Jiang, F., & Doudna, J. A. (2017). CRISPR-Cas9 Structures and Mechanisms. *Annu Rev Biophys*, *46*, 505-529. https://doi.org/10.1146/annurev-biophys-062215-010822

Jurivich, D. A., & Zhou, X. (2007). Stress: Physiological. In J. E. Birren (Ed.), *Encyclopedia of Gerontology (Second Edition)* (pp. 559-565). Elsevier. https://doi.org/https://doi.org/10.1016/B0-12-370870-2/00179-7

Jørgensen, S. M., Afanasyev, S., & Krasnov, A. (2008). Gene expression analyses in Atlantic salmon challenged with infectious salmon anemia virus reveal differences between individuals with early, intermediate and late mortality. *BMC Genomics*, *9*(1), 179. https://doi.org/10.1186/1471-2164-9-179

Kjøglum, S., Larsen, S., Bakke, H. G., & Grimholt, U. (2006). How specific MHC class I and class II combinations affect disease resistance against infectious salmon anaemia in Atlantic salmon (Salmo salar). *Fish & Shellfish Immunology*, *21*(4), 431-441. https://doi.org/https://doi.org/10.1016/j.fsi.2006.02.001

Klykken, C., Boissonnot, L., Reed, A. K., Whatmore, P., Attramadal, K., & Olsen, R. E. (2022). Gene expression patterns in Atlantic salmon (Salmo salar) with severe nephrocalcinosis. *Journal of Fish Diseases*, *45*(11), 1645-1658. https://doi.org/https://doi.org/10.1111/jfd.13687

Laplante, A. F., Moulin, V., Auger, F. A., Landry, J., Li, H., Morrow, G., Tanguay, R. M., & Germain, L. (1998). Expression of Heat Shock Proteins in Mouse Skin During Wound Healing. *Journal of Histochemistry & Cytochemistry*, *46*(11), 1291-1301. https://doi.org/10.1177/002215549804601109

Li, H., Yang, Y., Hong, W., Huang, M., Wu, M., & Zhao, X. (2020). Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. *Signal Transduction and Targeted Therapy*, *5*(1), 1. https://doi.org/10.1038/s41392-019-0089-y

Maisnier-Patin, S., Roth, J. R., Fredriksson, Å., Nyström, T., Berg, O. G., & Andersson, D. I. (2005). Genomic buffering mitigates the effects of deleterious mutations in bacteria. *Nature Genetics*, *37*(12), 1376-1379. https://doi.org/10.1038/ng1676

Matz, J. M., Blake, M. J., Tatelman, H. M., Lavoi, K. P., & Holbrook, N. J. (1995). Characterization and regulation of cold-induced heat shock protein expression in mouse brown adipose tissue. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *269*(1), R38-R47. https://doi.org/10.1152/ajpregu.1995.269.1.R38

Munir, D., Munro, E. S., Secombes, C. J., & Dooley, H. (2020). Atlantic salmon kidney (ASK) cells are an effective model to characterise interferon (IFN) and IFN-induced gene expression following salmonid alphavirus infection. *Fish Shellfish Immunol*, *106*, 792-795. https://doi.org/10.1016/j.fsi.2020.08.043

Muralidharan, S., & Mandrekar, P. (2013). Cellular stress response and innate immune signaling: integrating pathways in host defense and inflammation. *Journal of Leukocyte Biology*, *94*(6), 1167-1184. https://doi.org/10.1189/jlb.0313153

Peñaranda, M. M. D., Jensen, I., Tollersrud, L. G., Bruun, J. A., & Jørgensen, J. B. (2019). Profiling the Atlantic Salmon IgM(+) B Cell Surface Proteome: Novel Information on Teleost Fish B Cell Protein Repertoire and Identification of Potential B Cell Markers. *Front Immunol*, *10*, 37. https://doi.org/10.3389/fimmu.2019.00037

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*, *29*(9), e45. https://doi.org/10.1093/nar/29.9.e45

Pfaffl, M. W. (2006). *Real-time PCR (1st ed.)*. (M. Dorak, Ed. First edition ed.). Taylor & Francis.

