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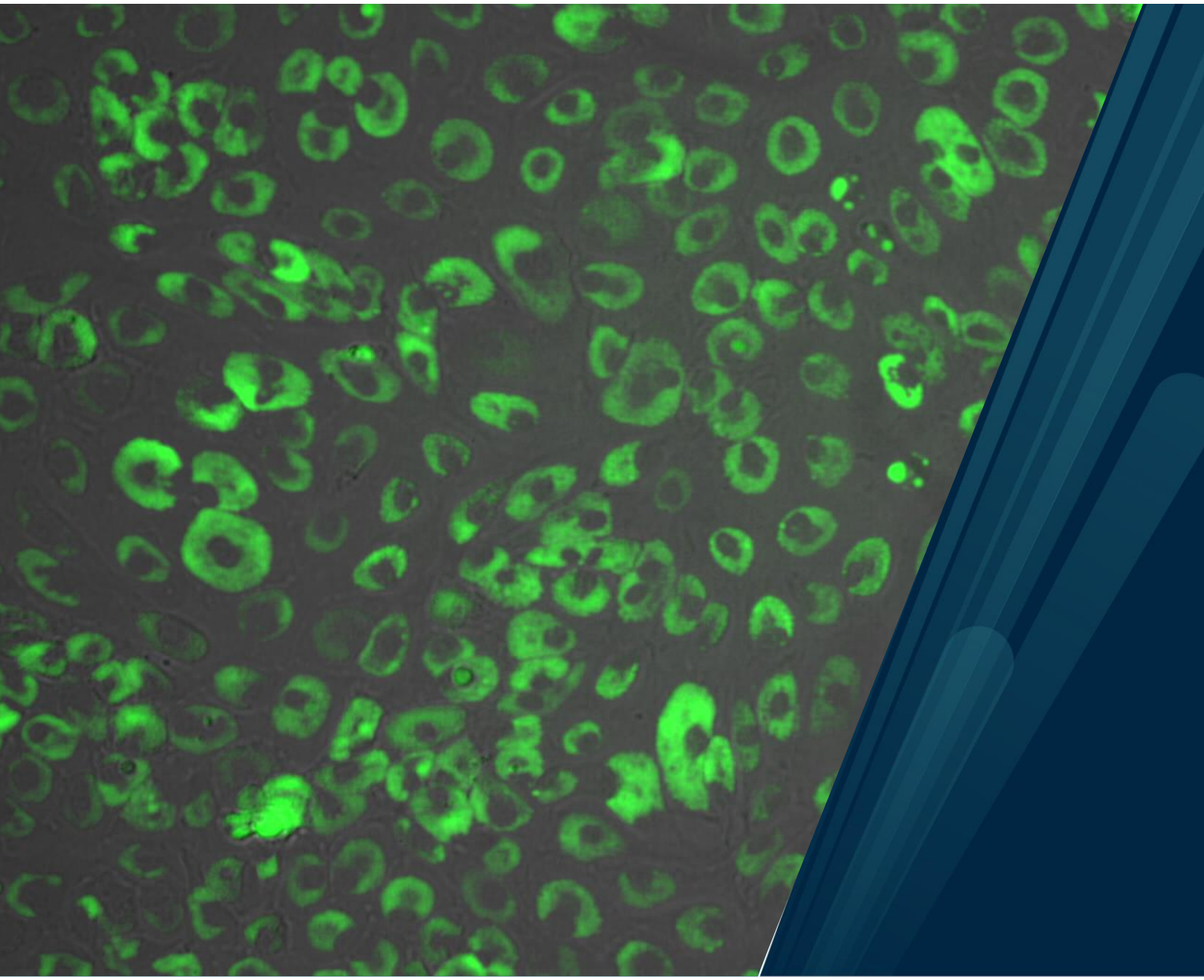

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Cover figure: ASK-1 cells electroporated with RNP complex (20x magnification);micrograph obtained two weeks after cell recovery. Photo: Trygve André Hagen Strømsnes

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Tromsø, May 2022
Trygve André Hagen Strømsnes

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Abbreviations

ASK-1	Atlantic salmon kidney
Cas 9	CRISPR associated protein 9; A nuclease protein
CHSE-214	Chinook salmon embryo 214
CRISPR	Clustered regulatory interspaced short palindromic repeats
DECODR	Deconvolution of complex DNA repair
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double strand brake
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
GFP	Green fluorescent protein
GMO	Genetically modified organism
HDR	Homology-directed repair
ICE	Inference of CRISPR edits
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISA	Infectious salmon anemia
ISAV	Infectious salmon anemia virus
LB	Lysogeny broth
NHEJ	Non-homologous end joining
NGS	Next generation sequencing
ODM	Oligonucleotide directed mutagenesis
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
SDN	Site directed nucleases
SDS	Sodium dodecyl sulfate
SDSA	Synthesis-dependent strand annealing

sgRNA	Single guide ribonucleic acid
TALEN	Transcription activator-like effector nuclease
ZFN	Zinc finger nuclease

Abstract

Background: The gene editing tool, CRISPR/Cas9, can potentially be applied in aquaculture to combat some of the problems of the farmed salmon industry including infectious diseases. The RNP (ribonucleoprotein)-based CRISPR/Cas9 strategy has recently been shown to be efficient in gene editing in diverse cell lines and organisms. Application of RNP strategy in salmon cell biology is still at its infancy. In this study, an in-house synthesized sgRNA (single guide ribonucleic acid) complexed to a Cas9-EGFP (EGFP; enhanced green fluorescent protein) was used to edit the *cr2* gene (complement receptor 2) which is an immune gene regulated during infection of Atlantic salmon (*Salmo salar*) by infectious salmon anemia virus. The target cells in this study were the salmonid ASK-1 and CHSE-214 cell lines. Strategies for isolation and expansion of edited single cell clones were also established.

Methods: Site specificity and cleavage potentials on the *cr2* gene of three sgRNAs (single guide ribonucleic acid) complexed to Cas9 were assessed using the *in vitro* cleavage assay prior to cell transfection of the complex. Transfection of the selected sgRNA/Cas9-EGFP complex was achieved by electroporation, and evaluation of *in vivo* activity was done using the T7 endonuclease 1 (T7E1) mismatch detection assay. Flow cytometry assisted cell sorting (FACS) enabled enrichment of the electroporated cells prior to detection of mutation by a combination of Sanger sequencing and the bioinformatic tools ICE and DECODR.

Results: The sgRNAs/Cas9 complex showed targeted *in vitro* cleavage activity of the *cr2* gene, which was supported by the *in vivo* T7E1 mismatch assay following transfection in ASK-1 and CHSE-214 cells. A high electroporation rate of the RNP complex (approx. 85%) was achieved. Gene edits were successfully detected in the genome of the edited cells although with variable editing efficiency (100% and 5%) due to the heterogenous nature of the FACS enriched cell population. Isolation and expansion of edited single cell clones was achieved but could not be reproduced within the timeframe of this project.

Conclusion: This project successfully applied the CRISPR/Cas9 RNP complex strategy to mutate the *cr2* gene in ASK-1 and CHSE-214 cells. The partial success in isolation and expansion of single clones of edited cells, and the identification of pitfalls as well as future perspectives related to the use of RNP complex in salmonids cells will contribute to extend this new frontier.

1 Introduction

1.1 Background

1.1.1 CRISPR/Cas versus other gene editing techniques

The clustered regularly interspaced short palindromic repeats/CRISPR-associated Cas protein (CRISPR/Cas) has become the most popular tool for gene editing. It has surpassed the other gene editing tools, namely zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), oligonucleotide directed mutagenesis (ODM), and meganucleases. The CRISPR/Cas, ZFN, TALEN, ODM and meganucleases are also known as site directed nucleases (SDN) because they target specific sites in a gene where they induce targeted mutation. Presently, CRISPR/Cas of which CRISPR/Cas9 is the commonest variant, is the most popular SDN because it is relatively easy to use, inexpensive and the most efficient. The CRISPR/Cas9 was derived from the *Streptococcus pyogenes* Type II CRISPR/Cas system, which is a component of the bacterial defense system against bacteriophages and plasmids (Cong et al., 2013, Jinek et al., 2012).

The CRISPR RNA (crRNA), which is a part of the bacterial Type II system, is the part that is homologous to the target DNA and combines with a transactivating CRISPR RNA (tracrRNA), the part that recruits the Cas protein (Jansen et al., 2002). In CRISPR/Cas9, both crRNA and tracRNA are fused/assembled to form the guide RNA (gRNA) (also termed sgRNA; single guide RNA) (Wei et al., 2015, Heler et al., 2015), which directs the Cas9 to the complementary region in the DNA. The sgRNA is approximately 20 – 24 base pair (bp) long and can be programmed to target any site in any gene which fulfils the requirement of the presence of a PAM (protospacer adjacent motif; 5´-NGG-3´ for the Streptococcus Cas9) site immediately downstream of the sequence region homologous to the gRNA. At this site, the Cas9 can make a DSB three nucleotides upstream of the PAM sequence. The DSB activates the cell´s DNA repair mechanisms through either of two pathways: the non-homologous end joining (NHEJ) or the homology directed repair (HDR) pathway. For gene knockout, the NHEJ repair pathway, which makes an error prone undirected nucleotide changes in the absence of a repair template, is desirable over the HDR where an exogenous DNA sequence template is needed to direct the repair mechanism leading to introduction of donor nucleotide sequences at target sites (Jiang and Doudna, 2017). The insertion and deletion (indels) mutations made by the error prone NHEJ can lead to knock out of the function of a target gene.

1.1.2 Applications of gene editing in aquaculture/salmon breeding

Fish aquaculture, in particular salmon breeding, is plagued by a plethora of challenges, including infectious diseases, reduced fertility and viability, slow growth, escapee fish and environmental pollution (Pérez-Sánchez et al., 2018, Okoli et al., 2022). The CRISPR/Cas9 technology has been added to the toolbox of potential strategies to combat these challenges (Okoli et al., 2022). Recently, CRISPR/Cas9 was used to induce increased growth in two fish species, namely, a tiger puffer (edited to grow 1.9 times heavier than conventional tiger puffers) and a red sea bream (edited to grow 1.2 times larger than conventional red sea bream (Biotechnology, 2022). The increased growth was achieved using CRISPR/Cas9 to disrupt the appetite-controlling leptin receptor gene in tiger puffer. In red sea bream, the myostatin protein, which suppresses muscle growth was disrupted allowing the fish to grow about 1.2 times larger on the same amount of food (Biotechnology, 2022). Other examples of the application of CRISPR/Cas9 in fish breading is the experimental creation of sterile (Wargelius et al., 2016), albino (Edvardsen et al., 2014) and high omega-3 producing Atlantic salmon (*Salmo salar)* (Jin et al., 2020). The albino and sterile gene-edited A. salmon were proof-of-principle experimental fish aimed at solving the escapee problem of farmed salmon.

