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Rapid Diagnostics for Salmonid Alphavirus 2 and 3 in Atlantic salmon.

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Abstract:

Salmon is an important export product for Norway in addition to being sold in large quantities within the country. Salmon diseases such as infectious salmon anemia (ISA) and salmonid alphavirus (SAV), that leads to pancreas disease (PD), cause large quantities of fish to be slaughtered every year and lead to large losses. If testing for these diseases becomes easier to access and easier to carry out, testing can act as a preventive measure to reduce the number of fish that must be slaughtered. Fish health and faster diagnostics are an important part of increasing food production in the world as the population increases. This can greatly reduce financial losses and lead to more growth. Reducing slaughter is also good for animal welfare and the environment.

In this project, an RT-qPCR kit form GeneMe was used to detect SAV2 and SAV3 in Atlantic salmon. The aim of the project was to optimize this kit for validation purposes for commercial testing by NordicDx. Infected salmon hearts were tested after being stored in various conditions, the results indicates that the GeneMe kit detects SAV2 and SAV3 (QnsP1 gene) in addition to an internal control, Elongation factor 1α (EF1AA) gene. More samples were positive after being stored in homogenization buffer over some hours, and the results were easier to interpret after using a TissueLyser for homogenization of the fish tissue and syringe filtration of the for removal of inhibitors. This indicates that the protocol could need further optimalization and could benefit from these results.

Acknowledgments

I would like to give my gratitude to Professor Mark van der Giezen, thank you for your guidance and for making this project possible. I would also like to give my sincerest thanks to laboratory supervisor Celine Lorentsen, for superior guidance in my writing and great help in the laboratory. Thank you for having patience with me throughout this thesis and being a great supervisor. A special thanks to everybody at NordicDx for making this project possible, all the talks during the breaks and help in the laboratory.

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Abbreviations

H – buffer: Homogenization buffer

N – buffer: Normalization buffer

Cq: Quantification cycle

RT – qPCR: Reverse Transcription Real-Time Polymerase Chain Reaction

PCR – mix: SAV test well

HIV/AIDS: Human immunodeficiency virus/acquired immunodeficiency syndrome

SARS: Severe Acute Respiratory Syndrome

COVID-19: coronavirus disease

1. Introduction

Figure 1.1. Atlantic salmon from Rogaland before dissection.

Fish is globally a large and important part of the diet, especially Atlantic salmon in Norway. Infection diseases linked to fish health are therefore an important topic to research as potential infectious diseases can affect humans. The Norwegian economy depends on fish farming and the export of Atlantic Salmon abroad (Misund, 2021). In addition, the Norwegian Directorate of Health recommends eating fish two to three times a week, and many choose Atlantic salmon (*Kostrådene*, 2016.). The fishing industry also leads to many jobs. In order to prevent big losses, the use of fast diagnostics can therefore be a great advantage. Faster diagnostics, together with the development of vaccines are important to prevent and slow down the spread of diseases.

1.1 Emerging infection diseases

Emerging infectious diseases are diseases that have just appeared, either in a population or spreading to new areas. There may be new unknown diseases or there may be known diseases that threaten to spread rapidly to new areas (*Emerging Infectious Diseases*, n.d.). In recent years, more and more of these diseases have appeared, which affect the world economy and world health. Some examples are HIV/AIDS, SARS, H1N1 influenza, and COVID-19. As a result of people travelling, these diseases affect the entire world and spread easily. These new diseases come either from mutations, diseases that spread to new areas, or old infections that get to spread because the health system collapses (Morens & Fauci, 2013). New infection diseases are constantly appearing among animals, and these can potentially be zoonotic diseases and infect humans too (*Zoonoses*, 2020.). Therefore, measures that monitor and

prevent infection are important. Covid-19 is an example where better monitoring of the virus could possibly have prevented the pandemic (See et al., 2021).

1.2 Virus in fish/salmon

According to SNL, fish farming is defined as raising fish in captivity. In Norway, the first farmed salmon was slaughtered in 1971 but has occurred for thousands of years around the world. Today, fish farming in Norway has a turnover of more than NOK 65 billion a year, making Norway the top producer of fish-farmed Atlantic Salmon (Misund, 2021).

Fish can be infected with viruses from most of the virus families such as rhabdoviruses, birnaviruses, herpesviruses, iridoviruses, reoviruses, orthomyxoviruses, and retroviruses (Kim & Leong, 1999). The two most common virus diseases among fish farming in Norway are Infectious salmon anemia (ISA) and SAV. These lead to losses in the farming-process and also results in a less beneficial effect from public control measures, which are meant to limit the infection and prevent the virus from establishing itself in new zones. None of the viruses are dangerous for humans or warm-blooded animals, but have serious consequences for the fish and the fish-farming because whole fish cages can potentially die if infected (Barentswatch, 2017.; *Infectious salmon anemia/veterinary institute (ISA)* , n.d.). Fish-farming is also useful to observe how different diseases in fishes spread among individuals, or between different cages. (Kim & Leong, 1999).

1.3 Salmonid Alphavirus and Pancreas disease in Atlantic salmon

SAV is a single-stranded RNA virus belonging to the family Togaviridae and genus Alphavirus. The virus affects Atlantic salmon and rainbow trout and was first detected in 1976 (Hodneland, 2006). There are 6 subtypes of the virus, where the variation is in the proteins E2 and nsP3 (Jansen et al., 2010). SAV1 and SAV4-6 are detected in Atlantic salmon mostly in Ireland and Scotland. There are two different subtypes of SAV2, FW, and MW. SAV2 FW causes a disease called sleeping disease (SD) found in France, England, Scotland, Germany, Italy, Spain, Poland, and Switzerland. SAV2 MW causes pancreas disease (PD) found in Norway and Scotland. SAV2 is the most common subtype in Norway, but SAV3 is also detected in some places and leads to PD (Hodneland, 2006.). SAV leads to high mortality, because of injuries to the muscles and pancreas among the fish which further leads to economic losses for companies around the world. In addition, SAV-free countries can refuse to import salmon from areas that are infected. SAV 3-6 has only been detected in

seawater whereas SAV1 and SAV2 have been detected in both sea- and freshwater (Deperasińska et al., 2018).

SAV3 has a higher mortality rate than SAV2 (Jansen et al., 2015). Fish farms with SAV2 can live with the infection for a long time and some only get detected due to routine checks. This is because the fish can be infected with the virus without disease outbreaks. Stress is a factor that can lead to outbreaks and is therefore important to avoid. Fish infected with SAV3 usually gets sick, and many fish dies before slaughter. (*Pankreassykdom (PD)*, n.d.).

In Norway, there are several measures to reduce the consequences of the PD disease, prevent it from spreading in monitored areas, and limit the spread of the various subtypes of the SAV virus. There are two surveillance zones: From Jæren to the border between Norway and Sweden, and from Skjemta, Flatanger to the border between Norway and Russia (Forskrift om tiltak for å forebygge, begrense og bekjempe PD hos akvakulturdyr, 2017). Salmon, trout, rainbow trout, and char living in aquaculture facilities with untreated seawater must be tested for SAV at least once a month, by analyzing 20 fish. If the fish is moved from facilities with untreated seawater, the fish must be tested for SAV during the last 3 weeks before they are moved. This also applies if fish are transported out of the PD zone and are being kept in seawater. Then the fish must be kept in the cage until the analysis results are ready. The result must clear within 7 days after the samples have arrived at the laboratory, and information about any vaccines must also be included. The samples are analyzed for SAV using PCR and the subtype must be analyzed by sequencing (Forskrift om tiltak for å forebygge, begrense og bekjempe PD hos akvakulturdyr, 2017). If PD is suspected in a facility where no disease has been previously detected, 10 fish samples must be analyzed. Organ samples are placed on formalin and spongy tissue from the heart's ventricle and mid-kidney are placed in RNA-later. If the infection is suspected in the surveillance zones, samples from 10 fish must also be analyzed. If positive samples are not confirmed after the analysis, samples from 60 fish shall be analyzed once a month for 4 months after the last positive sample (Forskrift om tiltak for å forebygge, begrense og bekjempe PD hos akvakulturdyr, 2017.). Fish released into facilities from Taskneset to Langøya must be vaccinated. Further, there are rules for the use of equipment and vessels that have been in the PD zone. There are different measurements for the fish farms that are diagnosed with SAV2 and SAV3. Unlike SAV2, SAV3 makes the fish much sicker, and therefore there are various measures after the diagnosis of either SAV2 or SAV3. For fish diagnosed with subtype 3, the Norwegian Food Safety Authority may order for the individuals to be put down. In some cases, the fish can be moved to the monitoring

zone for further growth if the risk of infection is low. This can also be ordered for SAV2 if the risk of infection is high, but usually but usually the fish will live until slaughter because with SAV2 the chance of a high volume of dead fish is much lower when infected with SAV3 (Forskrift om tiltak for å forebygge, begrense og bekjempe PD hos akvakulturdyr, 2017).

SAV transmits horizontally, not vertically, through the water and can spread to new localities through bodies of water, personnel, and equipment (Rimstad et al., 2011.). It has also been investigated that well boats are causes to infection, even though they are disinfected, and this can be the reason why the virus spreads and appears in new areas (Thomsen, 2018).

The first signs of PD with SAV3 and SAV2 infection are that the fish stop eating and swims closely against others in the surface against the direction of the current. The infection leads to injuries to the muscles and the heart, which affects the circulation of the blood. The injuries can affect the quality of the slaughtered fish, that is if the fish does not die before slaughter. The virus also affects the pancreas which leads to reduced growth. Some of the fish that survives the outbreak, become healthy again while some remains sick. After infection, it can take 2-3 weeks before the fish with SAV3 die (*Pankreassykdom (PD)*, n.d.). The fish cannot be infected again, and therefore, vaccines are very relevant. (Deperasińska et al., 2018; Skjold et al., 2016)

1.5 Economic consequences

Norway accounts for about half of the world's production of Atlantic salmon. The value of salmon exports was NOK 72.9 billion in 2019, which corresponds to 1.06 million tons of salmon. In addition, 1.28 million tons salmon are sold in Norway (*Steinset, 2020*). Rainbow trout and salmon farming account for 5.5 percent of export earnings (Misund, 2021). The consequences of viruses and diseases spreading among fish are therefore huge.

1.6 Polymerase chain reaction (PCR)

One effective way to detect RNA viruses is with reverse transcription real-time PCR (RT-qPCR) which uses pathogen-specific RNA to make cDNA directly from the tested samples. Reverse transcriptase, an enzyme, will make the cDNA from the target RNA. Further, PCR is used to amplify the DNA and the expression of the target RNA can be detected. The method detects and amplifies the cDNA simultaneously along the way. Because of this, it is not necessary for electrophoresis to confirm the amplification. (*Real-Time Polymerase Chain Reaction - an overview*, n.d.) (Stahl et al., 2017, p. 884)

There are three major steps in a standard PCR reaction. (Stahl et al., 2017, p 884). In the first step called denaturation, the template DNA is denatured by heating into two separate strands. The first step will happen above 90 °C. In the second step, called annealing, DNA primers are hybridized to the target sequence. The primers help identify the gene that will be detected and are designed especially for the gene of interest. The primers are hybridized before the cooling to ensure that most template strands are annealed to a primer, not to each other. This will happen at between 50 – 60 °C. In step three, called extension, DNA-polymerase extends the primers using the original DNA as the template by adding nucleotides to the 3`end of the primers, this usually happens at 70 – 78 °C. This way, DNA-polymerase will make copies of the target gene, called DNA replication. This cycle will be repeated until its copied as much as needed, for example in SAV detection, 40 quantification cycles are used. After the reaction, millions of copies of the target material can be present in the sample, depending on how much of the gene is present in the sample before the reaction, and how many cycles the PCR machine has done. The temperature profile vary and depend on the type of gene that is detected, and the primers and polymerase used. The DNA polymerase must withstand high temperatures and therefore the most common DNA polymerase used in PCR is Taq polymerase. Taq polymerase is isolated from *Thermus aquaticus*, a thermophilic bacterium, so it can tolerate high temperatures (Erlich, 2013; Primer, 2022; Stahl et al., 2017, p. 884).

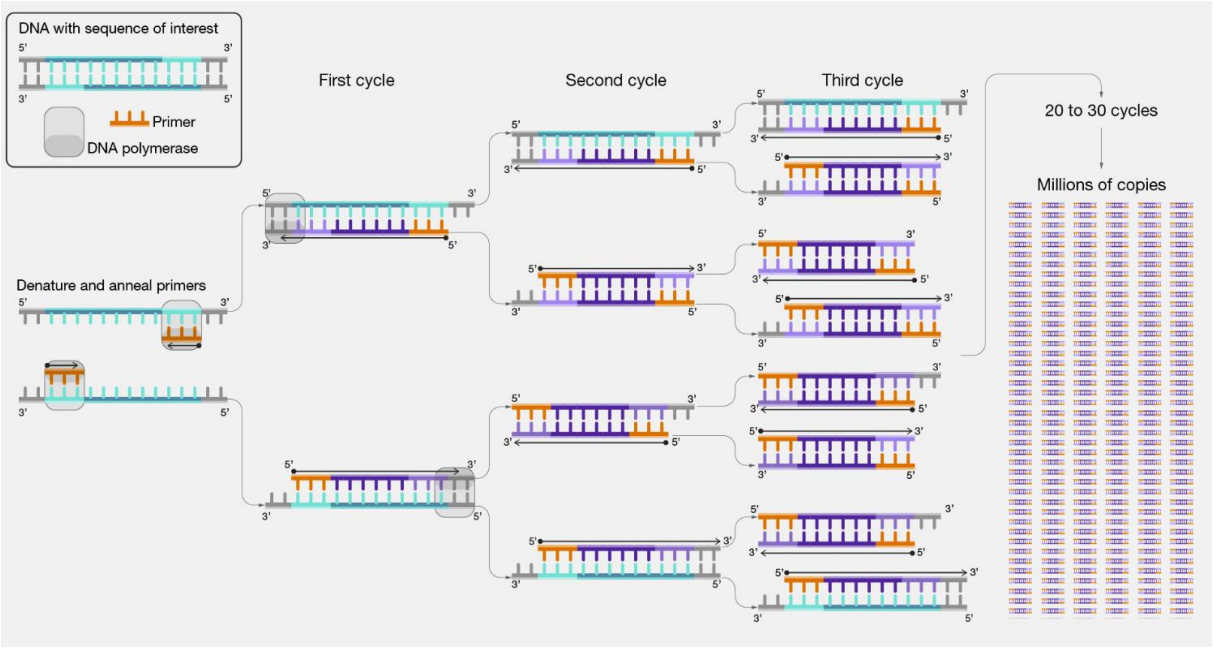

Figure 1.2. An overview of a PCR reaction. There are three major steps in a PCR-reaction. First is denaturation, then annealing and extension. The cycles are repeated and can make millions of copies. (Polymerase Chain Reaction (PCR), 2022).