Pfaffl, M. W., Tichopad, A., Prgomet, C., & Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnol Lett*, *26*(6), 509-515. https://doi.org/10.1023/b:bile.0000019559.84305.47

Poljšak, B., & Milisav, I. (2012). Clinical implications of cellular stress responses. *Bosn J Basic Med Sci*, *12*(2), 122-126. https://doi.org/10.17305/bjbms.2012.2510

Press, C. M., & Evensen, Ø. (1999). The morphology of the immune system in teleost fishes. *Fish & Shellfish Immunology*, *9*(4), 309-318. https://doi.org/https://doi.org/10.1006/fsim.1998.0181

Rolland, J. B., Bouchard, D., Coll, J., & Winton, J. R. (2005). Combined use of the ASK and SHK-1 cell lines to enhance the detection of infectious salmon anemia virus. *J Vet Diagn Invest*, *17*(2), 151-157. https://doi.org/10.1177/104063870501700209

Santoro, M. G. (2000). Heat shock factors and the control of the stress response. *Biochemical Pharmacology*, *59*(1), 55-63. https://doi.org/https://doi.org/10.1016/S0006-2952(99)00299-3

Smith, T. R., Tremblay, G. C., & Bradley, T. M. (1999). Characterization of the heat shock protein response of Atlantic salmon (Salmo salar). *Fish Physiology and Biochemistry*, *20*(3), 279-292. https://doi.org/10.1023/A:1007743329892

Strømsnes, T. A. H., Schmidke, S. E., Azad, M., Singstad, Ø., Grønsberg, I. M., Dalmo, R. A., & Okoli, A. S. (2022). CRISPR/Cas9-Mediated Gene Editing in Salmonids Cells and Efficient Establishment of Edited Clonal Cell Lines. *Int J Mol Sci*, *23*(24). https://doi.org/10.3390/ijms232416218

Svec, D., Tichopad, A., Novosadova, V., Pfaffl, M. W., & Kubista, M. (2015). How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. *Biomol Detect Quantif*, *3*, 9-16. https://doi.org/10.1016/j.bdq.2015.01.005

Svingerud, T., Holand, J. K., & Robertsen, B. (2013). Infectious salmon anemia virus (ISAV) replication is transiently inhibited by Atlantic salmon type I interferon in cell culture. *Virus Research*, *177*(2), 163-170. https://doi.org/https://doi.org/10.1016/j.virusres.2013.08.004

Teige, L. H., Kumar, S., Johansen, G. M., Wessel, Ø., Vendramin, N., Lund, M., Rimstad, E., Boysen, P., & Dahle, M. K. (2019). Detection of Salmonid IgM Specific to the Piscine Orthoreovirus Outer Capsid Spike Protein Sigma 1 Using Lipid-Modified Antigens in a Bead-Based Antibody Detection Assay [Original Research]. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.02119

Vabulas, R. M., Raychaudhuri, S., Hayer-Hartl, M., & Hartl, F. U. (2010). Protein folding in the cytoplasm and the heat shock response. *Cold Spring Harb Perspect Biol*, *2*(12), a004390. https://doi.org/10.1101/cshperspect.a004390

van Hees, M., Slott, S., Hansen, A. H., Kim, H. S., Ji, H. P., & Astakhova, K. (2022). New approaches to moderate CRISPR-Cas9 activity: Addressing issues of cellular uptake and endosomal escape. *Molecular Therapy*, *30*(1), 32-46. https://doi.org/https://doi.org/10.1016/j.ymthe.2021.06.003

Vazzana, M., Cammarata, M., Cooper, E. L., & Parrinello, N. (2002). Confinement stress in sea bass (Dicentrarchus labrax) depresses peritoneal leukocyte cytotoxicity. *Aquaculture*, *210*(1), 231-243. https://doi.org/https://doi.org/10.1016/S0044-8486(01)00818-3

Weaver, J. C. (1995). Electroporation Theory. In J. A. Nickoloff (Ed.), *Plant Cell Electroporation and Electrofusion Protocols* (pp. 3-28). Springer New York. https://doi.org/10.1385/0-89603-328-7:3

Zhang, S., Shen, J., Li, D., & Cheng, Y. (2021). Strategies in the delivery of Cas9 ribonucleoprotein for CRISPR/Cas9 genome editing. *Theranostics*, *11*(2), 614-648. https://doi.org/10.7150/thno.47007

Weaver, J. C. (1995). Electroporation Theory. In J. A. Nickoloff (Ed.), *Plant Cell Electroporation and Electrofusion Protocols* (pp. 3-28). Springer New York. https://doi.org/10.1385/0-89603-328-7:3

Zarate, J. (2006). The role of osmolyte transporters and heat shock proteins in adaptation of Atlantic salmon to selected stressors. ProQuest Dissertations Publishing.