Infectious disease is a pertinent problem that threatens the sustenance of the future expansion of the farmed fish industry. For example, the infectious salmon anemia (ISA) had a historic impact on salmon populations in Chile between 2009 and 2010, where there was a 54% reduction of farmed A. salmon from 211000 tons in 2009 to 98000 in 2010 (Asche et al., 2010). In Norway, there have been annual reports of between 1-25 outbreaks of ISA since 1993 (Jansen, 2021). Presently, there are no ISA vaccine on the Norwegian market that can induce full ISAV protection, exemplified with the increased outbreak of ISA in Norwegian aquaculture. However, CRISPR/Cas9 system can be used as a strategy to combat the problematic infectious disease of farmed aquaculture by targeting certain traits which may be responsible for virus entry, propagation and or genes central in antiviral protection. This technology has experimentally been used to increase viral resistance in several fish species. CRISPR/Cas has been exploited, for example, in grass carp (*Ctenopharyngodon idellus*) to create resistance against grass carp hemorrhagic virus (Zhong et al., 2002); homozygous rainbow trout (*Oncorhynchus mykiss*) strains which are resistant to infectious hematopoietic necrosis virus (IHNV) (Chiou et al., 2002); and expression of viral antisense genes to improve IHNV resistance in rainbow trout (Chiou et al., 2014). Gene editing can thus be combined with

selective breeding programs to enhance rate of genetic gain for disease resistance traits (Houston, 2017, Zenger et al., 2019, Houston et al., 2020).

1.1.3 Relevance of using CRISPR/Cas9 in cell biology

Studies of gene editing in salmonid-derived cells will contribute to elucidate functional mechanisms underlying cell response to e.g., pathogens as well as genetic factors responsible for disease resistance and susceptibility. Until the advent of CRISPR/Cas9, it was difficult to conduct studies in salmonid-derived cell-lines using available gene editing toolbox due to many factors including slow cell growth and transfection difficulty of the cells. Gene editing in salmonid cell-lines is still at its early stages although the introduction and use of CRISPR/Cas9 started ten years ago (Jinek et al., 2012). Using CRISPR/Cas9, Gratacap et al (Gratacap et al., 2020) efficiently edited ASK-1 and SHK-1 cells (cell lines originating from A. salmon) and CHSE-214 (derived from Chinook salmon); and Zoppo et al. (Zoppo et al., 2021) edited RTgutGC cell (cell of rainbow trout). Zoppo et al achieved a clonal cell-line selection, expansion and genotyping of the gene edited single cells. However, only a 34% gene editing efficiency was achieved, which was attributed to the nature of sgRNA, non-optimization of sgRNA using *in-vitro* cleavage assay, genomic target location and epigenetic accessibility (Zoppo et al., 2021). The Gratacap et al achieved 99-100% transfection of ASK-1, CHSE-214, SHK-1 cells with the ribonucleoprotein (RNP) (i.e., CRISPR & Cas9-EGFP) complex. Transfection being the first hurdle to overcome in any gene editing strategy, the high transfection result was remarkable given the intrinsic transfection difficulty of salmonid cells. The group also showed efficient editing of the cell-lines via the NHEJ pathway, but was unsuccessful in clonal cell-line selection, expansion, and genotyping of edited single cells (Gratacap et al., 2020).

Clonal line selection of gene edited cells and subsequent genotyping is necessary for elucidation of gene function, and at the end, development of vaccines and therapeutics. Further, establishment of platforms of gene editing in salmonids cell-lines will enable biosafety studies of the use of CRISPR/Cas in salmon breeding, such as off- and on-target effects of editing, adequacy of delivery systems and tools for detection and tracing of gene edited aquaculture products. The objective in the current study was to use the RNP CRISPR/Cas9 strategy to induce mutations in, the *cr2* gene in the ASK-1 and CHSE-214 cells. The *cr2,* codes for the complement receptor type 2 that plays an important role in A. salmon immune system and has been associated with susceptibility of the fish to ISA (Schiøtz et al., 2008, Abbas et al., 2019),

making it a good candidate for the study. *In-vitro* assay for testing the cleavage activities of sgRNAs was optimized together with methods for clonal expansion of edited single cells.

2 Aim of the thesis

The main aim in the project is to induce mutations in ASK-1 and CHSE-214 targeting the *cr2* gene, whereas the sub-aims were to: a) deliver the sgRNA and Cas9 (RNP) complex into the ASK-1 and CHSE-214 cells, b) *cr2* mutation analysis and c) establish method for clonal expansion of gene edited signal cells.

3 Materials and methods

3.1 Workflow

The workflow for this project is illustrated in the schematic (Figure 1) showing an overview of the pipeline for this thesis, followed by a more detailed explanation.

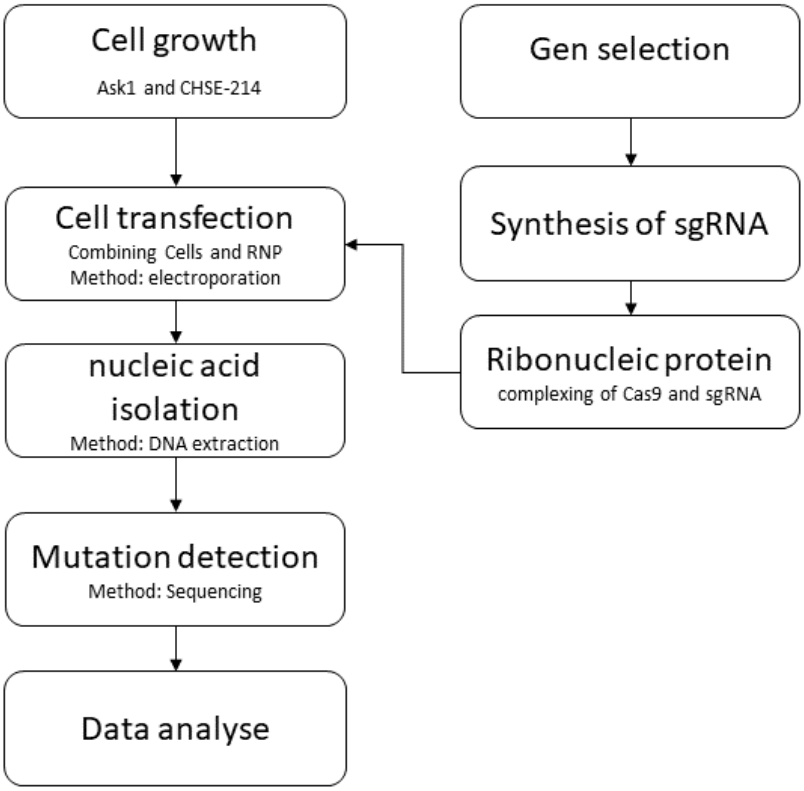

Figure 1. Workflow for this project. The project started out with identification of target gene, synthesis of sgRNA, complexing of Cas9 and sgRNA to form the RNP complex, transfection of the RNP complex into cells, DNA extraction, sequencing, and data analysis.

3.2 Cell lines used in the project

Atlantic salmon kidney (ASK-1) and Chinook salmon embryo 214 (CHSE-214) were used in the current study. ASK-1 was purchased from the Federal Research Institute for animal health, Germany, and CHSE-214 was purchased from Sigma-Aldrich, United Kingdom. The CHSE-214 is an adherent immortalized cell-line derived from Chinook Salmon (*Oncorhynchus tshawytscha)* embryo. The ASK-1 is also an adherent immortalized cell-line but derived from Atlantic salmon (*Salmo salar)* kidney. The ASK-1 and CHSE-214 are widely used for studies in cell biology. They are both susceptible to a wide range of viruses and are ideal for studies of salmon response to ISAV infection (Rolland et al., 2005).

3.2.1 Cell-culture

Vials of frozen cells were taken from liquid nitrogen, thawed in warm (70℃) water for less than 1 minute and aseptically transferred into a T-25 cm tissue culture flask containing the cell culture medium. The cell culture medium consisted of Leibovitz L15 (Gibco/USA) supplemented with 10% foetal bovine serum (FBS) (Biowest/Brazil). Cells were incubated in the dark (flasks wrapped in aluminium foil) between 22℃ - 22.1℃ until confluency. At confluency (>75% confluency), cells were passaged by aspirating culture media from flask, washed with phosphate buffered saline (PBS) (Sigma/UK) and detached by incubation in 0.25% trypsin (Sigma/USA) for 1-2 minutes (for ASK-1) and 6 minutes (for CHSE-214). Equal volumes of cell culture media w/FBS were added to the detached cells to neutralize the effect of trypsin. Cells were then pelleted by centrifugation at 1400 RPM for 5 minutes, resuspended in cell culture medium and split in 1:1 ratio into two new T-25 cm flasks. Flasks were incubated in the dark at between 22℃ - 22.1℃ until confluency. Passaging of cells was done at least three times before cells were used for subsequent experiments.

3.3 Choice of genes for CRISPR/Cas mutation

The gene selected for targeted CRISPR/Cas9 mutation was the complement receptor type 2-like, *cr2*. The *cr2* codes for complement receptor type 2, which plays an important role in A. salmon immune system (Schiøtz et al., 2008, Abbas et al., 2019) and has also been associated with the ISA disease.(Schiøtz et al., 2008)

3.4 Design and synthesis of sgRNA

Design of sgRNA for *cr2* was done using the Synthago sgRNA design website (https://design.synthego.com/#/) with *Salmo Salar* RefSeq ICSASQ V2 (Figure 2). Three sgRNA sequences (Table 1) from exon 2 were selected based on the software recommendations parameters of (i) early coding region; (ii) common exon; (iii) High activity; and (iv) minimal off-target. The three sgRNA sequences target different sections of the *cr2* gene.

Table 1. The three sgRNAs chosen for project

sgRNA Code	sgRNA sequences	PAM
sgRNA_cr2_01	AACGGCGCATCACATTTCGA	5´...TGG...3´
sgRNA_cr2_02	TGCGTGTGTGGATAGGACAA	5´...CGG...3´
sgRNA_cr2_03	CUCGAUUUGCGUGUGUGGAU	5´…AGG…3´

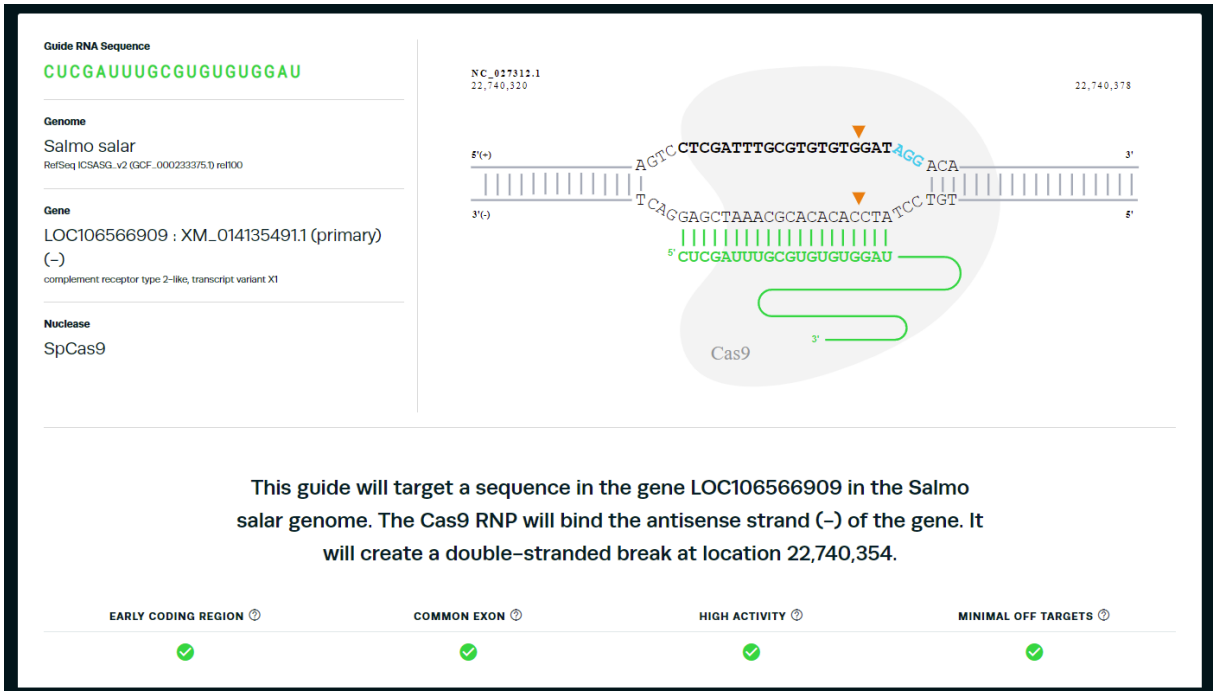

Figure 2. A view from https://design.synthego.com/#/ for a selected sgRNA showing the PAM in light blue and the alignment of the sgRNA sequence (green) to the homologous sequence. Orange arrows show the expected Cas9 cut site on both strands.