In order to detect the target material in the sample, fluorescently labelled PCR amplicons are often used, where the concentration of the fluorescence increase proportionally as the polymerization occurs. The sample gets a Cq at the point in the PCR run when the fluorescence of the PCR product overgoes the background fluorescence and crosses the threshold. The greater the RNA concentration in the sample before the reaction, the earlier the sample will get a Cq. (Real-Time PCR for mRNA Quantitation, 2005.) (Stahl et al., 2017, p. 884) (Mackay et al., 2004).

With innovative technology, results can be obtained within hours, compared to before when it took days to analyze the samples for the virus. This allows for fast diagnosis and allows for fast treatment or restrictions immediately. The method is highly accurate and sensitive.

1.7 Validation and verification

The test used in this project is a newly developed and innovative PCR test and is not commercially available yet. For newly developed tests to be approved they must go through a validation process. Validation is required to document that the method or test works for its purpose. In every new laboratory, the method or test must go through a verification process. This is a smaller process that confirms that the test works in a specific laboratory (*Validering/verifisering av analysemetoder, MBK,* n.d.).

The validation process normally contains 40 to 50 tests, where about 25 % are positive, 25 % are weak positive, and 50 % negative. If the agents are rare and difficult to provide the validation may consist of fewer tests. In a verification process, only half of the test are enough to be approved. (*Kvalitetskontroll av genteknologiske metoder*, 2018.)

Before the validation or verification, an exact plan must be composed. The plan must include a description of the method, the relevant material, controls, practical training, continuous observation, and the requirements for authorization. The protocol for the test or method needs to be adjusted and tested before the validation can start. *(Kvalitetskontroll av genteknologiske metoder*, 2018.)

The validation consists of the factors: analytic specificity, diagnostic sensitivity, accuracy, precision (repeatability and reproducibility), and detection capability. In a verification, measurements of accuracy and precision is sufficient. In both cases, a validation or verification report must be made. In the report, the performance of the test must be assessed

against the requirements that were set in advance, and the report must end with the conclusion "approved" or "not approved" (*Kvalitetskontroll av genteknologiske metoder*, 2018.)

1.9 Aim of thesis.

The main goal of this study is to examine the accuracy of the SAV test and collection kit (GeneMe) in detecting SAV2 and SAV3 viruses. The kit was used on isolated SAV3 RNA, fish with confirmed SAV infection, and healthy fish to certify that the kits could detect the internal control gene. This project is a part of a validation process of the kit from GeneMe. Here the stability of the virus is examined after storing in the freezer and the fridge over time, in addition to the stability in different buffers.

2. Materials and Methods

2.1 Materials

2.1.1 Fish tissue

Atlantic salmon hearts, from a fish slaughterhouse in Rogaland were collected 10. May 2022. The fish was picked up at night before 10. May and they were transported on a bluegill boat that killed the fish by cutting the gills. Therefore, the fish was already dead when they entered the fish slaughterhouse. The heart was taken out and brought to The University of Stavanger (UiS).

Whole dead salmon was picked up from a location in Rogaland and dissected at UiS 13. May 2022. Gills, feces, liver, heart, and brain were taken out and shock-frozen in nitrogen, and then put in a freezer at -80 °C. Then it was moved to a freezer at -75 °C after 24 h.

Positive control from the veterinary institute was received at 28. March 2022. The sample contained synthetic isolated SAV3 virus.

Five infected fish hearts, one infected liver sample, and one positive control were received from the veterinary institute at 24. March 2022.

2.1.2 SAV kit components from GeneMe

- SAV tests (each containing lyophilized PCR mix)
- Homogenization Buffer

- Normalization Buffer
- Positive Control (synthetic genes QnsP1 and EF1AA)

2.1.3 Equipment required in the laboratory

- Centrifuge for 1.5 ml tubes: VWR MiniStar whiteline, EU
- Ice to cool down the samples
- Pipette 50 µL
- Thermocycler (Bio-Rad CFX96 Touch) with Bio-Rad CFX Maestro 2.3 (version: 5.3.022.1030)
- Fridge
- Sterilizing cleaning agent: Chlorine
- Electronic weighing measuring mg
- 1.5 ml Eppendorf tubes
- Pasteur transfer pipettes
- Scalpel
- Metal homogenizer
- TissueLyser LT (QIAGEN)
- 5 mm stainless steel beads (Qiagen)
- Syringe filter (0.2 µm and 0.8 µm)

- Scissors
- Tweezers
- Scalpel
- Measuring mat
- Liquid nitrogen
- Freezer

2.2 Methods

2.2.1 Dissection of Atlantic Salmon

All equipment were washed with soap and hot water between each fish to prevent contamination. It was not washed between each collected organ. The fish was measured and weighed before the dissection. To start the dissection a scissor was cut into the anal vent and cut up between both sets of paired fins all the way up to the gills, it was not cut to deep to prevent the internal organs to be stabbed. Then it was cut vertical to the top of the fish from the anal vent and from the front gills. This loose part was cut totally of to get a better view of the fish before the dissection of the organs. Further the feces were collected into a test tube. Further a part of the liver was collected by cutting off a small part. To get to the heart, the other organs was cut away and the heart was found near the head of the fish, attached to the gills. To find the brain a cut was made diagonally behind the eyes, a bit tilted towards the eyes. Then it was cut in front of the eyes and backwards to the first cut. Then the skull was removed, and the brain could be taken out. Each sample needs to be at least 30 mg. The samples were shock freeze in nitrogen and then put in a -80 °C freezer and moved to -75 °C after 24 h. For an overview of the dissection, see figure 3.

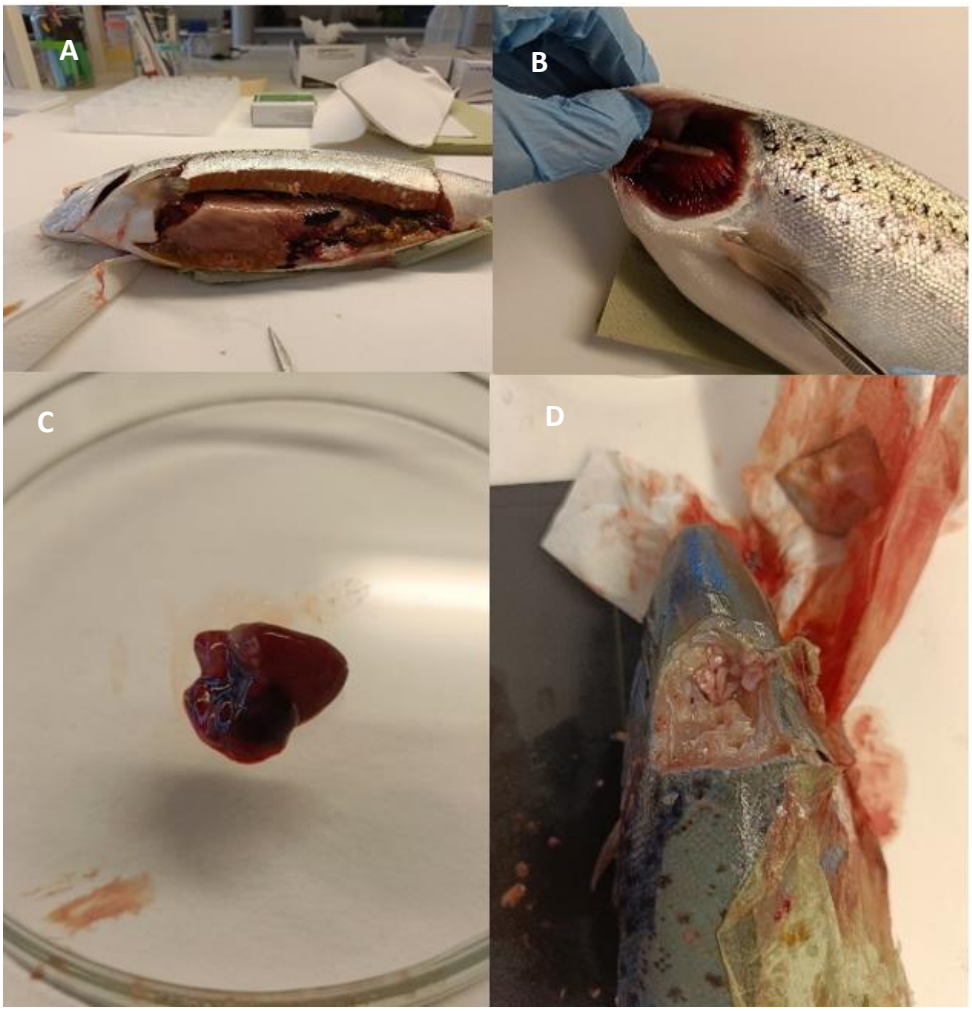

Figure 2.1. Dissection of Atlantic Salmon. Organs were collected to analyze further. A. Overview of initial removal of abdomen to access body cavity. B. View of gills. C Isolated heart. D. Top view of head to see brain.

2.2.2 Preparation for the PCR analysis

SAV test and collection kit (GeneMe, Black box system) was used. The PCR-mix contains innovated and patented thermostable Taq polymerase and specific primers that are 100 % compatible with the Salmonid alphavirus RNA gene sequence of the SAV2 and SAV3 QnsP1 genes. Normalizing buffer allows bringing RNA to favorable conditions for RT-PCR reaction. The homogenization buffer is designed to lysis the sample, disrupt the virus, and stabilize the RNA. The procedure for isolated RNA was used for the synthetic RNA sample provided by the Veterinary Institute. The procedure for the fish tissue was used on all the unknown samples. All the procedures were performed using RNase-free equipment.

Storing: The fish tissue was stored in the freezer before the homogenization. It could be stored at -20 °C up to one week. If it needed to be stored longer than one week it was frozen in -80 °C. Further the PCR-mixes were put in the fridge if it took more than 30 minutes to flood the samples into the PCR-mix. If the tissue were not frozen it was analyzed immediately. The samples could be stored in the refrigerator in several hours before analyzing.

Preparation with isolated RNA

Negative control was prepared by adding 50 µl Normalization buffer to a pre-aliquoted lyophilized PCR mix and the solution was resuspended. In order to prepare the unknown samples, 40 µl of Normalization buffer was added to the same PCR mix and resuspended. For the isolated RNA virus, 10 µl was added to the PCR mix. This was repeated for the unknown samples. A positive control tube was prepared by adding 50 µl Normalization Buffer to the lyophilized Positive Control master tube, containing synthetic SAV2 and SAV3 -genes, and resuspended. Then 50 µl of normalized positive control were then transferred to the PCR mix.

Preparation with salmon tissue

The tissue samples were first washed, in order to naturalize the conditions for the tissue samples which was stored in a salt rich buffer (RNA-later). The samples were washed five times in the homogenization buffer. Then the tissue was homogenized to extract the RNA-virus. The fish hearts were mainly homogenized using a manual steel homogenizer, however, 10 of the fish hearts were also analyzed after homogenized with TissueLyser LT using 5 mm stainless steel beads (Qiagen) for 2 min at 50 Hz. The tissue was frozen when homogenizing and RNAse-free Eppendorf tubes were pre-cooled together with the beads. Approximately 30 mg of fish tissue was cut off and transferred to an Eppendorf tube together with 300 µl of homogenization buffer. The mixture was homogenized by hand with a steel homogenizer. Further 500 µl fresh homogenization buffer was added and the solution was resuspended. The tubes were left on ice for at least 20 min. Then the samples were centrifuged (VWR MiniStar whiteline, EU) at 6000 rpm for 20 min. Some samples were filtrated through a syringe filter (0.2 µm and 0.8 µm). The prepared lysate was diluted 20x by adding 50 µl of the supernatant to 950 µl Normalization buffer.

Negative control was prepared by transferring 50 µl Normalization buffer to the pre-aliquoted lyophilized PCR mix and resuspended. The unknown samples were prepared by adding 50 µl of each normalized sample to tubes containing the PCR mix. Positive control was made by adding 50 µl of the Normalization buffer to the positive control tube. Then 50 µl of this mixture was added to a PCR mix.

Analysis of the unknown samples

The PCR tubes (all samples containing 50 µl) were further inserted into a thermal cycler (BIO-RAD CFX96 Real-Time System). The lid of the thermal cycler was set to a temperature of 105.0 °C. The temperature profile was divided into five sections. In section one the temperature was raised to 50.0 °C for 5 min. In sections two and three the temperature was set to 95.0 °C for respectively 18 sec and 3 sec. In section four the temperature was decreased to 60.0 °C for 9 sec, and at the end of each cycle, the relative fluorescence unit (RFU) was detected. Step three to five was repeated in 40 Quantification cycles (Cq), (see attached protocol section 1). Since the number of samples varied, the plate setup was therefore changed after this number. The setup always had one positive and one negative control. The fluorophores used were FAM for SAV virus detection and HEX for internal control. The internal control is the Atlantic salmon Elongation factor 1α (EF1AA) gene. The results were analyzed using Bio-Rad CFX Maestro 2.3 software.

The PCR runs were handled as valid if the appropriate signals were obtained for the controls in the reaction. The negative control had no signal for FAM and HEX. For the positive control to be valid Cq for FAM was under 30 and Cq for HEX was under 40 (Table 1).

Table 2.1. Interpretation of the controls. Assessment basis for whether the negative and positive controls are valid.

Channel	**Negative control**	**Positive control**	**Interpretation**
FAM	NO SIGNAL Cq undetermined	Cq < 30	VALID
HEX	NO SIGNAL Cq undetermined	Cq < 40	VALID

The test was negative for SAV2 and SAV3 if FAM Cq was undetermined and positive if Cq was under 40 (Table 2). The test was negative for HEX if Cq was under 40 and positive for Hex

if the Cq was under 40 or undetermined. The Cq value was determined at the level were the fluorescence signal crosses the threshold line.