Zhang, S., Shen, J., Li, D., & Cheng, Y. (2021). Strategies in the delivery of Cas9 ribonucleoprotein for CRISPR/Cas9 genome editing. *Theranostics*, *11*(2), 614-648. https://doi.org/10.7150/thno.47007

Appendix

Appendix I: RNA Isolation

Table 10. Total RNA concentration (ng/ul) and cDNA concentration (ng/ul) of Day 2 post-electroporation samples. *This table also shows the purity of extracted RNAs and cDNAs.*

Sample ID	RNA concentration (ng/ul)	A260/280 (1-8-2.1)	A260/230 (2.0-2.2)	cDNA concentration (ng/ul)	A260/280 (~1.8)	A260/230 (2.0-2.2)
2D Control 1	27.4	1.97	0.89	1765.8	1.81	1.99
2D Control 2	12.0	2.32	0.02	1853.6	1.82	1.82
2D Control 3	13.0	2.42	0.02	1694.0	1.82	1.51
2D Sham 1	8.0	1.94	0.02	1731.6	1.82	1.70
2D Sham 2	6.2	2.03	0.01	2044.2	1.79	1.71
2D Sham 3	4.9	1.81	0.04	1793.0	1.81	2.07
2D sgRNA 1	7.5	1.80	0.06	1554.4	1.83	2.04
2D sgRNA 2	10.6	1.68	0.56	361.9	1.82	1.48
2D sgRNA 3	4.8	1.83	0.04	358.0	1.82	1.47
2D Cas9 1	10.0	2.12	0.04	1693.3	1.81	1.89
2D Cas9 2	16.1	2.06	0.61	1688.2	1.83	2.20
2D Cas9 3	6.4	1.97	0.05	1592.0	1.83	2.20
2D RNP complex 1	31.9	1.54	0.49	1594.0	1.83	2.20
2D RNP complex 2	6.1	1.84	0.14	1644.6	1.83	2.17
2D RNP complex 3	6.6	1.80	0.05	1604.0	1.83	1.98

Table 11. Total RNA concentration (ng/ul) and cDNA concentration (ng/ul) of Day 7 post-electroporation samples. *This table also shows the purity of extracted RNAs and cDNAs.*

Sample ID	RNA concentration (ng/ul)	A260/280 (1-8-2.1)	A260/230 (2.0-2.2)	cDNA concentration (ng/ul)	A260/280 (~1.8)	A260/230 (2.0-2.2)
7D Control 1	3.3	1.71	0.33	1542.3	1.83	2.19
7D Control 2	4.5	2.09	0.02	1448.8	1.82	1.90
7D Control 3	12.3	1.60	0.24	1450.0	1.82	2.16
7D Sham 1	7.0	1.73	0.06	1426.3	1.83	2.05
7D Sham 2	8.1	1.54	0.68	1403.6	1.82	2.19
7D Sham 3	9.7	1.62	0.16	1154.3	1.82	2.09
7D sgRNA 1	7.0	1.81	0.23	1237.5	1.83	2.17
7D sgRNA 2	19.0	1.66	0.34	1339.0	1.83	2.14
7D sgRNA 3	13.1	1.73	0.48	1338.4	1.82	2.18
7D Cas9 1	8.1	1.60	0.31	1369.6	1.83	2.13
7D Cas9 2	18.1	1.70	0.80	1404.2	1.82	2.17
7D Cas9 3	10.5	1.75	0.23	1412.6	1.82	2.12
7D RNP complex 1	61.4	2.14	1.56	1368.5	1.80	2.14
7D RNP complex 2	19.5	2.26	1.03	1211.6	1.81	2.18
7D RNP complex 3	74.8	2.13	1.52	1503.8	1.81	2.16

Appendix II: DNA Isolation

***Table 12**. **Total genomic DNA concentration (ng/ul) 2-day and 7-day post-electroporation samples**. This table also shows the purity of extracted genomic DNAs.*