For synthesis of the sgRNA, a target-specific oligo was first designed in which the sgRNAs (Table 1) are appended to the 5' end of the T7 promoter sequence (TTCTAATACGACTCACTATA), and a 14 nucleotide overlap sequence (overlap with *S. pyogenes* Cas9 scaffold oligo) was appended to the 3' end such that the sgRNA sequence, which is preceded with a 'G' at the 5' end, is in the middle, i.e., the sgRNA is flanked by the T7 promoter sequence and the overlap sequence

(TTCTAATACGACTCACTATAG[N]$_{20}$GTTTTAGAGCTAGA). The synthesis was carried out in accordance with the manufacturer´s instructions by mixing the target-specific oligos with the manufacturer's (New England, Biolabs/UK) reaction mix (Table 2) at room temperature. The mixture was incubated at 37℃ for 1 hour. The resulting sgRNA contains the target-specific crRNA sequence as well as the tracrRNA. To ensure optimal activity, synthesized sgRNA was purified using the monarch RNA clean-up kit (New England Biolabs/UK) to remove proteins, salts, and unincorporated nucleotides. Concentration was measured using the nanodrop (nanodrop 2000c/ThermoFisher scientific/USA). Purified sgRNA was aliquoted and stored at -80℃ until use.

Table 2. Components for sgRNA synthesis

Components for sgRNA synthesis	**Volume**
Nuclease-free Water	2 µl
EnGEN 2x sgRNA Reaction mix *S. pyogenes* (NTP's dNTPs, *S. pyogenes* Cas9 scaffold oligo)	10 µl
Target-specific DNA Oligo (1 µM)	5 µl
DTT (0,1 M)	1 µl
EnGen sgRNA Enzyme mix (DNA and RNA polymerases)	2 µl

3.5 DNA extraction, RNA extraction and cDNA synthesis

For DNA isolation, confluent cells in 6-well or 12-well plates (or detached cell pellets) were lysed using 200 µl lysis buffer containing 20 µl proteinase K. DNA was subsequently isolated using the GenElute™ Mammalian Genomic DNA Miniprep Kit (SIGMA/USA) according to the manufacturer's instructions. For RNA isolation, confluent cells in plates (or pelleted cells in tubes) were lysed with 400 µl of lysis solution and RNA isolated using the RNAqueous®-4PCR Kit (Applied Biosystems/USA) in accordance with the manufacturer´s instructions. Reverse transcription of the isolated RNA to cDNA was done using the components of the High-Capacity cDNA reverse Transcription kit (ThermoFisher/USA) (Table 3) in accordance with manufacturer´s instructions and the PCR settings outlined in Table 4.

Table 3. High-capacity cDNA reverse transcription

Component	Volume
10x RT buffer	2.0 µl
25x dNTP mix (100nM)	0.8 µl
20X oligo dT (SO132)	1.0 µl
Multiscribe reverse TXase	1.0 µl
Nuclease free H_2O	5.2 µl
Total per reaction	10 µl

Table 4. End point PCR setting for conversion of RNA to cDNA

	Step1	**Step 2**	**Step 3**	**Step 4**
Temperature	25℃	37℃	85℃	4℃
Time	10 min	120 min	5 min	Hold

3.6 *In vitro* cleavage assay

The activities of the three synthesized sgRNAs were evaluated using an *in-vitro* cleavage assay system. Primers were designed to amplify the target region of interest, which is the region surrounding the sgRNA binding area. The expected amplicon size of the target region is 289 bp, and the sgRNA target region is approximately at the middle of the region as seen in Figure 3.

Figure 3. Region of interest (289 bp) showing the 3 sgRNA binding sites

Genomic DNA extracted from CHSE-214 cells served as the template for amplification. The reaction mixtures used for amplification is as listed in Table 5.

Table 5. Components used in the DNA amplification for in-vitro cleavage assay

Component	Quantity	Manufacturer
sgRNA	400-600 ng (z µl)	
Cas9 protein	1000 ng (y µl)	Sigma, USA
Target substrate	100-300 ng (x µl)	
10X NEB buffer	5,5 µl	New England biolabs, UK
10X BSA	1,5µ	New England biolabs, UK
Nuclease free water	to 15 µl 8-(x+y+z) µl	

The reaction mixture was incubated at 37℃ for 1h 30 minutes. To denature excess RNA, 1 ul RNAse was added to the mixture and incubated at 37℃ for 15 minutes, followed by addition of 1 µl STOP solution (30% glycerol, 1.2% SDS, 250 mM EDTA (pH 8.0)) and additional incubation at 37℃ for 15 minutes. The sgRNA 102 bp size was checked on 2% agarose gel.

3.7 RNP/Cas-9 complex formation

Prior to electroporation, sgRNA was diluted in nuclease-free 1x OPTIMUM media (Gibco/USA) and mixed with appropriately diluted Cas9-EGFP protein (SIGMA/USA) in a range between 37 pmol (1,2 µg) sgRNA per 26 pmol (5µg) Cas9 to 50pmol (1,6µg) sgRNA per 26 pmol 2:1 (sgRNA:Cas9) for 23700 -50000 cells. A ratio of 2:1 (sgRNA:Cas9) was used to form sgRNA/cas9 complex using the Synthego protocol (Synthego.com/resources). The mixture was incubated at room temperature for 10 minutes. The resulting complex was stored at -20℃ for not later than three months or until use.

3.8 Cell transfection by electroporation

3.8.1 Preparation of cells for electroporation

Cells at 50-70% confluency were trypsinized, transferred to a 15 ml Falcon tube and centrifuged at 100 x g for 5 minutes at room temperature. Old media were aspirated (or saved as spent media for subsequent use as conditioned media in later experiments). Cell pellets were resuspended in PBS and centrifuged at 100 x g for 5 minutes at room temperature. Thereafter cells were resuspended in 1ml Optimem medium (Gibco,USA) and transferred to a 1.5 ml

microcentrifuge tube to a final density between 1-8 x10^6 cells/ml and gently pipetted to obtain single cell suspension.

3.8.2 Cell electroporation

Electroporation of cells was done using the Neon Transfection system (Invitrogen/USA). The RNP-sgRNA complex was added to the cell suspension to a final concentration of 5 μM and mixed by gentle pipetting. Electrical pulse (1600 V, 10 ms, 3 pulses) was delivered to approximately 1.0 x 10^6 cells (ASK cells) or approximately 4.0 x 10^6 cells (CHSE-214 cells) in a 10 μl Neon tip. Electroporated cells were transferred to 12-well plates containing L15 media without antibiotics. Controls consisted of (i) sham-electroporated cells, i.e., cells electroporated with OPTIMEM resuspension medium, and (ii) cells electroporated with Cas9 protein. The setup was done in triplicate and incubated in the dark as previously described in 3.2.1. Cells were checked for viability and fluorescence after 24 h by fluorescence microscope (ZEISS AX10 vert.A1).

3.9 Fluorescence-activated cell sorting (FACS)

Fluorescence-activated cells sorting (FACS) (Figure 4) is a method for separation of cells of interest, in this case the enhanced green fluorescing ASK-1 and CHSE-214 cells, from a heterogenous cell population. Cells are separated based on the intensity of fluorescence, thus, enriching the population of desired cells while excluding undesired (non-fluorescent) cells. This is currently the state-of-the arts method of isolating desired single cells into, for example, a 96-well plate for clonal cell expansion.

3.9.1 Preparation of conditioned media for FACS:

Media from previous cell passages (spent media) was collected and centrifuged for 5 min at 100 x g to remove any floating cells and media were filter-sterilized with 0.20-micron syringe-adapted filter (VWR/USA). Spent media was mixed with equal volumes of fresh media and supplemented with 20% FBS to make the conditioned media.

3.9.2 Preparation of cells for FACS:

Following 72 h post transfection, cells were detached by trypsinization and washed in PBS as described in 3.2.1. The cells were resuspended in 1 ml conditioned media and kept on ice until FACS.

3.9.3 Cell sorting

Transfected cells were enriched via FACS on the BD FACSAria III cell sorter (BD/USA) using a130-micron nozzle (Figure 4). Single cells events were gated and the percentage of GFP-positive cells together with the intensity of GFP fluorescence from each cell was measured. EGFP-positive cells were sorted into a 96-well plate contain 200 µl of conditioned media or into a tube and later transferred to a 24-well plate or used directly for genomic DNA/RNA isolation. Cells sorted into 96-well plates were incubated for single cell colony expansion according to the conditions described in 3.2.1