Table 2.2. Assessment basis for interpretation of the unknown samples.

Channel	**Sample**	**Interpretation**
FAM	Cq undetermined	NEGATIVE
HEX	Cq < 40	NEGATIVE
FAM	Cq < 40	POSITIVE
HEX	Cq < 40 or undetermined	POSITIVE

3.Results

SAV virus and other emerging infectious diseases are a continuous threat to the Norwegian salmon industry. In this project, different salmon tissue was analyzed for SAV to optimize the protocol for SAV detection kit provided by GeneMe.

3.1 Positive control parallels

In order to examine how the Cq/virus is affected by being frozen a positive control with synthetic isolated SAV3 virus RNA from the Veterinary institute was frozen four times and analyzed with five parallels (figure 2). Median, average, and sample standard deviation were calculated from the five parallels (table 3). The threshold line was set, to approximately 20 RFU to compare the Cq values. The results of the four other experiments are provided in Appendix B, positive control parallels.

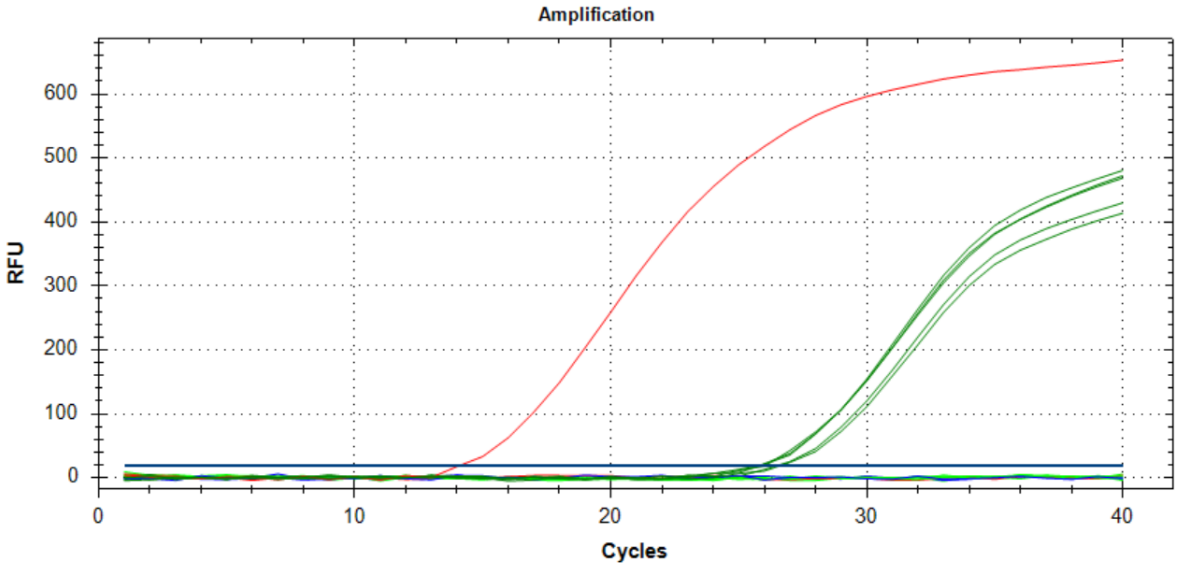

Figure 3.1. RT-qPCR analysis of SAV for determination of Cq for positive control with isolated SAV3 RNA by RT-qPCR. Four parallels were analyzed, the control was received from the Veterinary Institute 29. March 2022.

Table 3.1. Five parallels for four freezing cycles of positive control with isolated SAV RNA from the Veterinary Institute. The results are summarized by median, average and ±SD.

Parallell	***Freezing cycle 0 (fresh) (Cq)***	***Freezing cycle 1***	***Freezing cycle 2***	***Freezing cycle 3***	***Freezing cycle 4***
1	25.62	25.05	25.72	26.25	27.34
2	25.71	25.31	26.33	26.08	25.67
3	25.98	25.70	25.64	26.00	26.74
4	26.41	25.26	26.23	25.34	26.71
5	26.54	25.00	25.81	25.62	26.24
Median	25.98	25.26	25.81	26.00	26.71
Average	26.052	25.264	25.946	25.858	26.540
SD	0.41	0.28	0.31	0.37	0.62

Using R.studio (version 2022.02.2+485), the average Cq for each freezing cycle was compared and plotted (figure 3.2) . The plot shows the Cq values (blue dots) for the five parallels for each freezing cycle. The pink dots are the average Cq for each day.

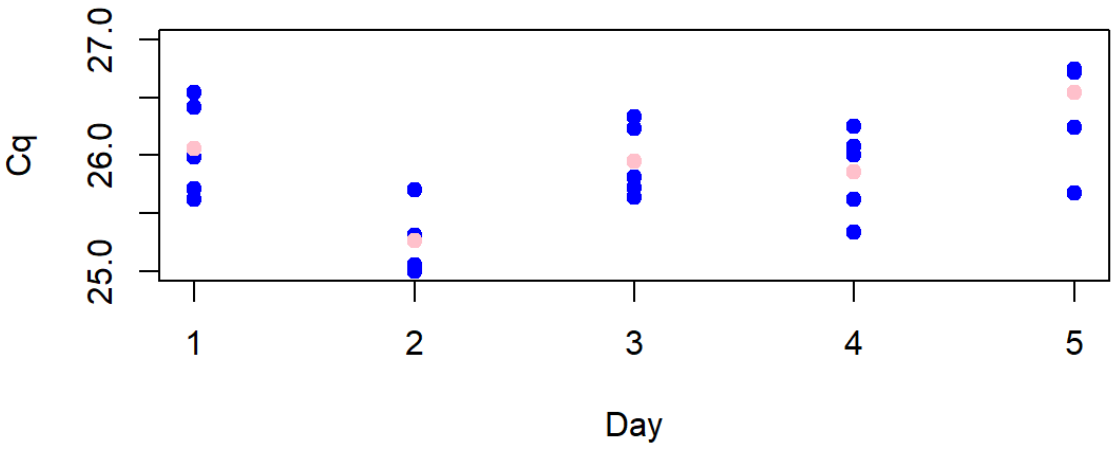

Figure 3.2. Plot of Cq-values for each of the five days and the average of each day. Pink: average Cq of each day. Blue: Measured Cq values.

3.2 Positive salmon hearts

Salmon hearts with confirmed SAV-virus were collected from a location in Rogaland and analyzed with various variables to optimize the protocol for SAV-analysis. Different conditions were examined to find out what worked best and were the most effective towards getting the best results possible. One batch of fish hearts was stored in H-buffer from GeneMe and one batch was stored in RNA-later. The hearts in RNA-later were stored for 24 h before being frozen at -20 °C. Ten of the hearts in RNA-later were washed three and five times in H-buffer to observe if it affected the results. Some of the graphs/results were difficult to interpret because of background noise due to inhibition and non-optimized homogenization in the graphs. Twenty hearts were stored in five different conditions (table 3.2). Heart 17 – 20 were stored 24 h in N-buffer before analyzed (figure 3.3). Heart 17 is the dark green graph that increases over the others towards the end. This is a good example of how a FAM positive graph can look like. FAM positive means that the FAM graph goes over the threshold line, so the sample gets a Cq. The lighter green graphs that are a bit lower is a good example of HEX positive graphs. This indicates that there is enough material to analyze and that the homogenization is successful. All results for positive salmon hearts are provided in Appendix B, infected fish hearts.

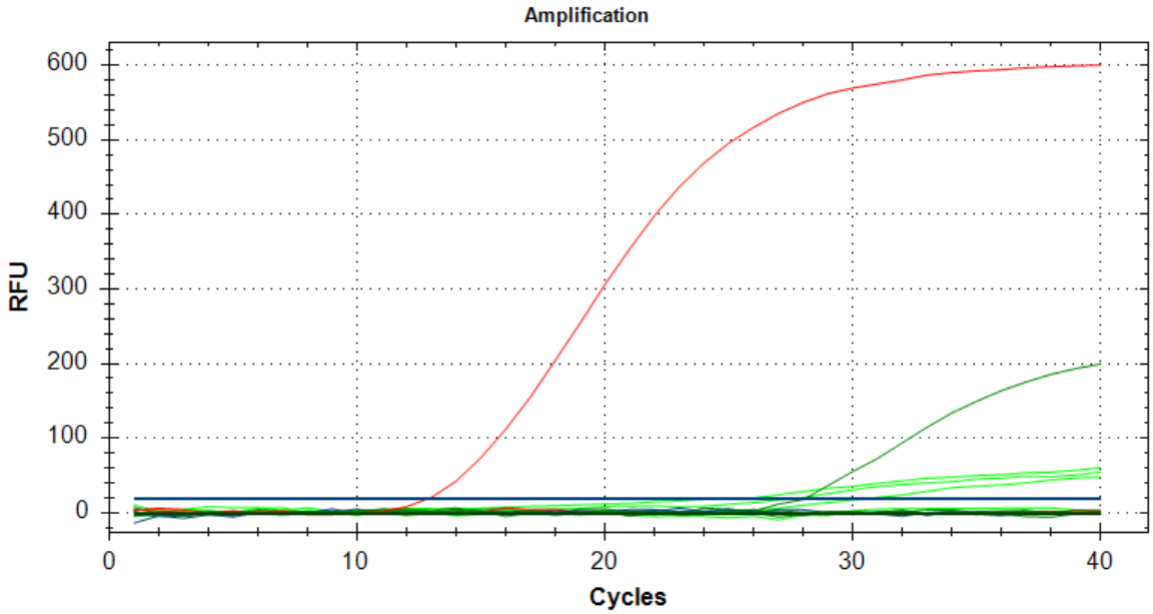

Figure 3.3. RT-qPCR analysis of SAV in heart 17 – 20 after 24 h in N-buffer. Red line is the positive control, dark green lines are each heart analyzed for FAM. Light green is each sample analyzed for HEX. The green line that increases around 28 cycles are sample 17, that is clearly positive and a good example of a FAM positive sample. The dark blue line is the threshold line.

Each of the 20 hearts was classified as positive or negative for FAM and HEX for all conditions (table 3.2). The hearts that are both negative for FAM and HEX are not detected because when there is not enough material a conclusion cannot be made due to the risk of false negative result. 7 of 20 hearts in H-buffer were positive for FAM. 9 of 20 hearts were positive for HEX. 8 of 20 hearts were inconclusive because they were both negative for FAM and HEX. After 24 h in the same H-buffer 14/20 hearts was positive for FAM and 12/20 was positive for HEX. 3/20 hearts were inconclusive. For the hearts stored in RNA-later and washed 3 times 3/10 were positive for FAM and 8/10 were positive for HEX. 2/10 hearts were inconclusive. When stored in RNA-later and washed five times 6/10 hearts were positive for FAM and 3/10 were positive for HEX, 3/10 hearts were inconclusive. Of the four samples taken from hearts stored in N-buffer for 24 h ¼ samples were positive for FAM and 4/4 were positive for HEX (table 3.2).

Heart 1-10 were also analyzed after being homogenized with a Qiagen Tissuelyser LT. The first batch were frozen in H-buffer and the other batch were frozen in RNA-later. The hearts were stored in a freezer at -20 °C for approximately one month before analyzed. The hearts stored in RNA-later were in addition filtered through a syringe filter (0.2 µm and 0.8 µm) and analyzed again. Heart 1 and 2 were filtrated through 0.2µm and heart 3-10 through 0.8µm. For the batch stored in H-buffer, heart 10 was positive for HEX, and 9 out of 10 hearts were inconclusive. For the batch stored in RNA-later 3/10 hearts were positive for HEX and 7/20 hearts were

inconclusive. The hearts stored in RNA-later and filtered through the syringe filter had 1/10 positive for HEX and 9/10 inconclusive. Heart 10 was positive for HEX at all conditions, and positive for FAM after being stored in RNA-later and not filtrated (table 3.2).

Table 3.2. Twenty fish hearts analyzed for SAV virus in different conditions. Each heart was classified as negative (N) or positive (P) for FAM and HEX. N: negative, P: positive. Fam (SAV) and HEX (internal control). - : not analyzed. Samples with N for both FAM and HEX are inconclusive.

Heart nr.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	
Condition																					
2 h in H-buffer	N	N	N	N	N	N	N	N	N	P	P	P	N	P	N	P	P	N	P	N	FAM
	P	P	N	P	P	N	N	N	N	P	N	N	N	P	P	P	P	N	N	N	HEX
24 h in H-buffer	P	P	N	P	P	P	P	P	P	P	N	P	P	N	N	P	P	N	P	N	FAM
	N	P	P	P	P	P	P	N	N	P	N	P	P	N	N	P	N	P	N	P	HEX
RNA-later, 2 h, washed 3 times	-	-	-	-	-	-	-	-	-	-	N	N	N	N	P	P	P	N	N	N	FAM
	-	-	-	-	-	-	-	-	-	-	P	P	P	N	P	P	P	P	P	N	HEX
RNA-later, 2 h, washed 5 times	P	P	P	N	N	P	P	N	N	P	-	-	-	-	-	-	-	-	-	-	FAM
	N	P	P	N	N	N	N	P	N	N	-	-	-	-	-	-	-	-	-	-	HEX
24 h in N-buffer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	N	N	N	FAM
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	P	P	P	HEX
RNA-later, 24 h, freezed, washed 5 times, Tissuelyser	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	FAM
	P	N	P	N	N	N	N	N	N	P	-	-	-	-	-	-	-	-	-	-	HEX
RNA-later 24 h, freezed, washed 5 times, Tissuelyser and filtrated	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*P*	-	-	-	-	-	-	-	-	-	-	FAM
	N	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*P*	-	-	-	-	-	-	-	-	-	-	HEX
2 h in H-buffer,freezed, Tissuelyser	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	-	-	-	-	-	-	-	-	-	-	FAM
	N	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*N*	*P*	-	-	-	-	-	-	-	-	-	-	HEX

3.3 Fresh fish

Fresh Atlantic salmon from a location in Rogaland were picked up right after slaying and brought to UiS. At UiS three fish were dissected and samples were taken from feces, liver, heart, gills, and brain. Samples were shock frozen in nitrogen and stored in a freezer at -80 ℃ for 24 h before moving to a -75 ℃ freezer. Feces, liver, heart, gills, and brain were analyzed

from two of the fish to investigate whether the homogenization method is good enough. The homogenization method was good enough if the internal control got a Cq value. Sample 1 and sample 10 had a small decrease in Cq, but the graphs were difficult to interpret due to disturbances in the signal. Sample 1 was later re-analyzed, but this time the sample passed through a sieve using a syringe (2 µm filter). After re-analyzing the sample it was negative for both FAM and HEX (Appendix B, Fresh fish).