	Day 2 post-electroporation			Day 7 post-electroporation		
Sample ID	**DNA concentration (ng/ul)**	**A260/280 (1-8-2.1)**	**A260/230 (2.0-2.2)**	**DNA concentration (ng/ul)**	**A260/280 (1-8-2.1)**	**A260/230 (2.0-2.2)**
2D Control 1	6.4	2.49	0.63			
2D Control 2	3.7	2.42	0.37			
2D Control 3	5.4	1.76	0.62			
2D Sham 1	-1.4	3.63	0.15	4.2	0.85	0.36
2D Sham 2	1.4	0.46	0.16	4.2	0.69	0.22
2D Sham 3	-0.9	0.79	0.07	3.0	0.51	0.15
2D sgRNA 1	3.1	2.10	0.36			
2D sgRNA 2	-0.5	1.88	0.04			
2D sgRNA 3	0.1	1.04	0.01			
2D Cas9 1	-2.1	1.69	0.17	3.5	2.53	0.42
2D Cas9 2	-0.1	0.11	0.01	4.4	2.31	0.80
2D Cas9 3	-1.1	-0.77	-0.45	3.2	0.61	0.20
2D RNP complex 1	2.8	3.35	-0.32	30.9	2.17	9.49
2D RNP complex 2	14.0	2.31	-3.28	15.1	1.95	18.29
2D RNP complex 3	6.3	1.08	0.77	20.1	1.97	6.64

Appendix III: Relative gene expression of the target genes

Table 13. Descriptive statistics, gene expression ratio and PCR Efficiency of hsp90 gene in Day 2 and Day 7 samples

Group	Gene expression ratio	Average gene expression ratio	Standard deviation	Standard error	PCR Efficiency	
					Target gene	EF-1α
2D Control 1	1.27	1.03	0.24	0.14	110	94
2D Control 2	0.83					
2D Control 3	0.98					
2D Sham 1	0.57	0.57	0.00	0.00	110	94
2D Sham 2	0.57					
2D Sham 3	0.57					
2D sgRNA 1	0.57	0.57	0.00	0.00	110	94
2D sgRNA 2	0.57					
2D sgRNA 3	0.57					
2D Cas9 1	0.66	0.42	0.20	0.12	110	94
2D Cas9 2	0.29					
2D Cas9 3	0.32					
2D RNP complex 1	3.96	3.50	0.41	0.24	110	94
2D RNP complex 2	3.17					
2D RNP complex 3	3.38					
7D Control 1	10.70	3.96	5.82	3.36	110	94
7D Control 2	0.60					
7D Control 3	0.63					
7D Sham 1	1.00	1.87	0.95	0.55	110	94
7D Sham 2	1.74					
7D Sham 3	2.89					
7D sgRNA 1	1.81	1.57	0.21	0.12	110	94
7D sgRNA 2	1.48					
7D sgRNA 3	1.43					
7D Cas9 1	0.99	1.10	0.10	0.06	110	94
7D Cas9 2	1.16					
7D Cas9 3	1.15					
7D RNP complex 1	5.70	10.10	4.81	2.78	110	94
7D RNP complex 2	15.24					
7D RNP complex 3	9.36					

Table 14. Descriptive statistics, gene expression ratio and PCR Efficiency of hsp70 gene in Day 2 and Day 7 samples

Group	Gene expression ratio	Average gene expression ratio	Standard deviation	Standard error	PCR Efficiency	
					Target gene	EF-1α
2D Control 1 2D Control 2 2D Control 3	0.94 1.02 1.07	1.01	0.17	0.1	92	94
2D Sham 1 2D Sham 2 2D Sham 3	5.70 5.70 5.70	5.70	0.0	0.0	92	94
2D sgRNA 1 2D sgRNA 2 2D sgRNA 3	5.70 1.09 2.41	3.07	2.38	1.37	92	94
2D Cas9 1 2D Cas9 2 2D Cas9 3	0.60 1.46 1.25	1.10	0.45	0.26	108	94
2D RNP complex 1 2D RNP complex 2 2D RNP complex 3	1.04 0.61 1.55	1.07	0.47	0.27	108	94
7D Control 1 7D Control 2 7D Control 3	2.5 0.67 0.87	1.34	1.12	0.65	109	94
7D Sham 1 7D Sham 2 7D Sham 3	5.14 7.78 12.01	8.31	3.47	2.0	109	94
7D sgRNA 1 7D sgRNA 2 7D sgRNA 3	0.98 0.58 1.47	1.01	0.45	0.26	109	94
7D Cas9 1 7D Cas9 2 7D Cas9 3	0.94 1.19 1.44	1.19	0.25	0.14	109	94
7D RNP complex 1 7D RNP complex 2 7D RNP complex 3	0.05 0.17 0.07	0.10	0.07	0.04	109	94