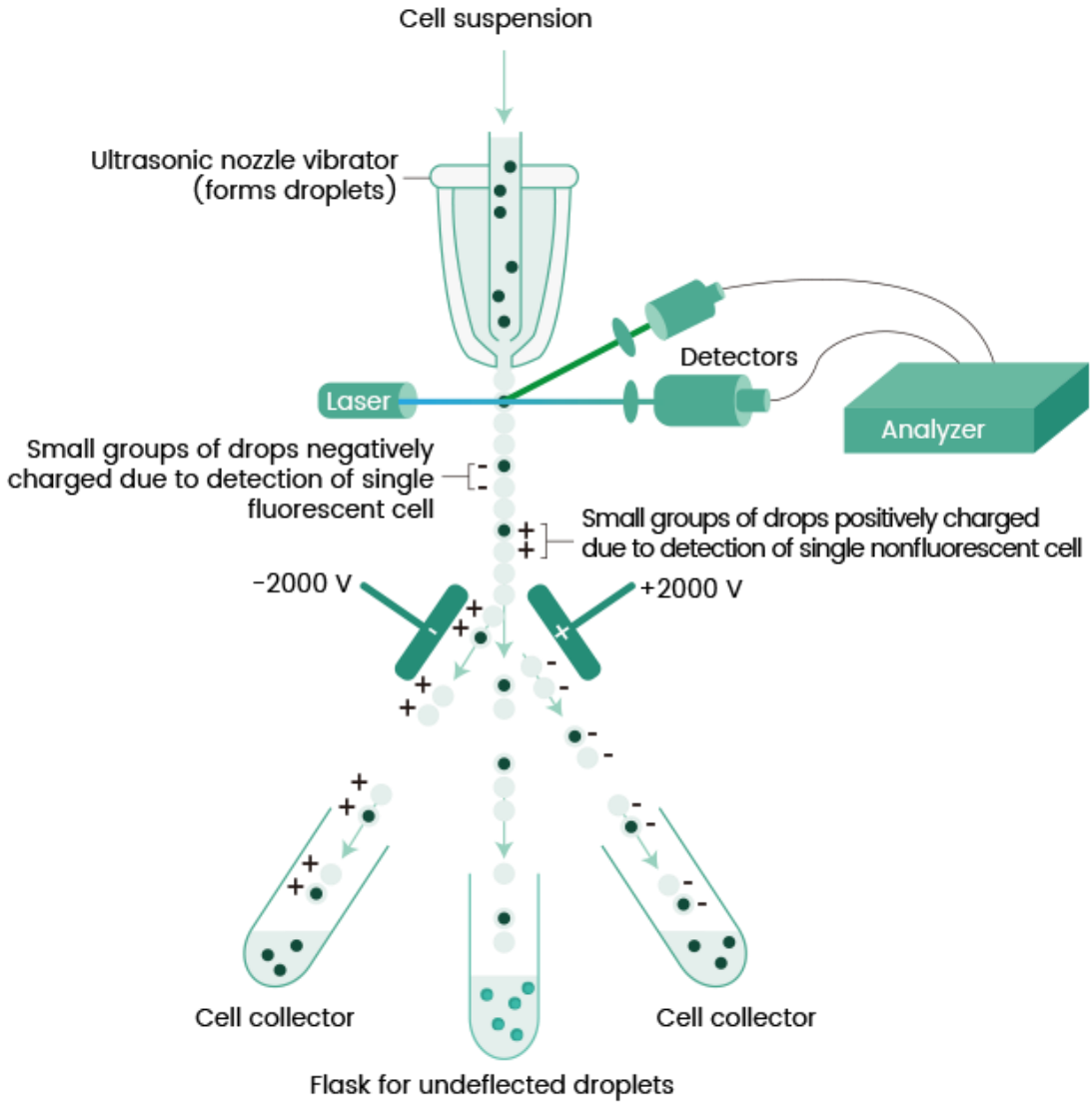

Figure 4. Illustration of FACS made by (SinoBiological, n.a) showing how cells from a mixed population is individually passed through a laser. Cells are sorted based on the analysis of a fluorescence detector, and fluorescing cells are attracted to a tube or well by electric current.

3.10 T7 Endonuclease 1 (T7E1) assay

Assessment of *in-vivo* cleavage ability of the RNP complex was performed on the amplified targeted region of the *cr2* gene. This was done using the T7E1 mismatch assay, which is a widely used method for evaluating the activity of site-specific nucleases. In the first step, the targeted region of the *cr2* was PCR-amplified using primers (Forward: CACTTGCCCTACATGCCTCA, Reverse: GCCACAACAACCTCATCCCA) and DNA template from sham-control and FACS-enriched RNP complex-treated cells, yielding a DNA fragment of approximately 487 bp. To determine whether mutations due to insertions and deletions were present, heteroduplexes were formed by denaturing and re-annealing the PCR product using the cycling steps presented in Table 6 in accordance with the manufacturer´s instructions (New England BioLabs/UK).

Table 6. T7 Assay amplification cycles

Cycle Step	**Temp**	**Time**	**Cycles**
Initial denaturation	98℃	5 min	1
Annealing	95-85℃	-2℃/sec	1
	85-25℃	-0.1 ℃/sec	1
Hold	4℃		1

Using the endonuclease I, which recognizes mismatches larger than 1 base, digestion of the annealed heteroduplexes was achieved by incubating the enzyme with the annealed PCR product in 1:19 ratio at 37℃ for 15 minutes according to the manufacturer´s instruction (New England BioLabs/UK). Thereafter, 1 ul of proteinase K was added to the mixture and incubated at 37℃ for 5 minutes to inactivate the T7 endonuclease I activity. Fragment analysis was done by electrophoresis of the mixture in a 2% agarose gel to provide an estimate of the in vivo efficiency of the RNP complex in ASK-1 and CHSE-214.

3.11 Detection of mutation by Sanger sequencing

To further determine the efficiency of gene editing and the exact type of gene edits, the targeted region in the *cr2* gene was amplified and sequenced as follows: Genomic DNA was extracted from cells transfected with the *cr2*_sgRNA/Cas9 complex and sham-transfected control cells as previously described in 3.8.2. The target region was amplified such that the amplicon size was approximately 289 bp. The primer sequences used for the amplification are (i) (forward primer: TTTGACACTTGATAATGCGACTGC)

(ii) (reverse primer: ACAAGGCAAAGTCCACTTTAACAC) and in accordance with the manufacturer's instructions. The cleaned-up PCR product was cloned into the pGEM-T Easy Vector System (Promega/USA), briefly 7 ul of PCR product was ligated into 1 µl (50 ng) pGEM-t Easy vector according to the reaction mixture in Table 7. The mixture was mixed by pipetting and incubated at room temperature for 1 hour. Table 7 shows the different constituents and volumes used in this setup.

Table 7. table for ligase reaction components

Component	**Volume**
Insert (DNA product)	7 µl
Vector	1 µl
T4 DNA ligase	2 µl
Rapid ligation buffer	10 µl
Nuclease free water if necessary	X µl
Total volume	20 µl

Transformation of ligated product was done using the JM109 High Efficiency competent cells (Promega/USA) according to standard protocol. Briefly, 3 µl of ligated product was mixed with 50 µl of JM109 competent cells in a sterile I7 x 100 mm polypropylene tube placed on ice for 30 min. Cells were heat-shocked for 2 min in a water bath at 42℃ without shaking, and tubes were immediately returned to ice for 2 minutes. Room-tempered SOC medium (250 µl) was added to the tubes containing transformed cells and incubated for 1.5 h at 37℃ with shaking (~230rpm). Thereafter, 200 µl transformed culture was plated onto LB/ampicillin/IPTG/X-Gal plate and incubated overnight. Plates were observed after overnight incubation for blue/white colonies; white colonies generally contain inserts.

For DNA extraction, single colonies were picked from both treated and control plates into LB ampicillin-containing broth and incubated overnight at 37℃. DNA extracted from pelleted overnight bacteria cultures was purified and served as template for PCR amplification used for Sanger sequencing according to the following protocol: Samples were diluted to 100 ng/µl, and 5 µl was mixed in a PCR tube with sequencing master mix, which contained (i) big dye, (ii) sequencing buffer, and (iii) M13 primer. The mixture was amplified by PCR according to the parameters listed below (Table 8).

Table 8. Settings used for sequencing PCR amplification

Steps	Temperature	Time	No. cycles
1	96℃	1 min	
2	96℃	30 sec	34
3	50℃	15 sec	
4	60℃	4 sec	
6	4℃		

3.12 Mutation analysis

Assessment of the nature and frequency of the CRISPR-mediated edits was conducted using the free online bioinformatic tool Inference of CRISPR Edits (ICE) ((https://ice.synthego.com) and Deconvolution of Complex DNA Repair (DECODR) ((https://decodr.org/analyze to analyse the Sanger sequences obtained from FACS enriched RNP complex electroporated cells in comparison with the sham electroporated control cells.

4 Results

4.1 Cell culture

The ASK-1 and CHSE-214 cells were slow-growing cells. A 1 x 10^6 CHSE-214 cells required approximately 7 days to reach about 60% confluency in a T-175 tissue culture flask. The ASK-1 cells grew slower requiring approximately 9 days to confluence when similar density of cells was seeded in the same T-175 tissue culture flask (Figure 5 and Figure 6).

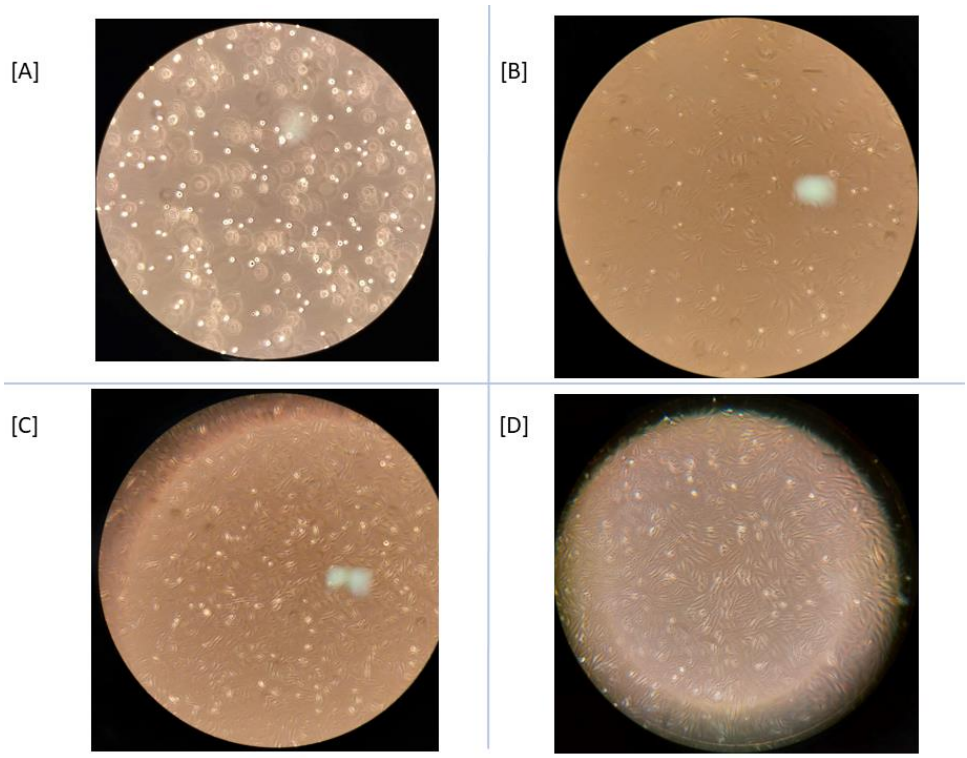

Figure 5. ASK-1 cells at different levels of confluency [A] Day 0 of cell culture; [B] Day 3 following cell culture, cells were at approximately 10% confluency in a T-175 tissue culture flask, [C] Day 9 following cell culture, cells were at approximately 60% confluency; [D] Example of cells at 100% confluency. Pictures are representative of microscope´s binocular phases taken from a T-175 tissue culture flask of ASK-1.