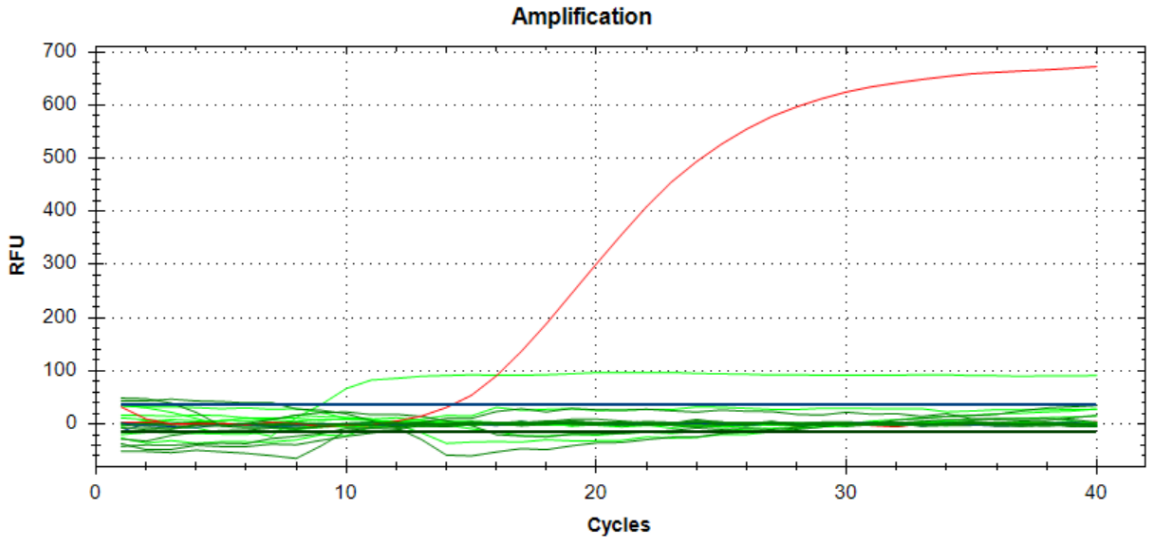

Figure 3.4. RT-qPCR analysis of SAV in ten samples of feces, liver, heart, gills, and brain from two different fish were analyzed. There were one sample of heart, liver, brain, feces and gills from each fish. Red line is the positive control, dark green lines are each sample analyzed for FAM. Light green lines are each sample analyzed for HEX. The dark blue line is the threshold line.

3.4 Fish hearts in ethanol

Four fish hearts, one positive control for SAV and one liver sample were sent from the Veterinary Institute. The hearts were stored in ethanol and confirmed positive from the Veterinary Institute. The hearts were washed one time in H-buffer and all samples were negative for FAM and HEX. The liver sample was not stored in ethanol, but fresh with no buffer. Regardless the sample was negative. The positive control had Cq 24.9 and GeneMe`s positive control had Cq 12.64.

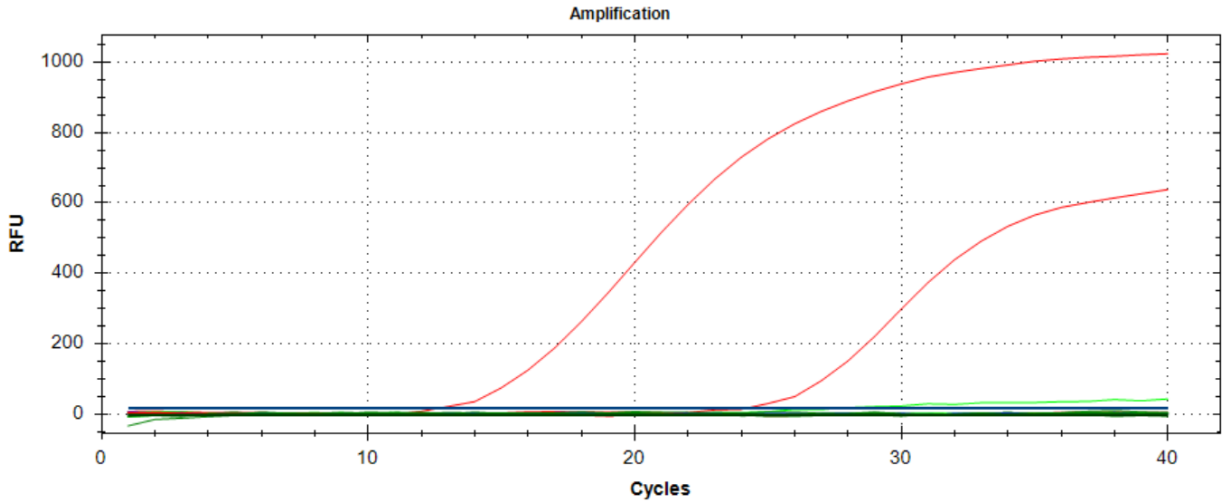

Figure 3.5. RT-qPCR analysis of SAV in four fish hearts stored in ethanol, one fish liver sample, and two positive controls. One positive control from GeneMe (red curve, left) and one from the Veterinary institute (red curve, right). Only the positive controls were positive. Red lines are the positive controls, dark green lines are each sample analyzed for FAM. Light green lines are each sample analyzed for HEX. The dark blue line is the threshold line.

4. Discussion

This thesis was done as a part of a validation process for NordicDx to approve GeneMe tests for SAV. The focus was to optimize the protocol for the validation process. Infected hearts were analyzed under different conditions to examine how these affect the results. Further, positive control with SAV RNA were analyzed over four freezing cycle to examine how it affects the Cq. In addition, organs from fish without suspicion of SAV infection were analyzed to confirm the internal control of the SAV test.

4.1 Positive control parallels

Positive control with SAV RNA from the veterinary institute were analyzed over four freezing cycles with five parallels each day to see if it affects the Cq. The average Cq for the five days was 26.052, 25.264, 25.946, 25.858 and 26.54. The average is very even, except a small increase for cycle 4. The standard deviation is higher for this day, which means that the average can be influenced by one value. In conclusion, the synthetic RNA from the Veterinary Institute can be used as a positive control after at least four freezing cycles.

Another thing to examine is the temperature of the samples when pipetting, because the reaction may start earlier for the samples with higher temperature. This may have happened when analyzing the positive controls. The sample was taken out from the freezer and put on ice, the

sample was colder when preparing the first parallel. Since the parallel taken last may be closer to room temperature and could possibly start the reaction in the PCR-mix before. It would have been interesting to examine the results when the whole procedure was done on ice.

4.2 Positive salmon hearts

From a slaughterhouse, 20 suspected infected hearts were collected. The hearts were cut in four, two of them were put in RNA-later, and two were put in H-buffer. The hearts were analyzed after being stored in five different conditions to evaluate the best outcome for protocol optimization. The first condition analyzed was the hearts that were put directly in H-buffer and analyzed after 2 h. The H-buffer with homogenized heart tissue was put in a fridge after analyzes, together with the N-buffer with heart 17-20. The hearts that were analyzed after 2 h had 7/20 positive for FAM and 9/20 positive for HEX. Comparing these numbers to the hearts stored for 24h in H-buffer and 24h in N-buffer in the fridge, there are more positive hearts for the two last conditions. When it stood 24 h in N-buffer it became positive for HEX for all samples. Only one of the samples is positive for FAM (sample 17) even though sample 19 also has been positive in H-buffer. This can be because the RNA virus may be degraded when stored, while the DNA that is more stable and contains the internal control will survive. Comparing the samples from hearts in H-buffer (2 h) and the samples that has been 24 h in H-buffer there are 7 more positive samples when the hearts in H-buffer has stayed in the fridge for 24 h. Compared to the hearts stored in N-buffer, the RNA is better conserved when stored in H-buffer, due to more positive results when analyzing samples from this condition. The best way to analyze them may be after a few h, but not too long as that might result in degraded RNA. Since the test kits are meant to be used in fast diagnostic, there may not be time to store the samples h before analyzing. This can be the solution if the result is inconclusive, or the graphs are difficult to interpret. Then it could be relevant to analyze the tests again from the H-buffer because then the H-buffer has homogenized the sample after 1 h.

There are more positive hearts for the samples washed 5 times after being stored in RNA-later against the ones only washed 3 times. This indicates that the RNA-later is a suitable way to store the fish hearts before analyzing because the RNA stays intact, however, the hearts should be washed at least five times to get rid of the RNA-later buffer which interferes with the PCR. The RNA-later makes it possible to store the tissue over time because it stabilizes and protects the RNA. This means that tissue could be taken out of the fish without being analyzed straight

away. This is very relevant for SAV testing because the hearts may be taken out far away from the test facility, and this way it is assured that the RNA virus is intact.

Heart 1-10 in H-buffer and heart 1-10 in RNA-later were analyzed again after being homogenized with a Qiagen TissueLyser LT. The hearts in RNA-later were also filtrated and analyzed again. This was done to evaluate if the manual homogenization procedure by hand could be improved by automatic high-speed shaking and to make the curves more readable by filtering out possible inhibitors from the samples. Heart 10 that was stored in RNA-later and filtrated, and was the only sample that came out positive for FAM. For HEX there were 5 hearts that were positive, one heart stored in H-buffer and one heart stored in RNA-later and filtrated. The rest of the hearts that were HEX-positive (3 samples) were stored in RNA-later without being filtrated. Heart 10 were positive for HEX for all three conditions, but only positive for FAM after being stored in RNA-later and not filtrated. This indicates that the best way to store the hearts over time is in RNA-later and that the syringe filter used here can possibly have fractured the unstable RNA-molecules because of the resistance (Precautions for Handling of RNA, 2016.). It would be interesting to filter the samples with a molecular sieve exclusively using the force of gravity, to observe if more samples then could contain intact RNA, the results were easier to interpret than before with smoother graphs due to the use of the TissueLyser and filtration.

4.3 Fresh fish without suspicion of infection

The results from the supposedly fresh fish were a bit difficult to interpret because some graphs increased sharply at the beginning and then decreased towards the end. The gills were interpreted as possibly positive, but the other organs from the fish were negative. It is difficult to say if the fish is sick with SAV or if the tissue and blood disturbed the reaction.

To make the result clearer to interpret the samples were analyzed again, but with a sieve. The result came back inconclusive with both negative for FAM and HEX and is therefore not a good indication of the real result. To make the samples clearer and to get more even and easier graphs to interpret an adapted sieve could be the solution. With a sieve the blood and all unwanted tissue will be separated to avoid inhibition and/or disturbance of the reaction. It is expected that a molecular sieve may affect the results.

This experiment also shows that hearts are the easiest organ to analyze. Gills are difficult to homogenize. Liver has too much blood and can prevent the reaction from happening. The feces have many bacteria that can prevent the reaction. The brain is difficult to dissect, and it is not

known if the virus is located in the brain. In conclusion, the heart of the fish is the optimal tissue for SAV analysis, because it contains less blood than in the liver and there are no bacteria that can inhibit the reaction. The heart is also easy to dissect from the salmon.

4.4 Fish hearts in ethanol

Five fish hearts, one liver sample and a positive control were sent from the Veterinary Institute. All the fish hearts were clearly negative with no signal for either FAM or HEX (figure 5). This indicates that storing fish tissue in ethanol is a poor storage method. Since the hearts were confirmed positive before analyzing them, this could be because the ethanol degrades the RNA when the homogenization destroys the cells, or due to the fact that residual ethanol may inhibit enzymatic reactions in the PCR reaction (Schrader et al., 2012). It could have helped with washing them more times, maybe five times to get all the ethanol cleaned away, but most likely the ethanol destroyed all present SAV-virus. The liver sample from the same batch was also negative even though it was stored without the ethanol. The explanation may be that the liver was full of blood, and it was difficult to make a sample without blood. Blood can disturb the PCR reaction so the result can be fake negative result with the liver.

4.5 Vaccines and faster diagnostics

To prevent the spreading of SAV it is important to know how the virus spreads and how it infects. This together with fast diagnostics, both histopathological examination and PCR, and further development of effective vaccines is important to keep the virus in check. Studies show that vaccines could be an effective way to stop the spreading because most fish that survives infection cannot be reinfected. Therefore, the development of vaccines is also an important factor (Deperasińska et al., 2018).

4.6 SAV2 and SAV3

Furthermore, it would be important to develop a test to distinguish between SAV2 and SAV3 virus (Jansen et al., 2015). If a fish cage gets a positive result for a SAV virus, the protocol is to sequence the virus to decide if it is SAV2 or SAV3. With a test kit that detects two unique genes, one for SAV2 virus and one for SAV3 virus, the test result could be clear within 1 hour instead of many days. Thus, restrictions can be initiated immediately if the result comes out positive for SAV3. It is also positive for cages with SAV2 to be detected early because then it can be monitored carefully and if the fish starts to get sick, they can be slaughtered before the whole cage are infected.

4.7 Future perspective

Sustainability is important as the population in the world grows and there is a growing shortage of food (Ranganathan et al., 2018). One way to solve this crisis is to eat more food from the ocean as an increasing world population lays more claim on the land for housing, transport, businesses, etc. So, there will be less and less agricultural land in the future, so the sea is a nice option to use but in a more sustainable manner. Therefore, it is important to continue the development of vaccines and methods of rapid diagnosis in order to prevent food waste and increase food production.

With climate change and a higher risk of new diseases emerging and spreading (Kurane, 2010), it would be desirable to be able to use this type of technology further to develop more rapid tests with high accuracy. Further it could be relevant to make the tests easier to analyze. This way the fish farmers could test the fish themselves and take appropriate action.

5.Conclusion

RT-qPCR kit from GeneMe was used to detect SAV2 and SAV3 in Atlantic salmon to optimize the kit. Infected salmon hearts were tested after being stored in various conditions, the results indicates that the GeneMe kit detects SAV2 and SAV3, but further optimalization could make the results easier to interpret. Fourteen of twenty hearts that were stored in H-buffer for 24 h were positive for SAV, this indicates that the test gives a better result after the samples have been stored in H-buffer, and it would be interesting to investigate the outcome after for example 12, 6 and 1 hour. The results from the samples that were homogenized with a TissueLyser and filtrated were easier to interpret. Hearts stored in RNA-later and washed five times in H-buffer before analysis had better results than hearts stored in H-buffer indicating that the H-buffer is not suited for long time storage. There were not possible to detect any SAV in hearts stored in ethanol. These are things to consider further in the optimalization.