Table 15. Descriptive statistics, gene expression ratio and PCR Efficiency of mhc I gene in Day 2 and Day 7 samples

Group	Gene expression ratio	Average gene expression ratio	Standard deviation	Standard error	PCR Efficiency	
					Target gene	EF-1α
2D Control 1	1.19	1.04	0.27	0.16	94	94
2D Control 2	0.78					
2D Control 3	1.17					
2D Sham 1	2.70	2.70	0.00	0.00	94	94
2D Sham 2	2.70					
2D Sham 3	2.70					
2D sgRNA 1	2.70	2.70	0.00	0.00	94	94
2D sgRNA 2	2.70					
2D sgRNA 3	2.70					
2D Cas9 1	2.89	1.78	0.96	0.56	94	94
2D Cas9 2	1.22					
2D Cas9 3	1.22					
2D RNP complex 1	1.94	2.51	0.50	0.29	94	94
2D RNP complex 2	2.89					
2D RNP complex 3	2.70					
7D Control 1	1.00	1.00	0.00	0.00	93.4	98
7D Control 2	1.00					
7D Control 3	1.00					
7D Sham 1	1.00	0.65	0.34	0.20	93.4	98
7D Sham 2	0.63					
7D Sham 3	0.32					
7D sgRNA 1	0.61	0.55	0.10	0.06	93.4	98
7D sgRNA 2	0.61					
7D sgRNA 3	0.44					
7D Cas9 1	0.58	0.46	0.11	0.06	91.1	93.4
7D Cas9 2	0.37					
7D Cas9 3	0.45					
7D RNP complex 1	0.11	0.29	0.21	0.12	91.1	93.4
7D RNP complex 2	0.52					
7D RNP complex 3	0.25					

Appendix IV: Target gene relative expression calculation

The relative gene expression was calculated using this formula. The PCR Efficiency % value varies, depending on the slope of the target gene. The GOI average CP and the HKG average CP are the average value of the 3 technical repeats. The ΔCP GOI and the ΔCP HKG were calculated by taking the average of the 3 technical repeats minus the CP value of the 1 repeat, e.g. (Average CP of Control 1 – Control 3) – Control 1 CP. The gene expression ratio was calculated by using the formula, R = (Converted E of GOI^ ΔCP GOI)/Converted E of HKG^ ΔCP HKG

Primer efficiencies	Efficiency %	Converted efficiency E
Gene of interest (GOI)	91	1.91
Housekeeping gene (HKG)	93	1.93

Sample	GOI average Ct	ΔCt GOI	HKG average Ct	ΔCt HKG	Gene expression ratio
Control 1	35	0.00	35	0.00	1.00
Control 2	35	0.00	35	0.00	1.00
Control 3	35	0.00	35	0.00	1.00
7D Complex 1	29.6	5.40	26.3	8.70	0.11
7D Complex 2	28.5	6.50	27.6	7.40	0.52
7D Complex 3	28.8	6.20	26.8	8.20	0.25

Group	Average	Standard deviation	Standard error
Control	1.00	0.00	0.00
Treated	0.29	0.21	0.12

GOI: Gene of interest or target gene; HKG gene: Housekeeping gene;

Appendix V: Standard curve of target and reference genes

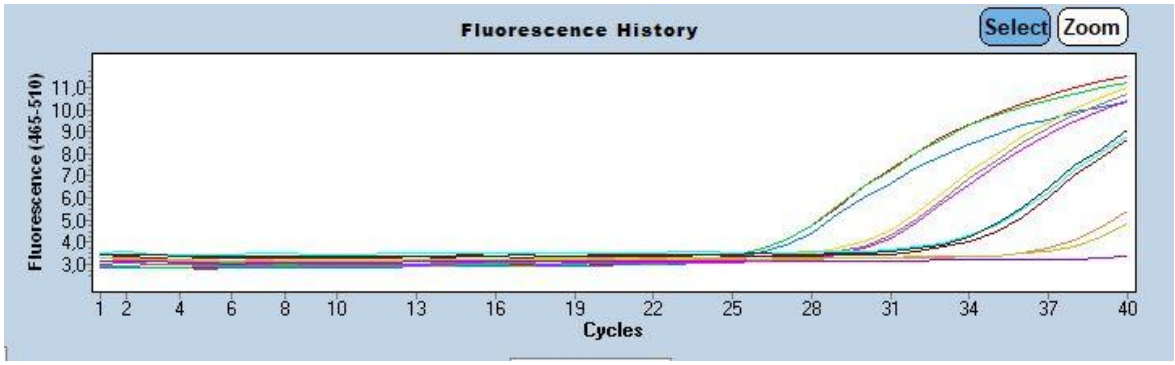

*Figure 23.**Standard curve of target gene hsp90 using ASK-1 cells as template***