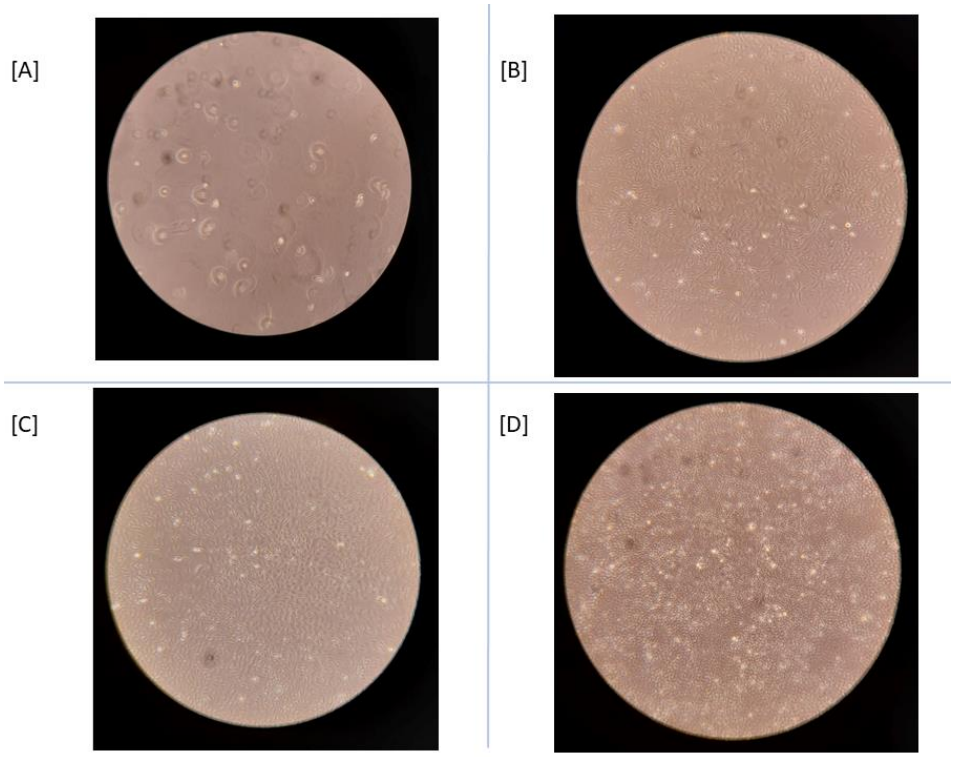

Figure 6. CHSE-214 cells at different levels of confluency [A] Day 0 of cell culture; [B] Day 3 following cell culture, cells were at approximately 40% confluency in a T-175 tissue culture flask, [C] Day 6 following cell culture, cells were at approximately 60% confluency; [D] Example of cells at 100% confluency. Pictures are representative of microscope´s binocular phases taken from a T-175 tissue culture flask of CHSE-214.

4.2 sgRNA synthesis and evaluation of sgRNA/Cas9 cleavage ability

As a cost-saving strategy, the three sgRNAs (cr2_01, cr2_02, cr2_03) targeting different sites in the *cr2* gene were synthesized in-house (Figure 7, Table 9). This approach showed that the three sgRNAs were made of high purity and in sufficient amounts (Table 9 and Figure 7).

Table 9. Nanodrop readings showing quantity and purity of synthesized sgRNA

Sample ID	Nucleic acid (DNA)	A260 (Abs)	A280 (Abs)	A260/ A280	A260/ A230
sgRNA Cr2_01	554,9 ng/µl	13,87	6,39	2,17	2,72
sgRNA Cr2_02	1285,1 ng/µl	31,13	14,890	2,16	2,72
sgRNA Cr2_03	1445,8 ng/µl	36,14	16,94	2,13	2,51

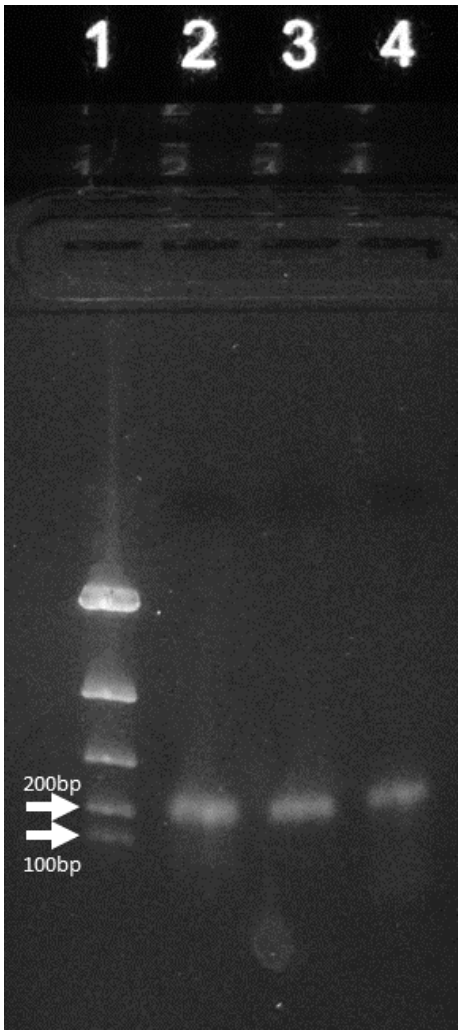

Figure 7. Bands of the three sgRNA on a 2% agarose gel. Electrophoresis was done with 1000 ng of heat-denatured sgRNA.

To determine the ability of the synthesized sgRNA to guide the Cas9 to the specific gene locus of interest and cause a DNA cleavage, an *in-vitro* cleavage assay was performed. The PCR amplicon (~ amplicon size 289 bp) of the *cr2* gene locus was incubated with sgRNA-Cas9 complex. Figure 8 shows two bands corresponding to the 160 bp and 129 bp sizes and a band at the 289 bp size (Lanes 1-6). Untreated controls (Lanes 7-11) with visible bands only at the 289 bp band size can be observed (Figure 8). This suggests an effective cleavage of the 289 bp amplicon into two smaller amplicons of 160 bp and 129 bp band sizes.

Components	1	2	3	4	5	6	7	8	9	10	11
Cas 9	+	+	+	+	+	+	-	-	-	-	+
sgRNA 1	+	+	-	-	-	-	-	+	-	-	-
sgRNA 2	-	-	+	+	-	-	-	-	+	-	-
sgRNA 3	-	-	-	-	+	+	-	-	-	+	-
DNA	+	+	+	+	+	+	+	+	+	+	+

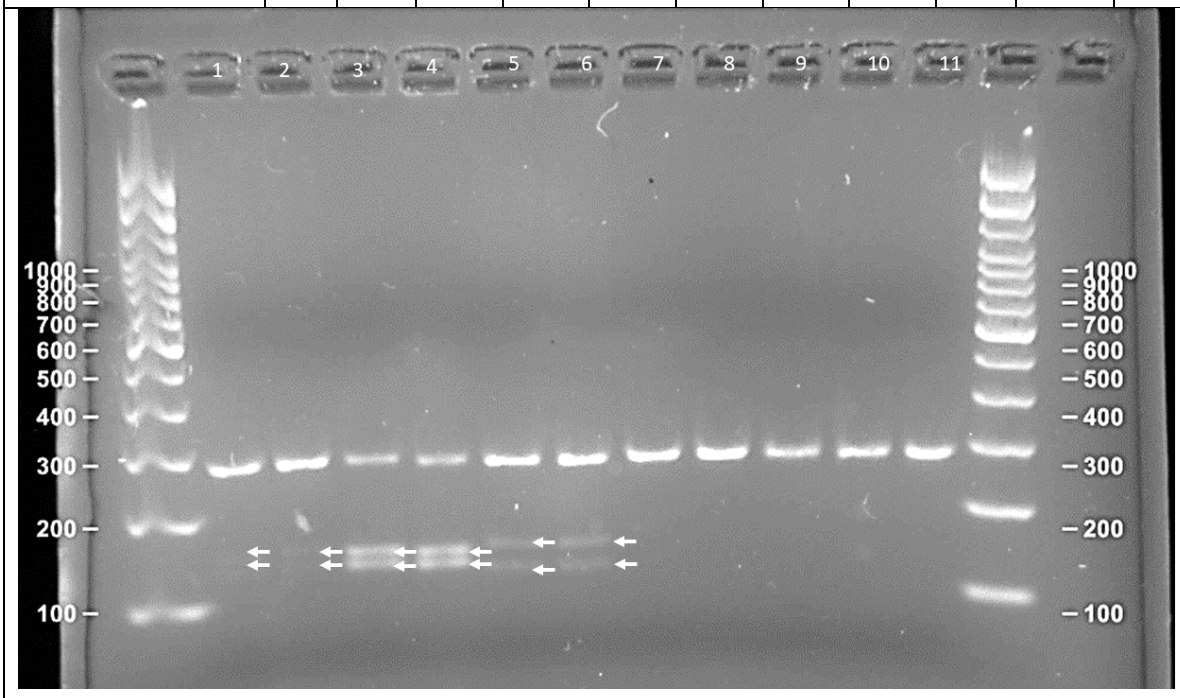

Figure 8. In-vitro cleavage assay. The gel shows bands in lanes 1-6 (experiment in duplicate) for Cas9-sgRNA complex; DNA template control (lane 7); sgRNA control (lanes 8-10); Cas9 control (lane 11). Cleavage products from the RNP complex cleavage are indicated with arrows.

4.3 Transfection of ASK-1 and CHSE-214 cells with sgRNA/Cas9 complex

Gene editing of the *cr2* gene in ASK-1 and CHSE-214 via the complexed sgRNA and Cas9 was achieved in the salmonids-derived cells ASK-1 and CHSE-214 via transfection with the sgRNA *cr2*_03/Cas9 complex. The *cr2*_03 was selected from the three most promising sgRNA candidates generated by the Synthago web tool (https://design.synthego.com/#/). For practical reasons, including availability at the time of study, the sgRNA *cr2*_03 was chosen for further investigation in this study, although sgRNA *cr2*_02 showed better *in vitro* assay cleavage result. The electroporation parameters, 1600 V 10 ms 3 pulses, which have previously shown high (99.9-100%) result in ASK-1 and CHSE-214 (Gratacap et al., 2020) were used. The results from electroporation in our experiments were approximately in the range between 75 – 80% with the RNP complex (i.e., sgRNA/Cas9-EGFP), and approximately 80-90% with the Cas9-EGFP. Cells with green fluorescence in comparison to non-fluorescing cells showed successful

transfection with either the sgRNA/Cas9-EGFP complex (Panel A) or Cas9-EGFP (Pane B) (Figure 9 and Figure 10). Following transfection, cells were incubated for up to 72 hours to allow cell recovery.

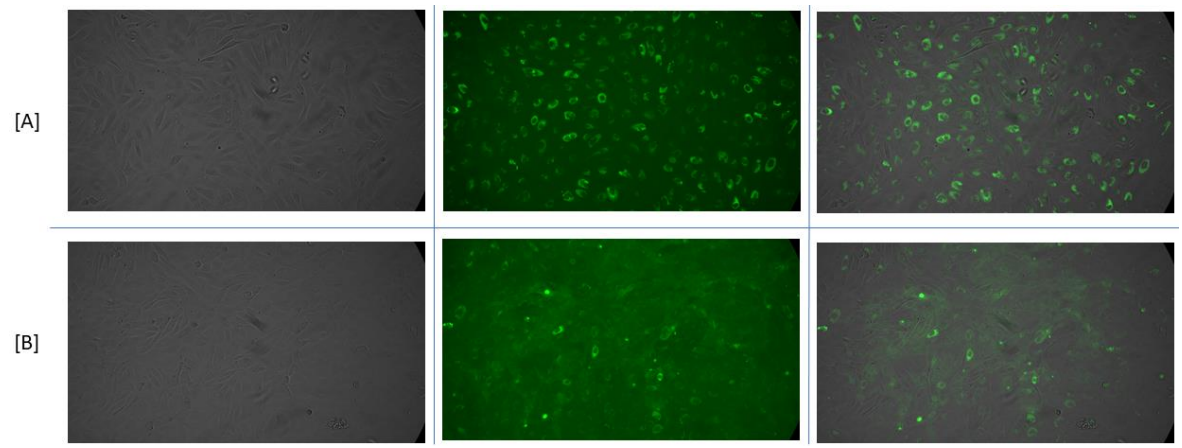

Figure 9. ASK-1 cells after electroporation [A] are cells electroporated with the RNP complex [B] cells electroporated with Cas 9 only. Left panels show column light micrographs, middle panels show cells using fluorescence microscopy. Whereas the right panels show overlay of left and middle panels.