It would be beneficial to develop similar methods as the one used in this thesis for other infectious diseases, as both the health sector and the food industry can benefit from better monitoring of threatening pathogens.

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Appendices

Appendix A: protocols

SAV protocol:

Preparation of samples for the SAV test

SAV kit components

- SAV tests

- Homogenization Buffer

- Normalization Buffer

- Positive Control

- molecular sieve / molecular filter

- 1.5 ml eppendorf tubes

- Pasteur transfer pipettes

- scalpel

- metal homogenizer

Equipment required in the laboratory

- centrifuge for 1.5 ml tubes

- ice in a container or a cooler of 0 degrees Celsius for 1.5 mL tubes

- pipette 50 uL

- tips with filters for pipette 50 uL

- Bio-Rad thermocycler

- fridge

- sterilizing cleaning agent: for example Line-antybacteria 70

- electronic weighing scale measuring milligrams

Procedure

I. Study with the use of isolated RNA

1. Prepare a negative control by adding 50 µl of normalization buffer to the SAV test well

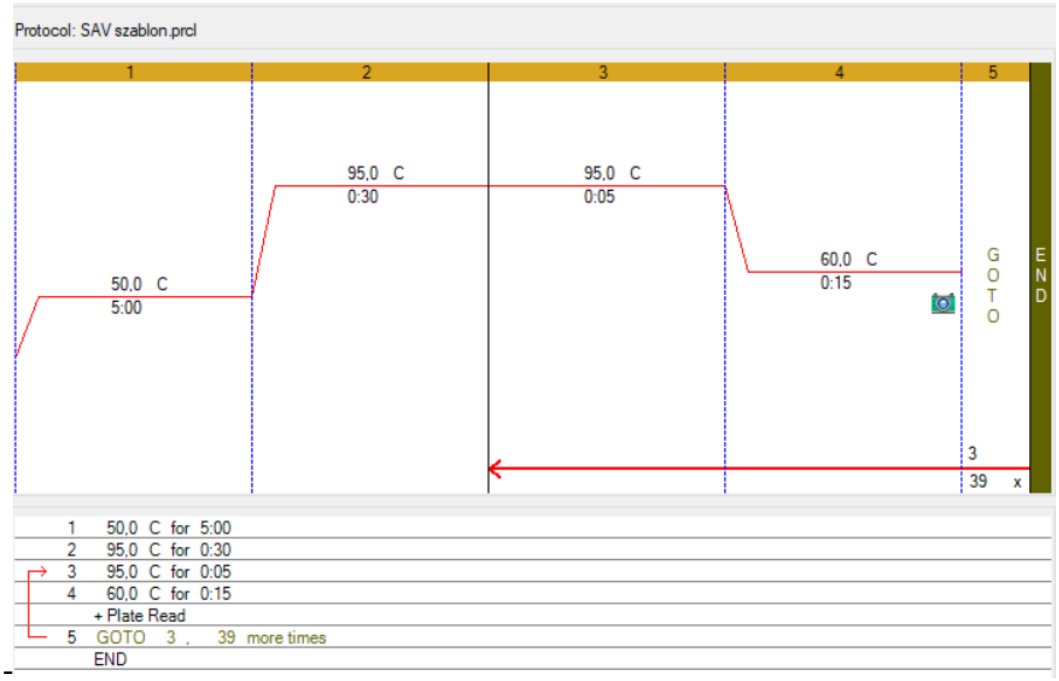
2. Add 40 µl of normalization buffer to the SAV test well and pipette 5 times.

3. Add 10 µl of the isolated virus RNA to the same well.

4. Repeat steps 2 and 3 for all RNA samples you intend to test.

5. Prepare a positive control by transferring 50 µl of Normalization Buffer to the Positive Control Tube. Transfer 50 µl of the mixture from the Positive Control Tube to the open SAV test well.

6. Insert the test into the thermal cycler and set the temperature-time profile:



Set the reading on the FAM (SAV virus detection) and HEX (internal control) channel.

II. Examination with the use of fish tissues

Homogenization of samples

1. By using a scalpel, cut approximately 30 mg of fish tissue.
2. Transfer to an Eppendorf tube with 300 ul of homogenization buffer (H).

3. Thoroughly homogenize the tissue sample with a steel homogenizer.

4. Fill the sample with 500 µl of homogenization buffer (H) and mix by pipetting several times

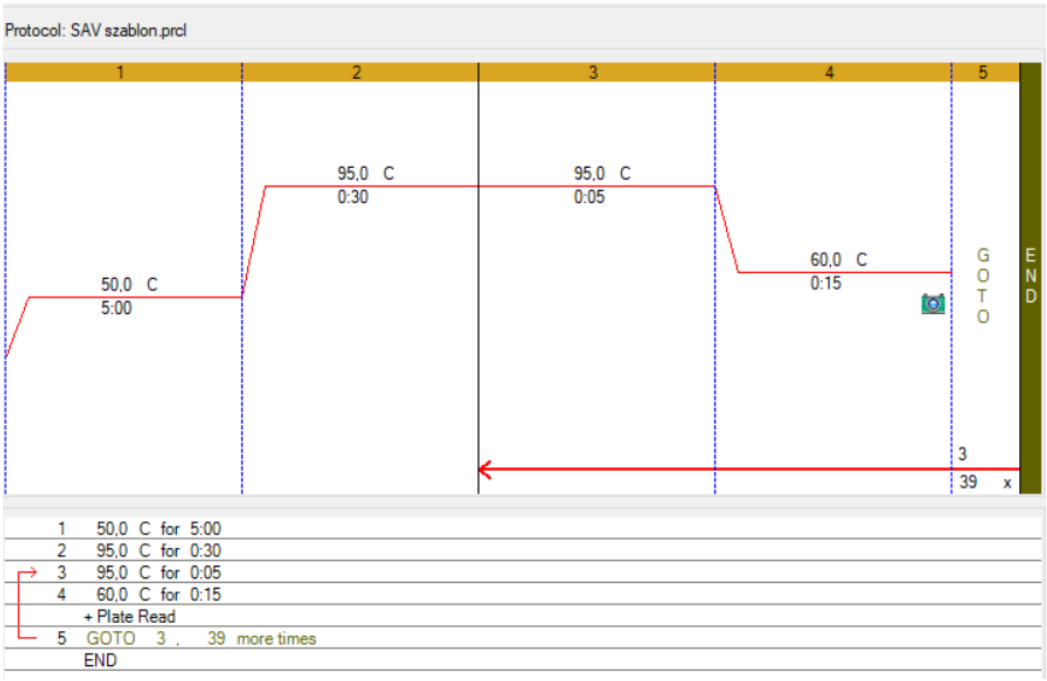
5. Leave on ice for 20 minutes.

6. Centrifuge for 10 minutes at a maximum speed of approx. 13,000 rpm.

7. Dilute the prepared lysate (without sediment) 20x in normalizing buffer (N1): add 50 µl of sample to 950 µl of normalizing buffer.

SAV test of samples

1. Prepare a negative control by adding 50 µl of normalization buffer to the SAV test well.
2. Add 50 µl of homogenized fish tissue to a SAV test well.
3. Repeat step 2 for all samples you intend to test.
4. Prepare a positive control by transferring 50 µl of Normalization Buffer to the Positive Control Tube. Transfer 50 µl of the mixture from the Positive Control Tube to the open SAV test well.
5. Insert the test into the thermal cycler and set the temperature-time profile:



Set the reading on the FAM (SAV virus detection) and HEX (internal control) channel.

Interpretation of the results

The correct test procedure and the ability to interpret the results are only possible if the appropriate signals are obtained for the controls in the reaction. When analysing SAV test data, please use the decision matrix below:

Interpretation of the controls:

Channel	NEGATIVE CONTROL	POSITIVE CONTROL	INTERPRETATION
FAM	NO SIGNAL Cq undetermined	Cq < 30	VALID
HEX	NO SIGNAL Cq undetermined	Cq < 40	

Interpretation of the testing samples:

Channel	Sample	INTERPRETATION
FAM	Cq undetermined	NEGATIVE
HEX	Cq < 40	
FAM	Cq < 40	POSITIVE
HEX	Cq < 40 or undetermine	

Tissue Lyser LT (QIAGEN) protocol:

Homogenization procedure for frozen fish tissue using Tissue Lyser LT (QIAGEN)

1. Place 2 ml microcentrifuge tubes containing 1 stainless steel bead (5 mm mean diameter) on dry ice for at least 15 min. Keep the insert of the TissueLyser LT Adapter at room temperature (15–25°C).

2. Transfer up to 30 mg fresh or frozen tissue to the precooled tubes and incubate for another 15 min on dry ice. If handling tissue samples stabilized with RNAlater RNA Stabilization Reagent or Allprotect Tissue Reagent, cooling on dry ice is not necessary.

3. Place the tubes into the insert of the TissueLyser LT Adapter, and incubate at room temperature for 2 min to avoid freezing of lysis buffer in step 4. Do not incubate for longer than 2 min, otherwise the tissue will thaw, resulting in potential RNA degradation.

4. Immediately add the appropriate volume of lysis buffer (e.g., Buffer RLT, Buffer RLT Plus, or QIAzol Lysis Reagent) to each tube. Note: If using Buffer RLT Plus, we recommend adding Reagent DX to prevent excessive foaming. For details, see “Disruption and homogenization in Buffer RLT Plus” (page 16).

5. Place the insert with sample tubes into the base of the TissueLyser LT Adapter, which is attached to the TissueLyser LT. Place the lid of the TissueLyser LT Adapter over the insert, and screw the knob until the lid is securely fastened.

6. Operate the TissueLyser LT for 2–5 min at 50 Hz. The duration of disruption and homogenization depends on the tissue being processed and can be extended until no tissue debris is visible. If processing fiber-rich tissues, complete disruption and homogenization may sometimes not be possible. However, small amounts of debris have no effect on subsequent RNA purification with QIAGEN kits and are usually digested in the proteinase K step.

7. Proceed with RNA, DNA/RNA, or DNA/RNA/protein purification. Do not reuse the stainless steel beads. (*(EN) - TissueLyser Handbook - QIAGEN*, u.å.)

Appendix B: PCR results

Positive controls

Positive control before freezing, arrived from the the Veterinary Institute at 29. Mars 2022.

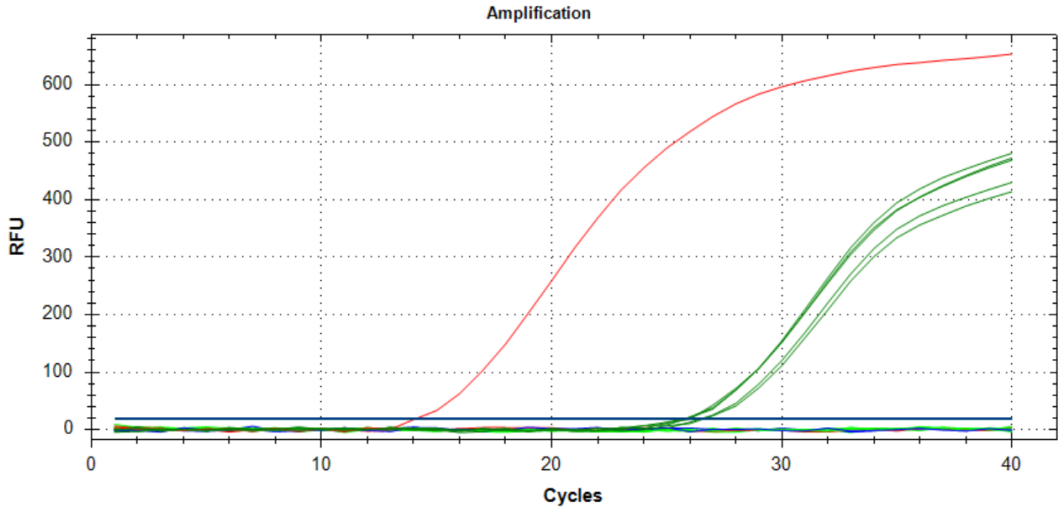


Figure B-1. Positive control from the Veterinary Institute 29. March 2022.

Table B-1. Cq values for positive control from the Veterinary Institute.

Well	Fluor	Content	Cq
B06	FAM	Unknown	25.62
C06	FAM	Unknown	25.71
D06	FAM	Unknown	25.98
E06	FAM	Unknown	26.41
F06	FAM	Unknown	26.54
G06	FAM	Pos Ctrl	14.02
A06	HEX	Neg Ctrl	N/A
B06	HEX	Unknown	N/A
C06	HEX	Unknown	N/A
D06	HEX	Unknown	N/A
E06	HEX	Unknown	N/A
F06	HEX	Unknown	N/A
G06	HEX	Pos Ctrl	N/A

Positive control, freezing cycle one, 29.04.2022

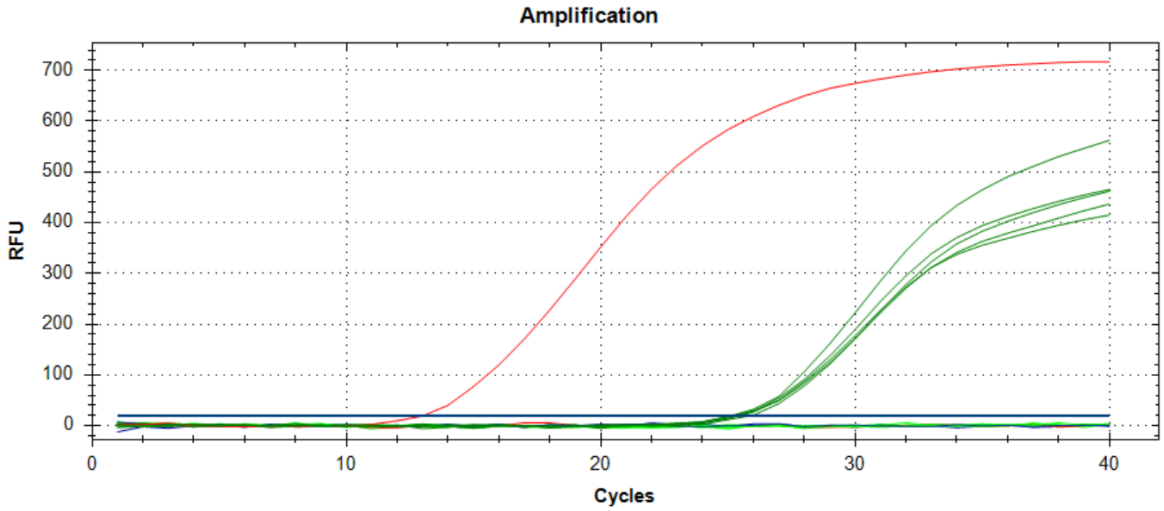


Figure B-2. Positive control from the Veterinary Institute 29. April 2022. Freezing cycle one.