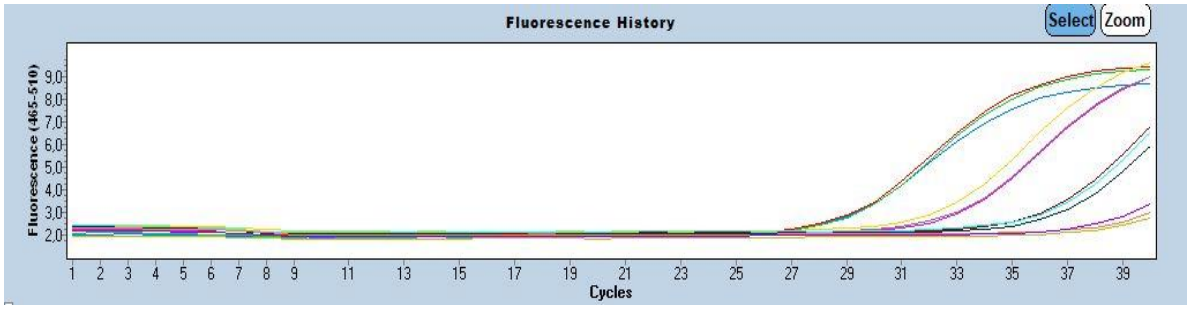

*Figure 24. **Standard curve of target gene hsp70 using ASK-1 cells as template***

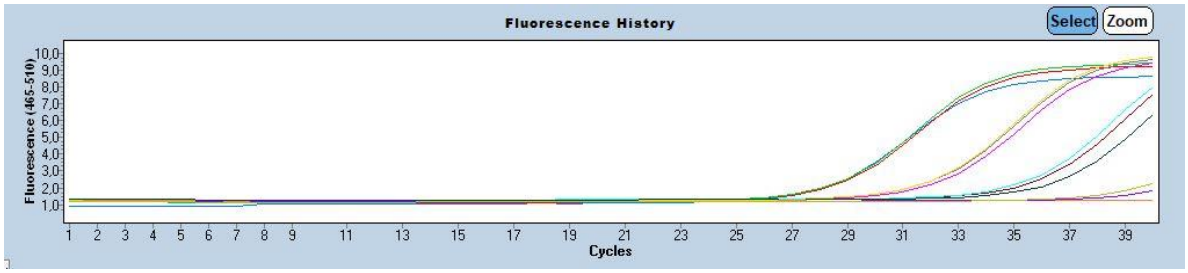

*Figure 25. **Standard curve of target gene hsp70 using ASK-1 cells as template***

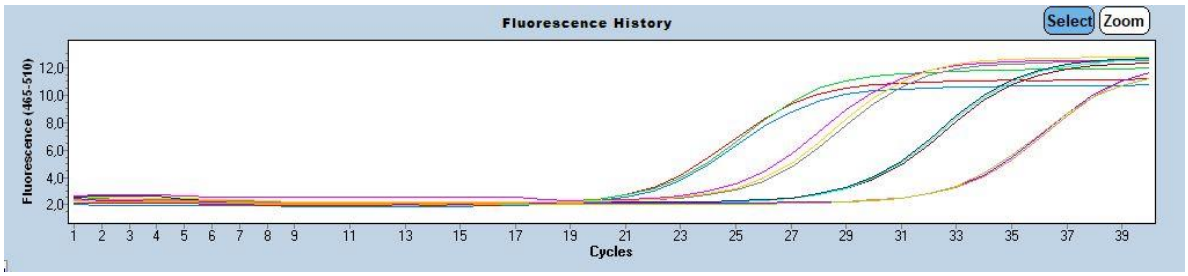

*Figure 26. **Standard curve of reference gene ef-1a using ASK-1 cells as template***