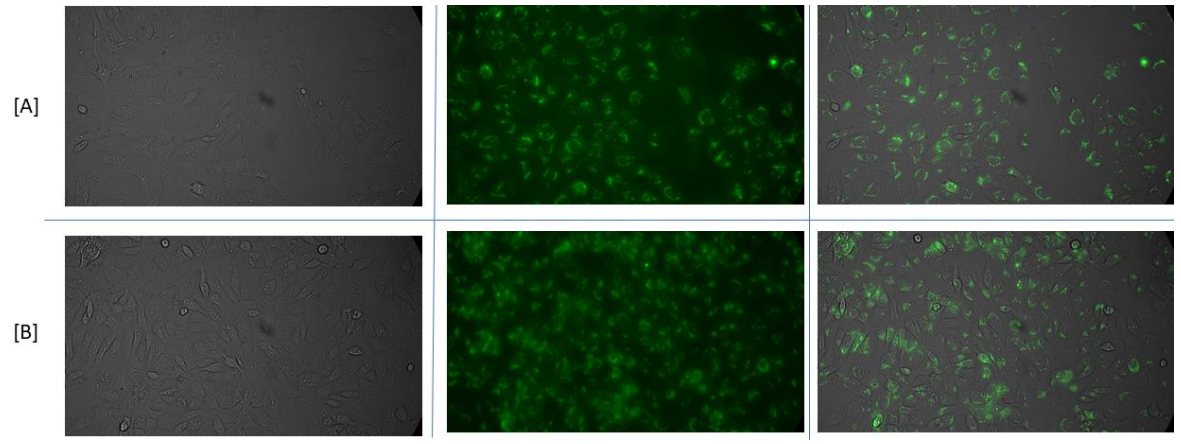

Figure 10. CHSE-214 cells after electroporation [A] are cells electroporated with the RNP complex [B] cells electroporated with Cas 9 only. Left panels show column light micrographs, middle panels show cells using fluorescence microscopy. Whereas the right panels show overlay of left and middle panels.

4.4 FACS-enrichment of transfected cells

Enrichment of transfected cells was done within 72 hours post electroporation using FACS. Single cell sorting of EGFP-positive cells into a 96-well plate was done before the rest of the EGFP-positive cells were sorted into a tube and subsequently transferred into a 24-well plate. SHAM-electroporated cells and Cas9-EGFP electroporated cells were used as background controls and were analyzed first. This served as a stringent control that enabled appropriate setting of gates for the isolation of only fluorescent cells from the RNP complex-electroporated cells. As a result, only about 10% of the fluorescent cells were sorted and were too few to be

propagated into monolayer when transferred into 24-well plates. A mitigation strategy was to collect the EGFP positive cells directly from FACS, without seeding. However, this resulted in too low DNA concentration for subsequent genotyping.

4.4.1 Clonal expansion of transfected cells

Limited cell growth was detected in the 96-well plates after 14 days for ASK-1 (Figure 11) and after 4 days for CHSE-214 (Figure 11). However, due to bacterial infection the cells were not processed further, and the timeframe of the project did not allow for repetition of the experiment.

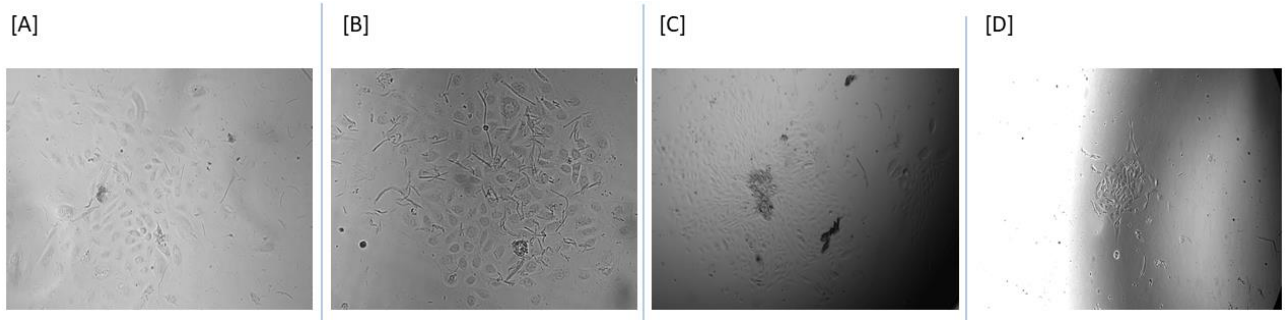

Figure 11. Micrographs show expansion of single cell clones. [A]-[C] ASK-1 cells in a 96-well plate at day 14 (20X magnification) (A) 18 (20X magnification) (B) and 27 (5X magnification) (C) respectively after FACS. Micrograph D shows CHSE-214 cells 4 days after FACS (10x magnification).

4.5 *In vivo* mismatch cleavage assay

The *in vivo* gene editing efficiency of the selected sgRNA was evaluated using the T7E1 assay. Fragments analysis on 2% agarose gel of the digested heteroduplexes clearly revealed two cleavage band at band sizes 281 and 207 in the samples containing RNP Complex-treated Cells (Lanes 3 and 4). No bands of similar sizes were found in the control samples of cham-electroporated cells (lanes 1 and 2). This corroborated the *In vitro* cleavage assay (Figure 12). Section 4.2 of the sgRNA and provided justification for evaluation of nature of edits by Sanger sequencing.

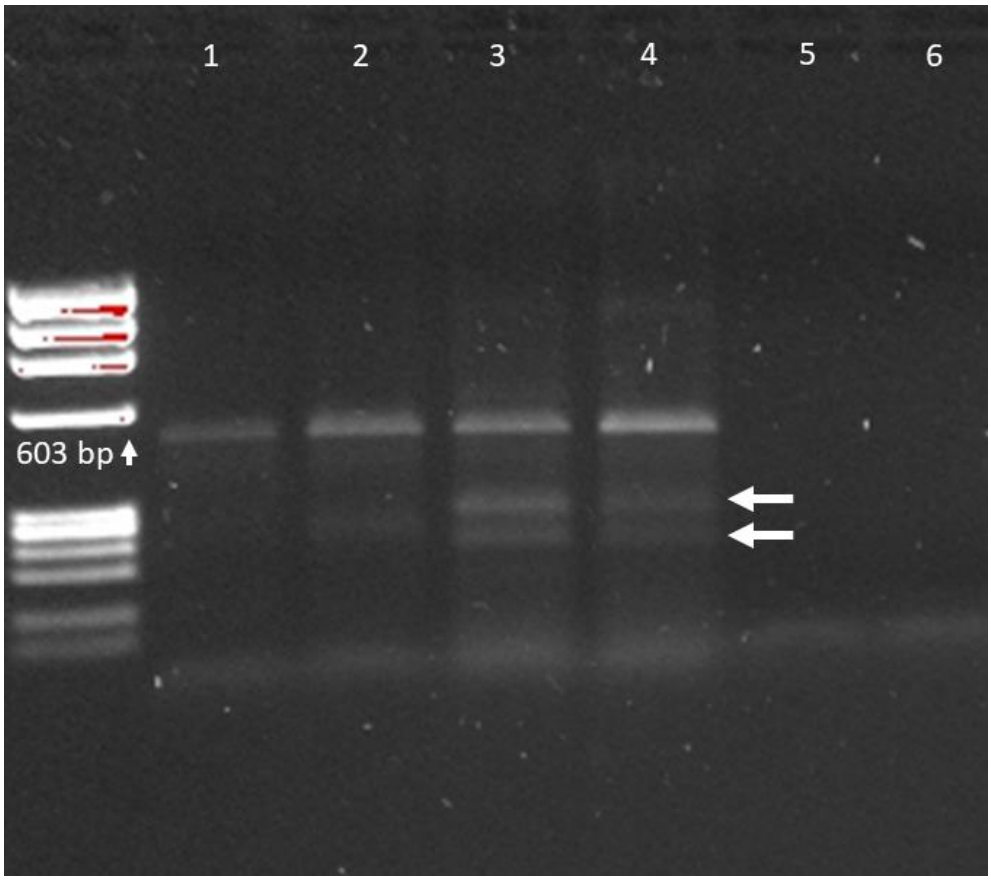

Figure 12. T7E1 endonuclease assay samples per line is line 1-2 are gDNA from FACS enriched sham-electroporated cells of CHSE-214 treated with T7E1, lanes 3-4 are gDNA from FACS enriched RNP complex-treated cells treated with T7E1. Cleavage bands are indicated with white arrows; 5-6 negative control.

4.6 Mutation detection

One of the objectives of the project was to assess the nature and frequency of the different types of mutation/edits present in the population of mixed wild-type and mutated ASK-1 and CHSE-214 cells caused by a CRISPR/Cas9 DSB. To achieve this, DNA from several positive bacteria transformants containing the amplified region of the *cr2* targeted by the sgRNA/Cas9 complex was subjected to Sanger sequencing. However, only 2% (2 out of approximately 100) samples submitted for sequencing showed successful ligation of the targeted region (amplicon size: 289 bp). A high percentage (80%) of sequenced samples showed ligated products of amplicon sizes in the range of 77 – 280 bp, where approximately 90% showed the 77 bp amplicon size. The 77 bp ligated product, which constituted about 90% of the ligated product different from the target region showed high homology (80% identity) with the *hnRNP R* (heterogeneous nuclear ribonucleoprotein R) gene of A. salmon (Figure 14), suggesting a possible insertion mutation during the DSB repair by the cells. This was rather surprising since insertion mutation of greater than few base pairs by the NHEJ repair pathway is a rare event. To confirm this, several transformants from the control plate was submitted for sequencing, however, the same 77 bp product was identified in some of the controls, indicating that this was an unspecific product that was amplified and ligated into the sequencing vector alongside the target product (Figure 15). This problem can be circumvented by gel purification of visible bands in the 289 bp band size region for sequencing; the limited timeline of this project did not allow for this to be done.

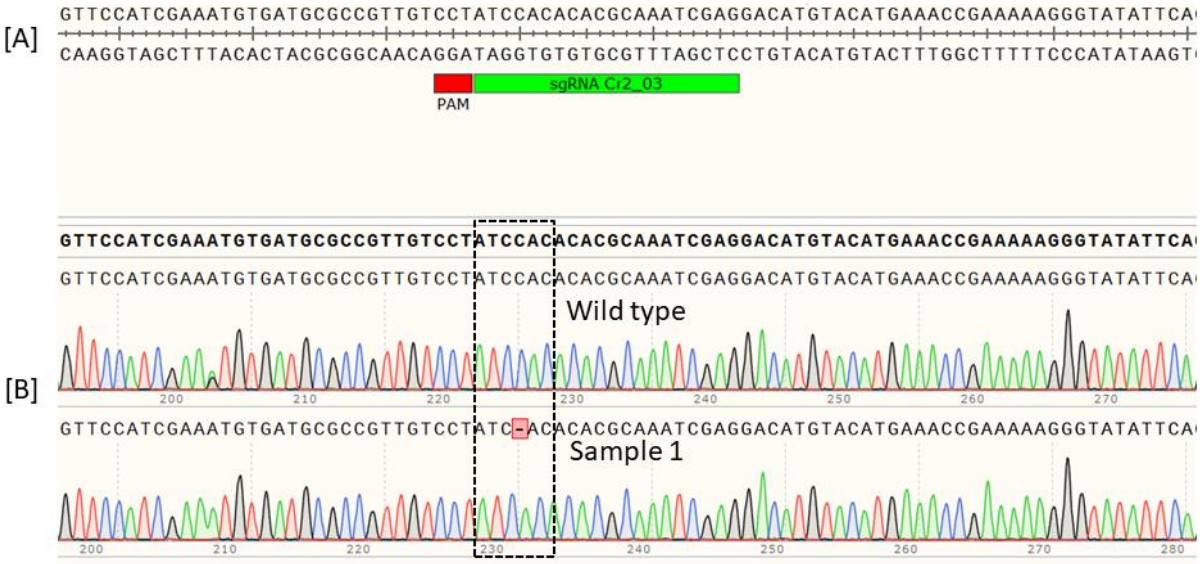

Figure 13. A representative Sanger sequence electropherogram. The block line shows [A] the PAM (red) region and the region homologous to the sgRNA (green). Wild-type sequence upper electrophoregram [B] is aligned to the experimental sequence (sample 1). A mismatch in the electropherogram is noticed in the indel region (dotted square).