Table B-2. Cq values for positive control from the Veterinary Institute. Freezing cycle one

Well	Fluor	Content	Cq
B06	FAM	Unknown	25.05
C06	FAM	Unknown	25.31
D06	FAM	Unknown	25.70
E06	FAM	Unknown	25.26
F06	FAM	Unknown	25
G06	FAM	Pos Ctrl	12.88
A06	HEX	Neg Ctrl	N/A
B06	HEX	Unknown	N/A
C06	HEX	Unknown	N/A
D06	HEX	Unknown	N/A
E06	HEX	Unknown	N/A
F06	HEX	Unknown	N/A
G06	HEX	Pos Ctrl	N/A

.

Positive control, freezing cycle 2.

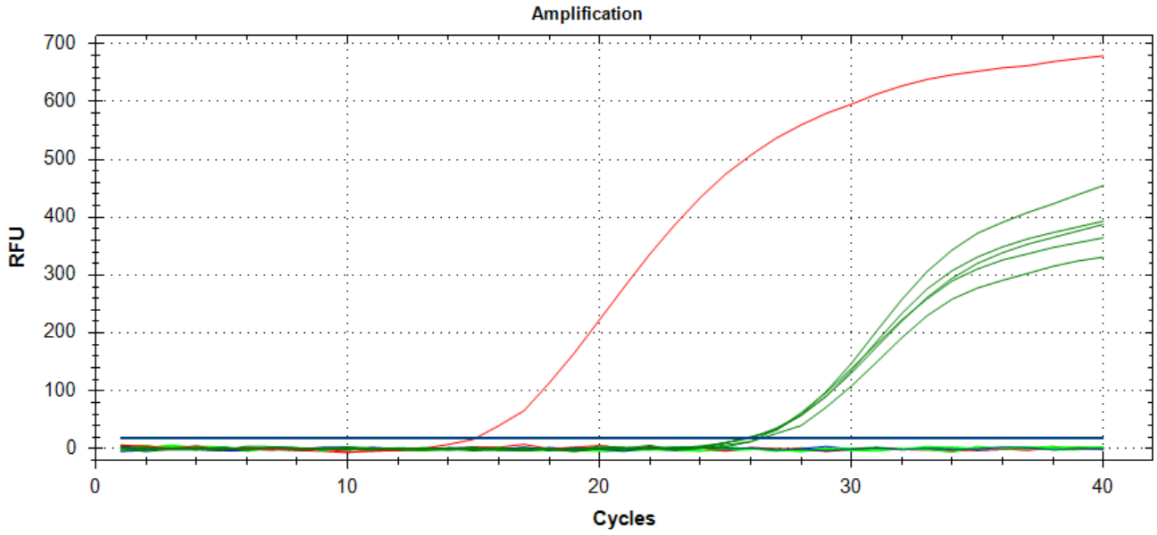


Figure B-3. Freezing cycle two. 2. May 2022.

Table B-2 Cq values for positive control from the Veterinary Institute freezing cycle two.

Well	Fluor	Content	Cq
B06	FAM	Unknown	25.72
C06	FAM	Unknown	26.33
D06	FAM	Unknown	25.64
E06	FAM	Unknown	26.23
F06	FAM	Unknown	25.81
G06	FAM	Pos Ctrl	15.03
A06	HEX	Neg Ctrl	N/A
B06	HEX	Unknown	N/A
C06	HEX	Unknown	N/A
D06	HEX	Unknown	N/A
E06	HEX	Unknown	N/A
F06	HEX	Unknown	N/A
G06	HEX	Pos Ctrl	N/A

Positive control freezing cycle 3.

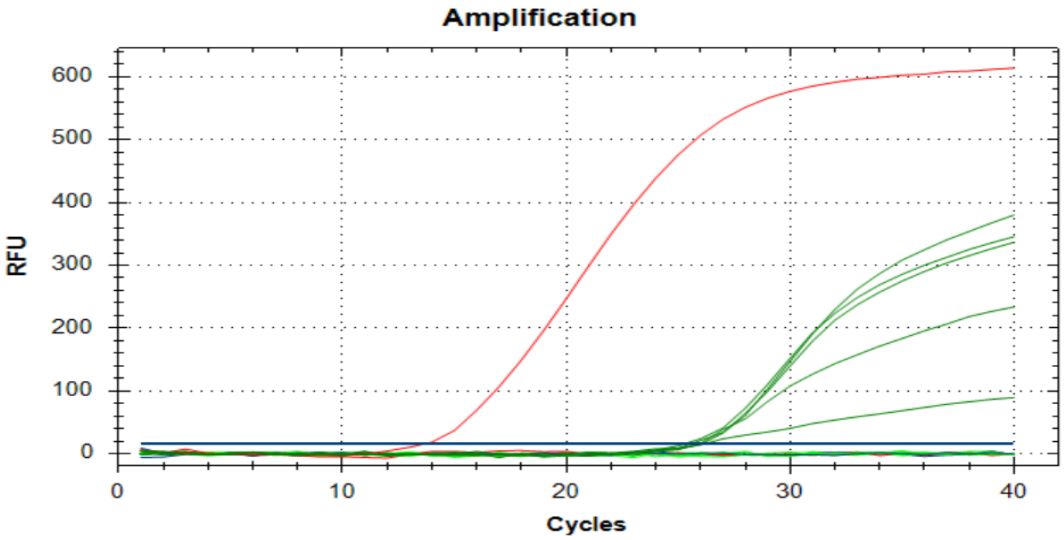


Figure B-4. Freezingcycle three. 4. May 2022.

Table B-3. Cq values for positive control from the Veterinary Institute. Freezing cycle three.

Well	**Fluor**	**Content**	**Cq**
B06	FAM	Unknown	26.25
C06	FAM	Unknown	26.08
D06	FAM	Unknown	26.00
E06	FAM	Unknown	25.34
F06	FAM	Unknown	25.62
G06	FAM	Pos Ctrl	13.76
A06	HEX	Neg Ctrl	N/A
B06	HEX	Unknown	N/A
C06	HEX	Unknown	N/A
D06	HEX	Unknown	N/A
E06	HEX	Unknown	N/A
F06	HEX	Unknown	N/A
G06	HEX	Pos Ctrl	N/A

Positive control freezing cycle 4.

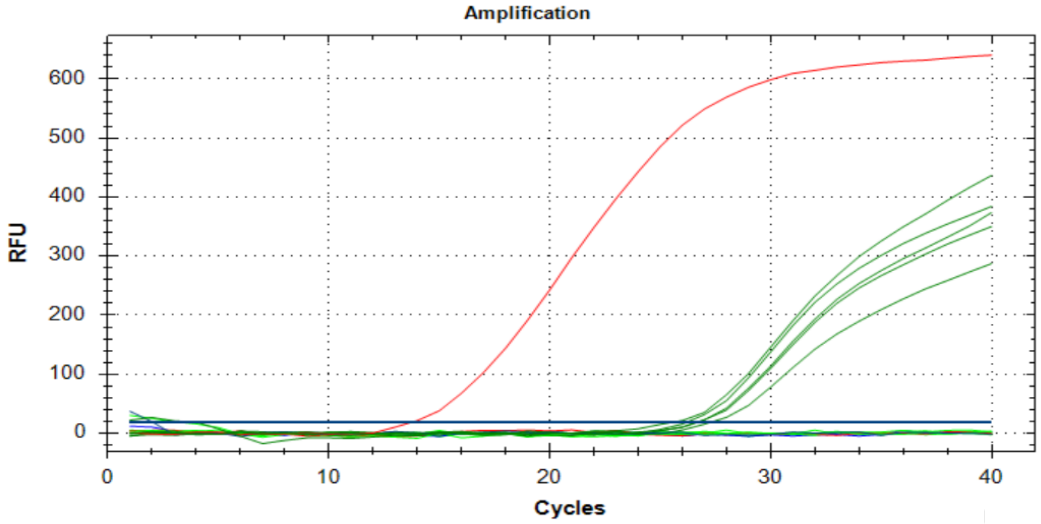


Figure B-5. Freezingcycle four. 4. May 2022.

Table B-4. Cq values for positive control from the Veterinary Institute. Freezing cycle four.

Well	Fluor	Content	Cq
B06	FAM	Unknown	27.34
C06	FAM	Unknown	25.67
D06	FAM	Unknown	26.74
E06	FAM	Unknown	26.71
F06	FAM	Unknown	26.24
G06	FAM	Pos Ctrl	13.82
A06	HEX	Neg Ctrl	N/A
B06	HEX	Unknown	N/A
C06	HEX	Unknown	N/A
D06	HEX	Unknown	N/A
E06	HEX	Unknown	N/A
F06	HEX	Unknown	N/A
G06	HEX	Pos Ctrl	N/A

Infected fish hearts

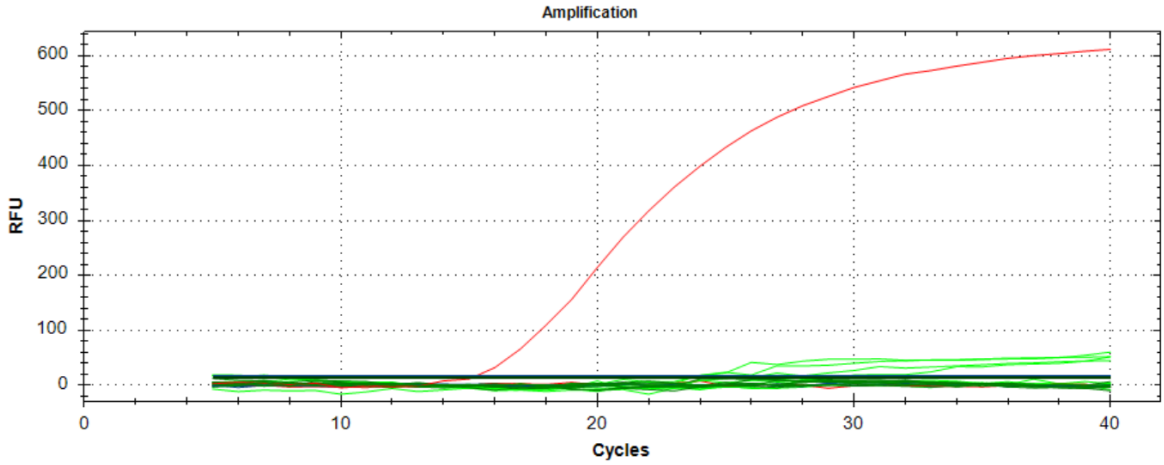


Figure B-6. Heart number 1 – 8 in homogenization buffer. All are negative with no Cq for the FAM channel. 10. May

Table B-5. Cq values for heart number 1-8 in H-buffer.

Well	Fluor	Content	Cq
A06	FAM	Neg Ctrl	N/A
A07	FAM	Unkown	N/A
B06	FAM	Unknown	N/A
B07	FAM	Unknown	N/A
C06	FAM	Unknown	N/A
C07	FAM	Pos Ctrl	15.27
D06	FAM	Unknown	N/A
E06	FAM	Unknown	N/A
F06	FAM	Unknown	N/A
G06	FAM	Unknown	N/A
A06	HEX	Neg Ctrl	N/A
A07	HEX	Unknown	N/A
B06	HEX	Unknown	23.74
B07	HEX	Unknown	N/A
C06	HEX	Unknown	29.46
C07	HEX	Pos Ctrl	N/A
D06	HEX	Unknown	N/A
E06	HEX	Unknown	23.7
F06	HEX	Unknown	26.04
G06	HEX	Unknown	N/A

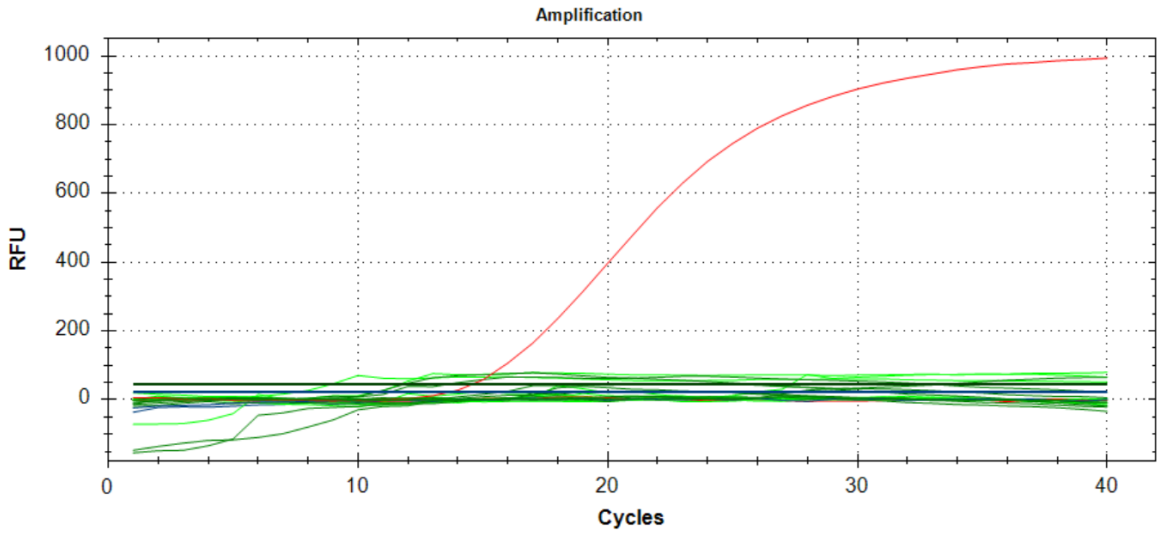


Figure B-7. Heart 9 – 16 in H-buffer. 10. May 2022

Table B-6. Cq values for heart 9 – 16 in H-buffer.