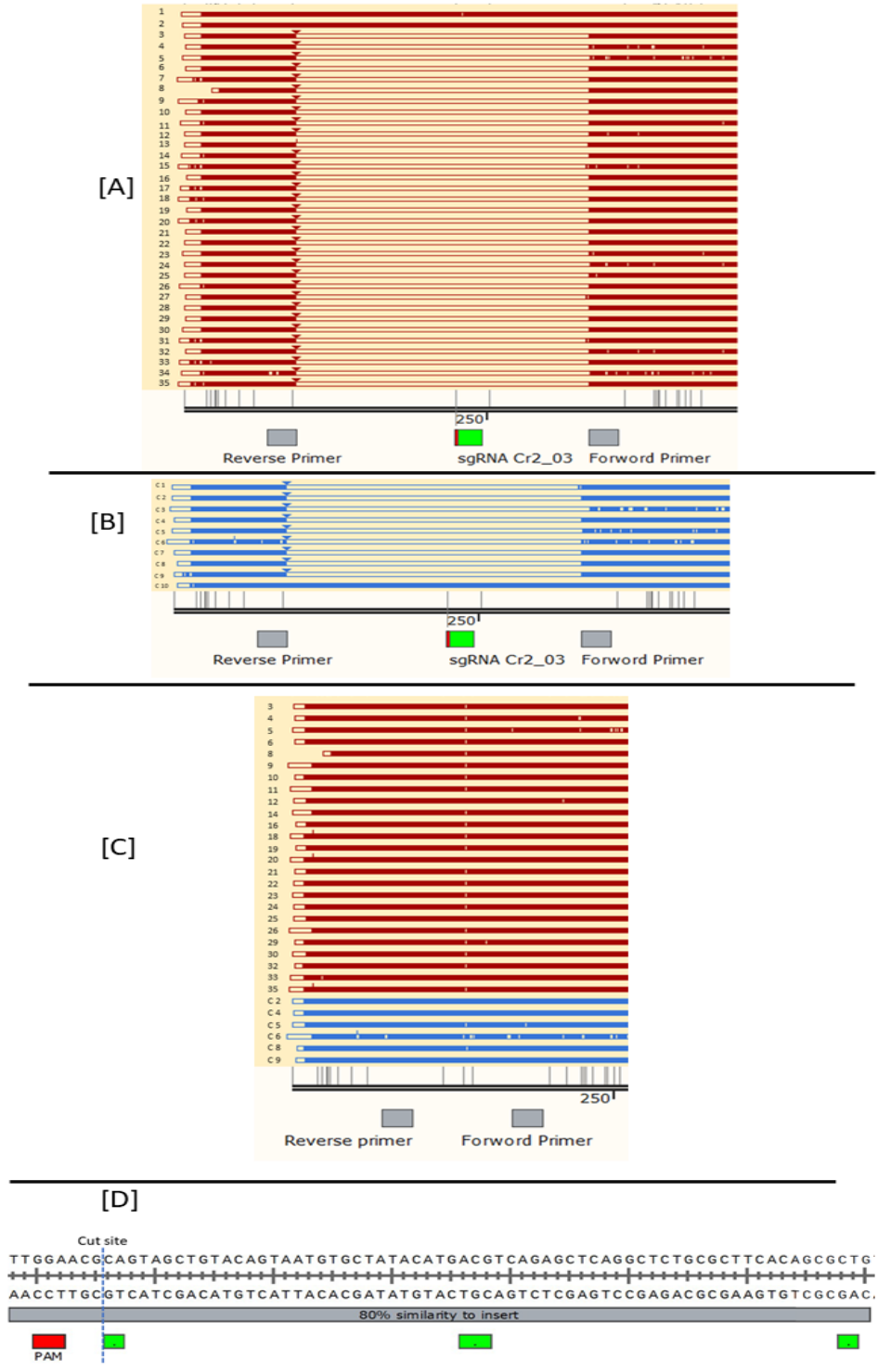

Figure 14. [A] Alignment of the Sanger sequences from the fluorescing RNP-complex transfected cells. The first two alignments were from colony samples containing the target region. The rest of the samples do not possess this target region. [B] Alignment of sequences from the control (sham-electroporated cells) also missing the target region. [C] Alignment of the sequences (77 bp insert) that were preferentially incorporated in place of the target region. Blue denotes control sequences; red denotes the 77 bp insert. [D] Alignment of the 77 bp insert with a homologous region, the hnRNP R (heterogeneous nuclear ribonucleoprotein R) from salmon. The 77 bp insert has high homology (80%) to the hnRNP R. Fragments of the sgRNA (green block) and a PAM site (red block) were found flanking the region, suggesting the hnRNP R is an off-target site for the sgRNA, and that a DSB would have occurred in this site leading to the insertion of the 77 bp insert. However, the same insert was found in some control samples, which leads to the conclusion that the insert was not the result of a CRISPR/Cas9-induced mutation.

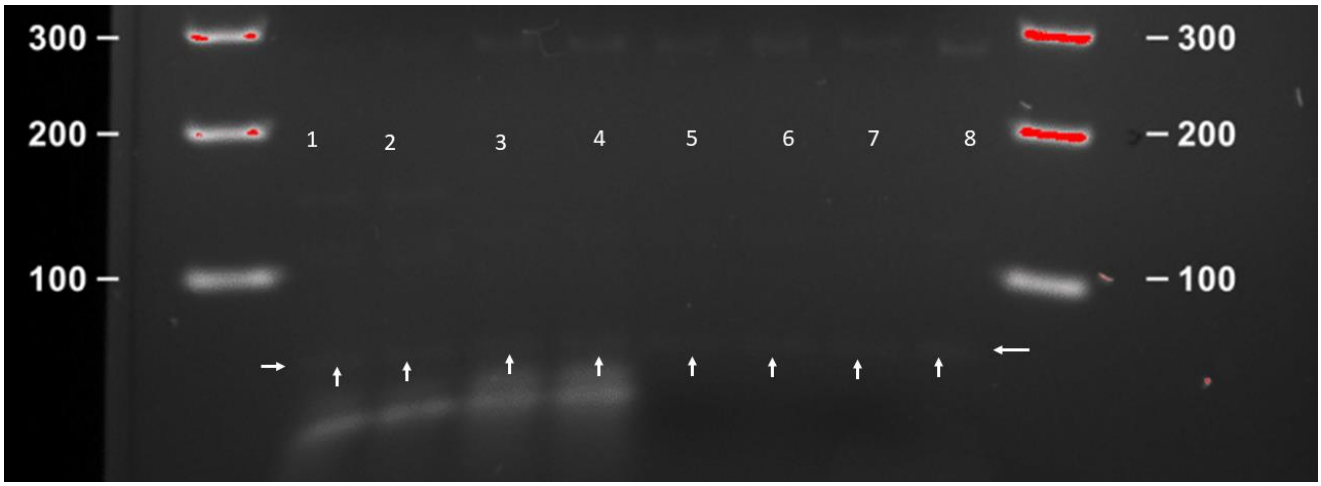

Figure 15. Agarose gel (2%) of the amplified products from the non-target region. The most visible band is in the 77 bp band size region, indicating that this product was preferentially ligated into the sequencing vector.

Electropherograms from the sequencing of control (sham-transfected) cells showed clean and evenly spaced peaks in comparison with the electropherograms of the RNP transfected cells, which showed overlapping peaks in the region targeted by the sgRNA (Figure 13) in the two samples. This indicated that indels occurred in the targeted region in these samples. Further analysis of CRISPR edits with ICE and DECODR showed presence of indels (-1) at the sgRNA/Cas9 targeted region with editing efficiency of 100% by ICE analysis (or 92.3% by DECODR analysis) in one sample and 5% by ICE analysis (or 7.7% by DECODRE analysis) (Figure 16). The difference in editing efficiency is expected from a mixed population of cells, because the NHEJ repair pathway of CRISPR/Cas9-induced DSB is not generalized but cell dependent, i.e., the repair is different for each individual cell. Thus, the editing efficiency of the sgRNA *cr2*_03 in one cell was 100% (or 92.3% by DECODR analysis) but was 5% (or 7.7% by DECODRE analysis) in the other cell. The respective R^2 of 1 and 0,99 showed good correlation between the Sanger sequence data and the indel distribution proposed by the two bioinformatic analysis. The observed indel mutations of 1 bp deletion and insertion in the 2 samples are compatible with the NHEJ repair pathway. (Figure 16).

Figure 16. Output of ICE and DECODRE analysis of the RNP transfected cells. The type of insertions and deletions (indels) detected in the analysed samples and their respective frequencies are indicated on the left side. The wild-type and edited sequences and aligned on the right side. On top of the wild-type sequence (in block line) is the region of the sgRNA (green) and PAM (red). [A]&[B] ICE analysis of two samples; sample in [A] showed a 100% efficiency, and sample in [B] showed a 5% efficiency. [C] & [D] DECODR analysis of the same samples showing 92.3% efficiency and 7.7% efficiencies respectively.

5 Discussion

This project aimed to induce knock-out mutations in ASK-1 and CHSE-214 cell-lines using the RNP-based CRISPR/Cas9 strategy. The project further aimed to analyze the specific type of nucleotide changes induced in the target gene by this type of mutation, and to isolate single cell clones harboring specific type of mutations. The targeted gene was the *cr2*, which codes for the complement receptor type 2 that plays an important role in A. salmon immune system and has been associated with the ISA disease of A. salmon (Schiøtz et al., 2008, Abbas et al., 2019). Furthermore, it has been shown in mammals that *cr2* can bind viral DNA e.g., Epstein Barr virus and HIV (Kumar et al., 2020, Lukácsi et al., 2020), making *cr2* a good candidate for such a study. This study is part of a larger project that aims to create new knowledge about gene editing enabling CRISPR/Cas9 to be applied safely and sustainably in salmon breeding. The ASK-1 and CHSE-214 are salmonids-derived cell-lines which represent relevant cells to study gene functions in salmon.