Well	Fluor	Content	Cq
A06	FAM	Neg Ctrl	N/A
A07	FAM	Unknown	10.8
B06	FAM	Unknown	18.97
B07	FAM	Unknown	N/A
C06	FAM	Unknown	27.63
C07	FAM	Unknown	17.47
D06	FAM	Unknown	11.36
D07	FAM	Pos Ctrl	13.75
E06	FAM	Unknown	15.91
F06	FAM	Unknown	N/A
A06	HEX	Neg Ctrl	N/A
A07	HEX	Unknown	11.66
B06	HEX	Unknown	N/A
B07	HEX	Unknown	8.91
C06	HEX	Unknown	27.32
C07	HEX	Unknown	N/A
D06	HEX	Unknown	N/A
E06	HEX	Unknown	N/A
F06	HEX	Unknown	N/A

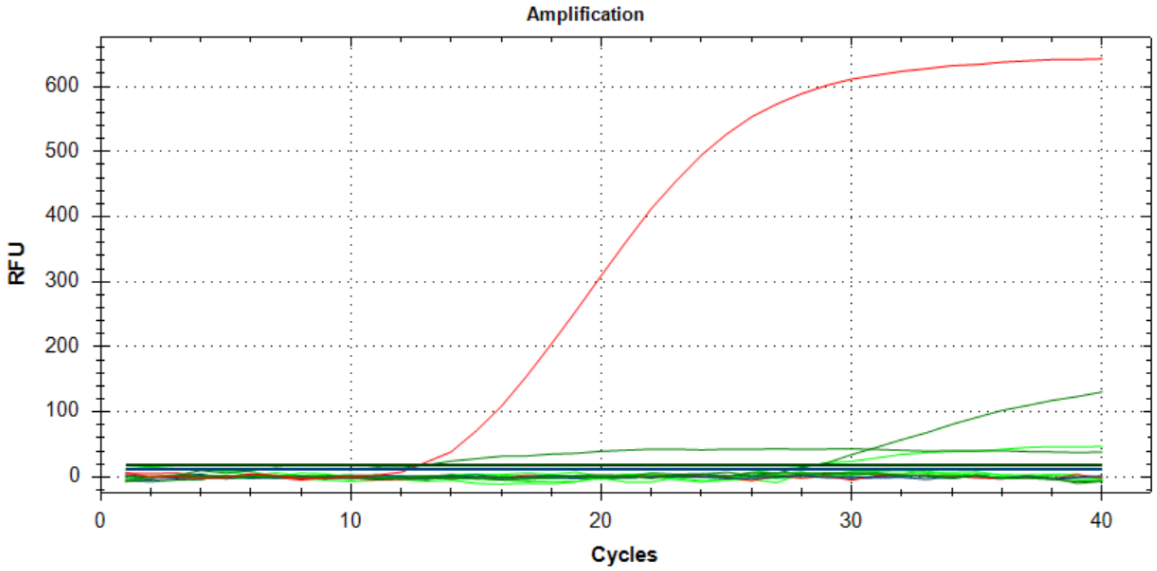


Figure B-8. Heart 17 – 20 in homogenzation buffer. 10. May 2022.

Table B-7. Cq values for heart 17-20 in H-buffer.

Well	Fluor	Content	Cq
A06	FAM	Neg Ctrl	N/A
B06	FAM	Unknown	27.53
C06	FAM	Unknown	N/A
D06	FAM	Unknown	N/A
E06	FAM	Unknown	10.06
F06	FAM	Pos Ctrl	12.22
A06	HEX	Neg Ctrl	N/A
B06	HEX	Unknown	28.47
C06	HEX	Unknown	N/A
D06	HEX	Unknown	N/A
E06	HEX	Unknown	N/A
F06	HEX	Pos Ctrl	N/A

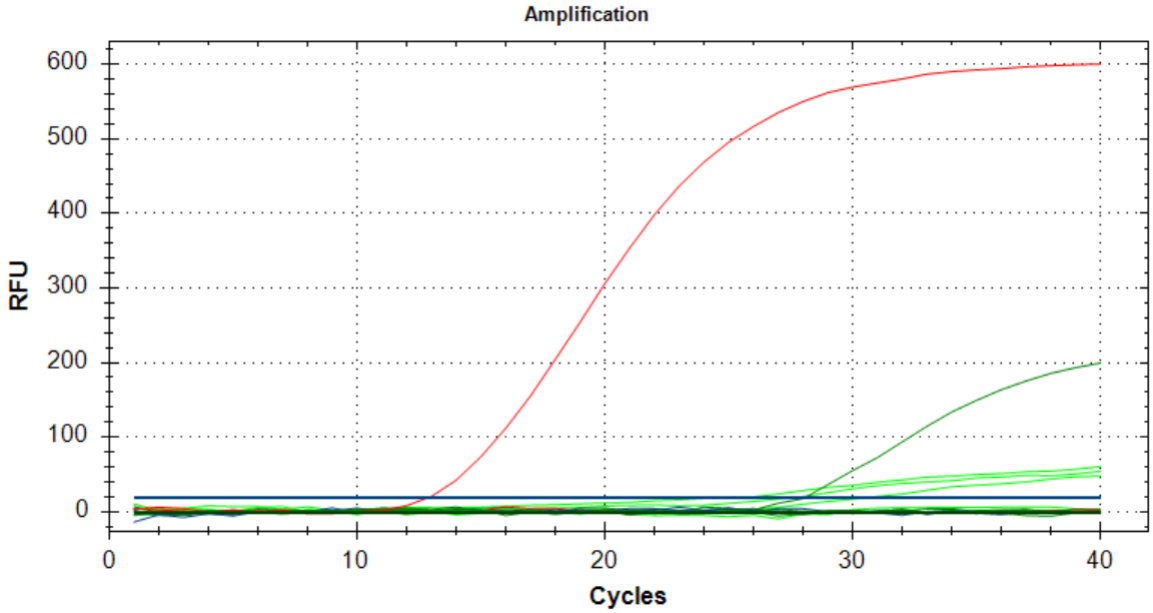


Figure B-9. Prøve 17 – 20, straight from normalization buffer taken the day before. 11. May 2022

Table B-8. Cq values for heart 17-20 stored in N-buffer.

Well	**Fluor**	**Content**	**Cq**
A06	FAM	Neg Ctrl	N/A
B06	FAM	Unknown	28
C06	FAM	Unknown	N/A
D06	FAM	Unknown	N/A
E06	FAM	Unknown	N/A
F06	FAM	Pos Ctrl	12.77
A06	HEX	Neg Ctrl	36.62
B06	HEX	Unknown	14.77
C06	HEX	Unknown	12.67
D06	HEX	Unknown	10.35
E06	HEX	Unknown	29.34
F06	HEX	Pos Ctrl	21.64

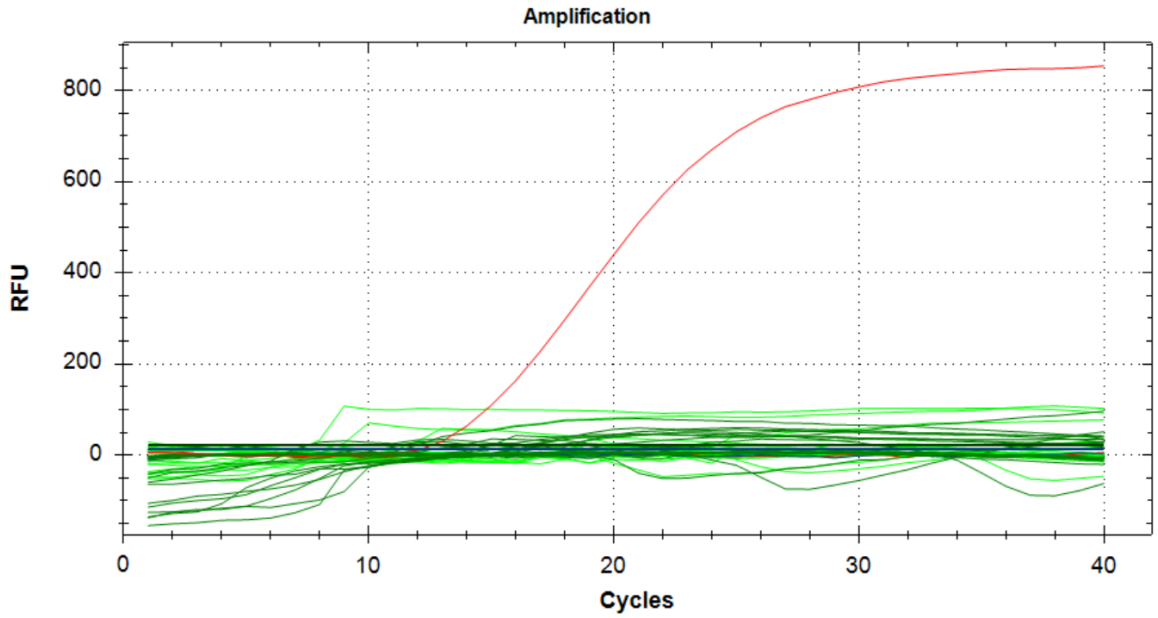


Figure B-10. Heart 1 -20 after 24 h in H-buffer. 11. May 2022.

Table B-9. Cq values for heart 1-20 stored for 24 h in H-buffer.

Well	Fluor	Content	Cq
A06	FAM	Neg Ctrl	N/A
A07	FAM	Unknown	26.89
A08	FAM	Unknown	11.32
B06	FAM	Unknown	20.57
B07	FAM	Unknown	12.54
B08	FAM	Unknown	18.13
C06	FAM	Unknown	29.26
C07	FAM	Unknown	35.71
C08	FAM	Unknown	N/A
D06	FAM	Unknown	N/A
D07	FAM	Neg Ctrl	N/A
D08	FAM	Unknown	14.97
E06	FAM	Unknown	14.33
E07	FAM	Unknown	15.76
E08	FAM	Unkonwn	N/A
F06	FAM	Unknown	14.20
F07	FAM	Unknown	36.06
F08	FAM	Pos Ctrl	12.09
G06	FAM	Unknown	16.28
G07	FAM	Unknown	N/A
H06	FAM	Unknown	15.95
H07	FAM	Unknown	N/A
A06	HEX	Neg Ctrl	N/A
A07	HEX	Unknown	N/A
A08	HEX	Unknown	11.52

B06	HEX	Unknown	N/A
B07	HEX	Unknown	N/A
B08	HEX	Unknown	N/A
C06	HEX	Unknown	24.13
C07	HEX	Unknown	15.33
C08	HEX	Unknown	7.49
D06	HEX	Unknown	8.63
D07	HEX	Unknown	N/A
D08	HEX	Unknown	18.55
E06	HEX	Unknown	17.98
E07	HEX	Unknown	28.83
E08	HEX	Unknown	N/A
F06	HEX	Unknown	15.81
F07	HEX	Unknown	37.20
F08	HEX	Pos Ctrl	N/A
G06	HEX	Unknown	21.71
G07	HEX	Unknown	N/A
H06	HEX	Unknown	18.81
H07	HEX	Unknown	N/A

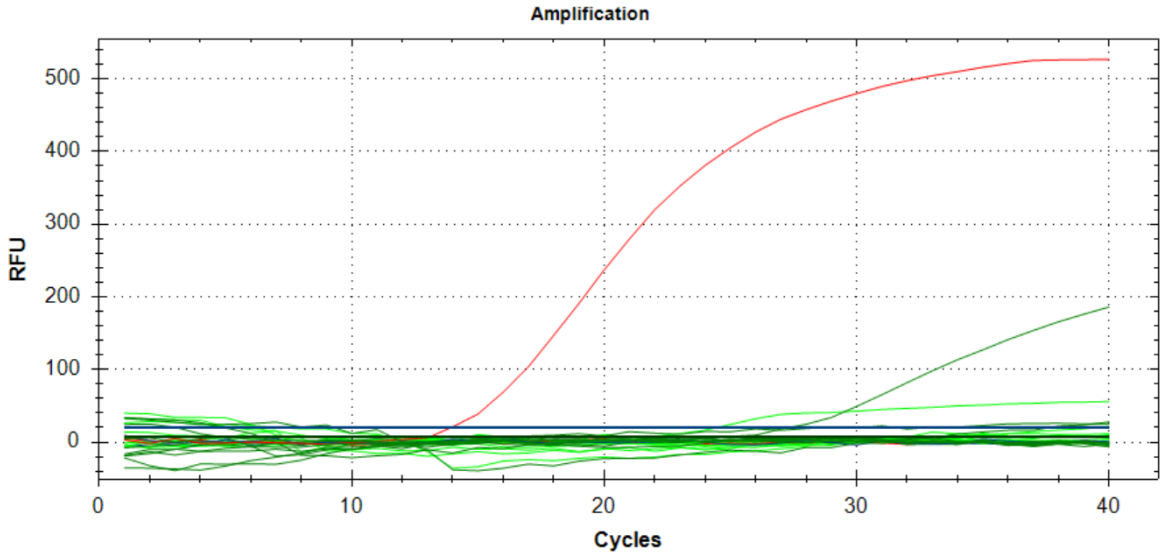


Figure B-11. Heart 11 -20, washed 3 times after stored in RNA – later for 24 h. 11. May 2022

Table B-10. Cq values for heart 11-20 stored in RNA-later and washed three times before analysis.