The RNP complex was transfected into the cells by means of electroporation. Salmonid cells including ASK-1 and CHSE-214 cell lines are intrinsically difficult to transfect (Collet et al., 2018) due to the low incubation temperature, and high saturation of the phospholipids in the cellular membrane compared to mammalian cells (Lopez et al., 2001). In the present study, approximately 70-80% of the ASK-1 and CHSE-214 cells were effectively transfected with the RNP complex, which is slightly less than the efficiency (85-90%) observed with the control cells that were electroporated with Cas9. The RNP complex being a bigger molecule than Cas9, expectedly transfected with less efficiency. Although this electroporation yielded high efficiency, it was less than the 99.9 -100% efficiency recorded in the same cells by Gratacap et al. (Gratacap et al., 2020) not withstanding that the present study used the same electroporation parameters optimized by Gratacap et al. (Gratacap et al., 2020). This difference can be attributed to differences in composition of the guide RNAs. Chimeric single gRNA was used in the present study which is different from the crRNA/tracrRNA used by Gratacap et al. However, the non-inclusion of electroporation with Cas9 control in their experimental setup precludes direct comparison with the result of the present study. Nonetheless, the result supports the efficient transfection of the hitherto difficult-to-transfect salmonids cells, ASK-1 and CHSE-214, with RNP complexes reported by Gratacap et al. (Gratacap et al., 2020).

Validation of cleavage activities of the three sgRNAs that target different sites of the *cr2* gene revealed the *cr2*_sgRNA_02 as having the best cleavage activity, indicating that this sgRNA may result in the highest *in-vivo* editing efficiency, although *in-vitro* cleavage activity does not always correlate with *in-vivo* efficiency. However, for practical purposes, the sgRNA *cr2* _03 was chosen for further studies although it showed a weaker *in-vitro* cleavage activity. This sgRNA has been used in a related study by the research group at NORCE, and as such would provide opportunity for comparison of results. Nonetheless, the positive T7E1 sgRNA *cr2*_03 further shows a correlation between the *in-vitro* cleavage assay and the *in-vivo* cleavage viability of the sgRNA *cr2*-03/Cas9 RNP complex. Due to time limitation, the T7E1 assay was performed only with genomic DNA derived from sgRNA cr2_03/Cas9-transfected CHSE-214 cells, but it was expected that the positive cleavage viability of the RNP complex could be extrapolated to the ASK-1 cells that were also transfected with the same RNP complex. Thus, both positive *in-vitro* and *in-vivo* cleavage assays provided enough rationale for further analysis of mutation by sequencing.

Sequence alignment of the Sanger sequencing data revealed base pair mismatches in the target region between the sham-control and the RNP transfected cells, indicating gene editing in this region. Both ICE and DECODRE algorithms confirmed presence of edits and listed the CRISPR edits profile to constitute mostly of 1 bp indels, which is in concordance with the nature of NHEJ editing. In the absence of a template that is homologous to the cut site of CRISPR/Cas9, the cell repair of DSB in DNA follows the NHEJ pathway (Jiang and Doudna, 2017). The NHEJ is error prone and results in base pair mismatches, which constitute mostly in few base pair indels (Jiang and Doudna, 2017). However, the range of editing efficiency of 5% and 100% (ICE estimate), and 7% and 92.3% (DECODRE estimate) of the sgRNA *cr2*_03 in this study was wide. The 5% represented the lowest editing efficiency while 100% represented the highest efficiency that could be achieved by the sgRNA *cr2*-03/Cas9 complex in the experimental system of the project. The FACS-enriched cells were a mixed cell population. It was expected that each mutated cell contained a unique type of mutation given that the NHEJ repair system is cell dependent and varies from cell to cell. Other studies also recorded variable gene editing frequencies, for example, Gratacap et al (Gratacap et al., 2020) recorded 100% editing frequencies, but Zoppo et al. reported just 34% editing frequency (Zoppo et al., 2021). These studies presented only the highest detected representative editing frequencies.

The variability in mutation per cell necessitates clonal isolation and expansion of cells with specific mutation for functional characterization of the importance of the mutation and understanding of protein function. Single cell isolation and clonal expansion was successful via a combination of FACS and sequential single cell expansion using conditioned media containing growth factors secreted. This is remarkable given that previous attempts were unsuccessful with salmon-derived cell-lines (Gratacap et al., 2020), although Zoppo et al. 2021succeeded with the rainbow trout cell RTgutGC cell-line using a tedious and difficult-to-reproduce approach (Zoppo et al., 2021). Salmonid-derived cells are slow growing, and growth can be further slowed or completely inhibited by antibiotics. This necessitated incubation of 96-well plates for several weeks with the attendant top-up of growth media before patches of clonal cells are observed and/or serially expanded. During this period cell cultures often get contaminated. This obstacle can be overcome by introduction of antibiotics in incremental concentration after the single cells have attached and started to proliferate.

The Sanger sequencing showed positive results in only two samples out of approx..100; thus, highlighting the less than optimum robustness of Sanger sequencing compared to next generation sequencing (NGS) for detection of NHEJ-mediated indels, especially for a heterogenous population of gene edited cells (Sentmanat et al., 2018). The number of FACS-enriched cells was very low due to the stringent gating using both negative (non-EGFP electroporated cells) control and positive (Cas9 electroporated cells) controls. The requirement for positive control during the FACS gating was necessary to ensure non-interference of Cas9-EGFP in the sorting of fluorescence cells, because some Cas9-EGFP adhered to the cell surfaces and could not completely be washed off with several washes of PBS prior to FACS. The low number of FACS-enriched cells resulted in low DNA yield, which led to sub-optimal PCR amplification and ligation of the target region into the pGEM®-T Easy vector for Sanger sequencing. The problem related to low cell number can be overcome by sequential expansion of the low FACS-enriched cells first in several 96 wells, then pooling the cells and transferring into a 24-well for further sequential expansion until enough cells are generated for genotyping.

A 77 bp-insert which is highly homologous (80% identity) to the *hnRNP R* (heterogeneous nuclear ribonucleoprotein R) was identified in the amplified region of interest in 25 experimental samples following sequencing, and it was tempting to attribute this as an insertion mutation. However, the identification of the same insert in 6 control samples derived from the sham-control cells showed that the insertion was not caused by NHEJ of the RNP induced DSB, but by ligation into the cloning vector of an amplicon resulting from non-specific primer

amplification in a non-target region sequel to Sanger sequencing. Zoppo et al. (Zoppo et al., 2021) attributed similar phenomenon in their study to an insertion mutation via the synthesis-dependent strand annealing (SDSA) pathway, which is a type of HDR repair mechanism. The SDSA repair pathway is rare where no repair template is provided in a CRISPR/Cas-induced mutation. However, non-inclusion, by Zoppo et al., of data from control samples precluded a direct comparison of this phenomenon. The partial success at isolation and expansion of edited single cells of ASK-1 and CHSE-214 was attributed to both none use of antibiotics in the growth media and the slow growth characteristics of the cells. Till date, single clone isolation and expansion of edited ASK-1 and CHSE-214 cells have not been reported. It is noteworthy that the only published attempt (Gratacap et al., 2020) recorded an unsuccessful outcome. Thus, the partial success recorded in this study is a giant leap towards attempt for establishment of platforms of gene edited salmonids cell clones harboring specific mutations. Such platforms are important for phenotypic and immunological studies as well as functional mechanisms underlying host response to salmonid pathogens.

5.1 Limitations of study

- **Slow cell growth and proliferation:** as stated in Section 4.1, ASK-1 and CHSE-214 are slow growing cells taking a CHSE-214 cell number of 1 X 10^6 up to 6 days (or 9 days for ASK-1) to get to about 60% confluency. The cells also require relatively high cell density per space to proliferate. For example, CHSE-214 requires a minimum of 5 X 10^4 cells in a 6-well plate for proliferation (data not presented in this thesis, but experiment was done in the research group). These characteristics of the two cells affected the rate of cell recovery after electroporation and FACS as well as ability to isolate and expand single edited cells for genotyping. Overall, these factors impacted on ability to optimize methods and parameters within the limited timeframe of this project.
- **Limitations of Sanger sequencing for detection of NHEJ-mediated mutation:** identification, quantification, and analysis of gene editing efficiency by Sanger sequencing has previously been shown to have a high concordance with results from next generation sequencing (NGS) analysis (Sentmanat et al., 2018). However, Sanger sequencing can sequence only one fragment per run. This necessitates: (i) use of a cloning vector (pGEM-T Easy® in this study) to amplify the target region with the attendant bacteria transformation; (ii) sequencing of several individual positive transformants to identify mutation. For a mixed population of FACS enriched cells (as

is the case with this project) containing both cells in which gene editing was not achieved and cells in which gene editing was successful, several transformants will harbor insert-sequences from either gene-edited cells or none-gene edited cells. Picking of the right colonies with the desired inserts (i.e., sequence inserts from edited cells) is a matter of probability and is time-consuming. Sanger sequencing is cheap and widely available, but the upstream sample preparation, especially for mixed population of cells containing both gene edited, and none-gene edited cells, the method is unpredictable and laborious. An alternative is the NGS, which is more robust and offers a higher sequence resolution (Sentmanat et al., 2018).

- **Use of immortalized cell lines and only two cell-types:** this will limit the impact of the present study given that immortalized cell lines may not depict the actual impact(s) of specific mutations in the animal/organism. This, thus, highlights the limitations on the impact of the study also imposed by using only two types of cell lines. Increasing the number of cell lines as well as use of primary cell lines will better mirror the actual impacts of specific mutations on the fish.
- **Relevance:** CRISPR/Cas holds great promises as a tool that can be used to combat the problematic infectious diseases of farmed salmon. The tool can also be deployed to fine-tune the hitherto underexplored genetics and functional genomics of salmon. Information on application of CRISPR/Cas strategies on salmon cell biology is few and at its infancy. This project extends the existing knowledge on application of the CRISPR/Cas9 RNP strategy on ASK-1 and CHSE-214 salmon cell lines.
- **Future direction:** establishment of clonal cells containing specific mutation is key for genetic and functional genomic studies of salmon. A future perspective of the current study is the optimization of methodologies for establishment of single clones of cells harboring specific targeted mutation. Approaches such as use of coated 96-well plates and introduction of growth factors in the cell growth media can be tested. The established gene edited cell lines will serve as a platform for phenotypic and immunological studies, e.g., reduced or increased virus production due to the specific mutation(s).

6 Conclusion / final remarks

This project provided new knowledge on the application of the CRISPR/Cas9 RNP strategy for targeted gene editing in the salmonids cell lines ASK-1 and CHSE-214. Our approach of in-house sgRNA synthesis provided a cost-effective (approximately 30%-50% price reduction per reaction) approach to this strategy. Further, the systematic testing of the sgRNA activities both *in-vitro,* (by the *in-vitro* activity cleavage assay), and *in-vivo* (by the T7E1 assay) provided preliminary evaluation of the efficiency of the system before being subjected to the resource-demanding sequencing platform. The high electroporation result of both the sgRNA/Cas9 complex (75-80%) and the Cas9-EGFP control (80-90%) of the hitherto difficult-to-transfect salmonid cells both supported and extended the study of Gratacap et al. (Gratacap et al., 2020). Unlike the Gratacap study which circumvented enrichment of edited population (having achieved a 99.9%-100% electroporation), this study introduced a FACS-based enrichment of edited population - a necessary step towards establishment of single clonal gene edited cells with unique phenotype. Although the success of establishment of clonal gene edited cells was short-lived due to contamination, nonetheless and within the lifespan of the project, a proof-of-concept was provided using ASK-1 and CHSE-214 cells. Overall, the works described in this project has extended the state-of-the-art.

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