Well	**Fluor**	**Content**	**Cq**
A06	FAM	Neg Ctrl	N/A
A07	FAM	Unknown	N/A
B06	FAM	Unknown	N/A
B07	FAM	Unknown	N/A
C06	FAM	Unknown	N/A

C07	FAM	Unknown	N/A
D06	FAM	Unknown	N/A
D07	FAM	Pos Ctrl	13.87
E06	FAM	Unknown	N/A
F06	FAM	Unknown	32.38
G06	FAM	Unkonwn	37.45
H06	FAM	Unknown	27.15
A06	FAM	Neg Ctrl	N/A
A07	FAM	Unknown	13.98
B06	FAM	Unknown	5.11
B07	FAM	Unknown	33.64
C06	FAM	Unknown	19.48
C07	HEX	Unknown	N/A
D06	HEX	Unknown	14.54
D07	HEX	Pos Ctrl	N/A
E06	HEX	Unknown	N/A
F06	HEX	Unknown	39.04
G06	HEX	Unknown	31.88
H06	HEX	Unknown	35.02

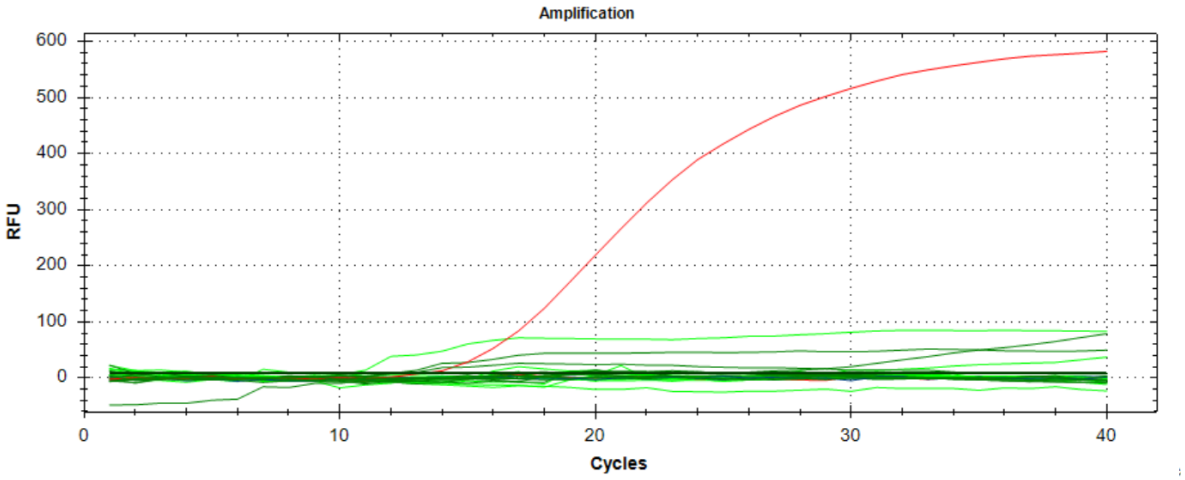


Figure B-12. Heart 1 -10, in RNA-later, washed five times in H-buffer. 12. May 2022.

Table B-11, Cq values for heart 1-10 stored in RNA-later, washed five times in H-buffer.

Well	**Fluor**	**Content**	**Cq**
A06	FAM	Neg Ctrl	N/A
A07	FAM	Unknown	N/A
B06	FAM	Unknown	N/A
B07	FAM	Unknown	N/A
C06	FAM	Unknown	N/A
C07	FAM	Unknown	N/A
D06	FAM	Unknown	N/A
D07	FAM	Pos Ctrl	13.34

E06	FAM	Unknown	N/A
F06	FAM	Unknown	N/A
G06	FAM	Unknown	23.17
H06	FAM	Unknown	18.86
A06	FAM	Neg Ctrl	N/A
A07	FAM	Unknown	29.27
B06	FAM	Unknown	N/A
B07	FAM	Unknown	9.93
C06	FAM	Unknown	10.40
C07	HEX	Unknown	29.88
D06	HEX	Unknown	15.77
D07	HEX	Pos Ctrl	N/A
E06	HEX	Unknown	N/A
F06	HEX	Unknown	2.76
G06	HEX	Unknown	18.86
H06	HEX	Unknown	18.86

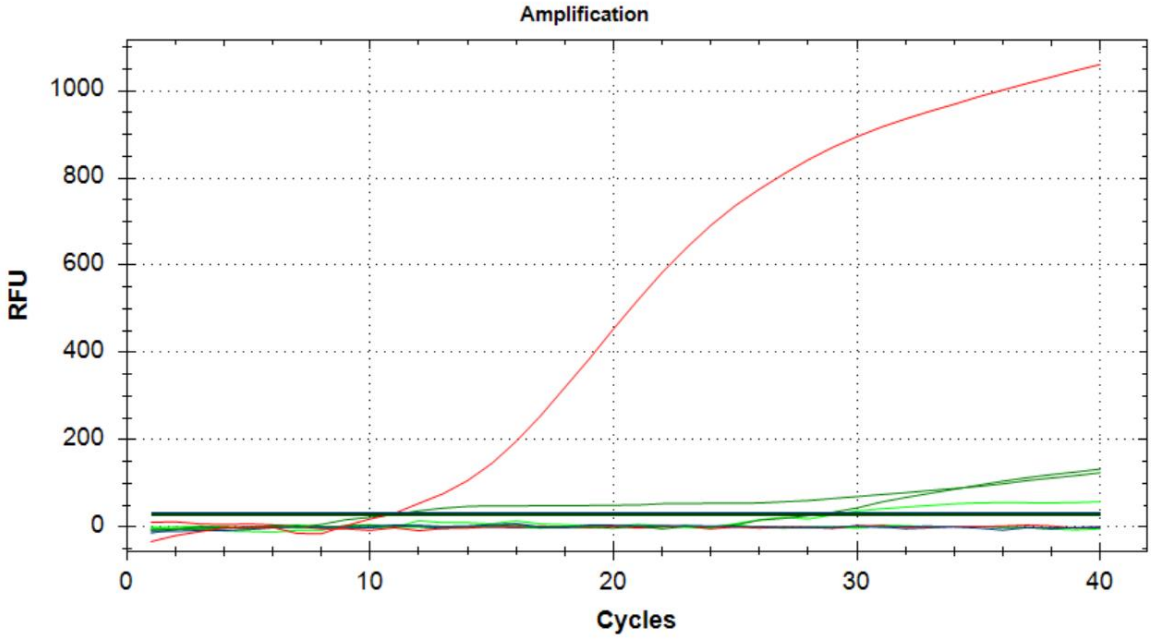


Figure B-13. Sample 17 washed three and five times. Taken after from the same sample as made before. 19. May 2022

Table B-12. Cq values for heart 17, stored in RNA-later. One of them were washed three times, and one was washed five times.

Well	**Fluor**	**Content**	**Cq**
A06	FAM	Neg Ctrl	N/A
A08	FAM	Unknown	28.67
B08	FAM	Pos Ctrl	10.98
H07	FAM	Unknown	11.31
A06	HEX	Neg Ctrl	N/A
A08	HEX	Unknown	28.84

B08	HEX	Pos Ctrl	N/A
H07	HEX	Unknown	N/A

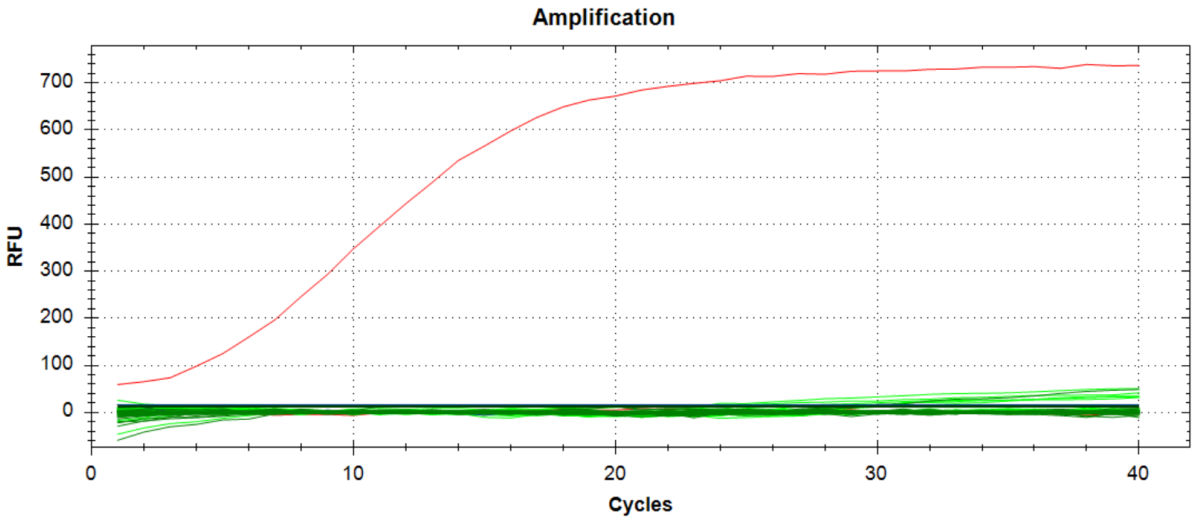


Figure B-14. RT-qPCR analyze of heart 1-10 in different conditions for SAV. Heart 1-10 in both RNA-later and H-buffer was analyzed after being homogenized with a Tissuelyser.

Table B-13. Cq values of heart 1-10 in three different conditions. H: frozen in H-buffer, R: frozen in RNA-later, RS: frozen in RNA-later and filtered.

Well	Fluor	Content	Cq
A06	FAM	Neg Ctrl	N/A
A07	FAM	8 H	N/A
A08	FAM	6 R	N/A
A09	FAM	4 RS	N/A
B06	FAM	1 H	N/A
B07	FAM	9 H	N/A
B08	FAM	7 R	N/A
B09	FAM	5 RS	N/A
C06	FAM	2 H	N/A
C07	FAM	10 H	N/A
C08	FAM	8 R	N/A
C09	FAM	6 RS	N/A
D06	FAM	3 H	N/A
D07	FAM	1 R	N/A
D08	FAM	9 R	N/A
D09	FAM	7 RS	N/A
E06	FAM	4 H	N/A
E07	FAM	2 R	N/A
E08	FAM	10 R	N/A
E09	FAM	8 RS	N/A
F06	FAM	5 H	N/A
F07	FAM	3 R	N/A
F08	FAM	1 RS	N/A
F09	FAM	9 RS	N/A
G06	FAM	6 H	N/A
G07	FAM	4 R	N/A

G08	FAM	2 RS	N/A
G09	FAM	10 RS	29.81
H06	FAM	7 H	N/A
H07	FAM	5 R	N/A
H08	FAM	3 RS	N/A
H09	FAM	Pos Ctrl	N/A
A06	HEX	Neg Ctrl	N/A
A07	HEX	8 H	N/A
A08	HEX	6 R	N/A
A09	HEX	4 RS	N/A
B06	HEX	1 H	N/A
B07	HEX	9 H	N/A
B08	HEX	7 R	N/A
B09	HEX	5 RS	N/A
C06	HEX	2 H	N/A
C07	HEX	10 H	26.15
C08	HEX	8 R	N/A
C09	HEX	6 RS	N/A
D06	HEX	3 H	N/A
D07	HEX	1 R	28.75
D08	HEX	9 R	N/A
D09	HEX	7 RS	N/A
E06	HEX	4 H	N/A
E07	HEX	2 R	N/A
E08	HEX	10 R	24.63
E09	HEX	8 RS	N/A
F06	HEX	5 H	N/A
F07	HEX	3 R	23.19
F08	HEX	1 RS	N/A
F09	HEX	9 RS	N/A
G06	HEX	6 H	N/A
G07	HEX	4 R	N/A
G08	HEX	2 RS	N/A
G09	HEX	10 RS	30.1
H06	HEX	7 H	N/A
H07	HEX	5 R	N/A
H08	HEX	3 RS	N/A
H09	HEX	Pos Ctrl	N/A

Fresh fish

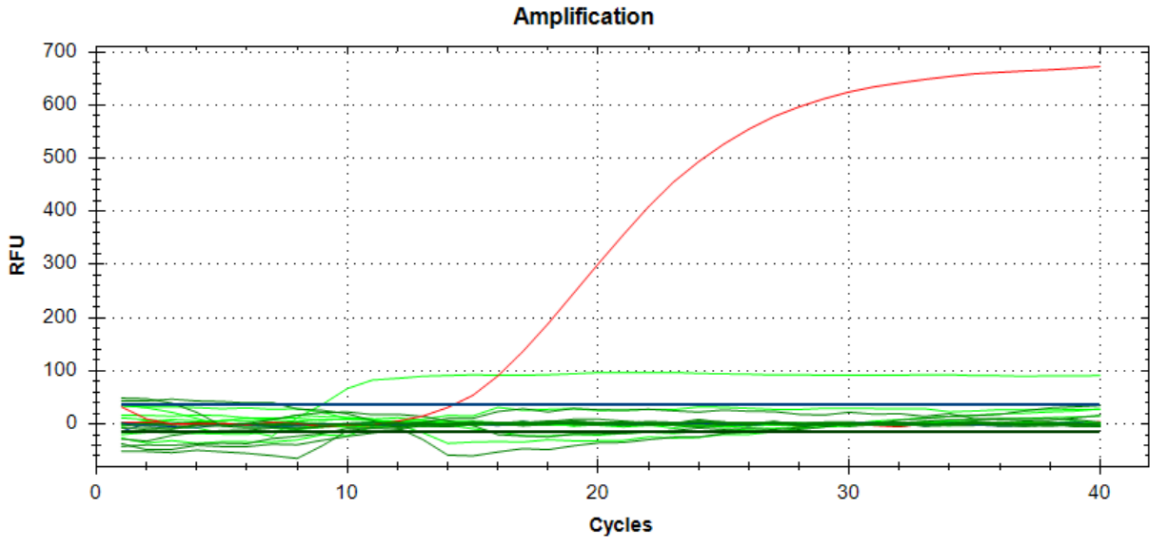


Figure B-15. RT-qPCR analyze of 2 healthy fish for SAV. Collected from a location in Rogaland. 16. May 2022.

Table B-14. Cq values for heart, brain, gills, feces and liver from 2 healthy fish,

Well	Fluor	Content	Cq
A06	FAM	Neg Ctrl	N/A
A07	FAM	Unknown	N/A
B06	FAM	Unknown	N/A
B07	FAM	Unknown	N/A
C06	FAM	Unknown	N/A
C07	FAM	Unknown	N/A
D06	FAM	Unknown	N/A
D07	FAM	Pos Ctrl	14.23
E06	FAM	Unknown	N/A
F06	FAM	Unknown	N/A
G06	FAM	Unknown	N/A
H06	FAM	Unknown	N/A
A06	HEX	Neg Ctrl	N/A
A07	HEX	Unknown	6.83
B06	HEX	Unknown	N/A
B07	HEX	Unknown	N/A
C06	HEX	Unknown	N/A
C07	HEX	Unknown	27.12
D06	HEX	Unknown	24.08
D07	HEX	Pos Ctrl	N/A
E06	HEX	Unknown	7.26
F06	HEX	Unknown	N/A
G06	HEX	Unknown	10.48
H06	HEX	Unknown	N/A

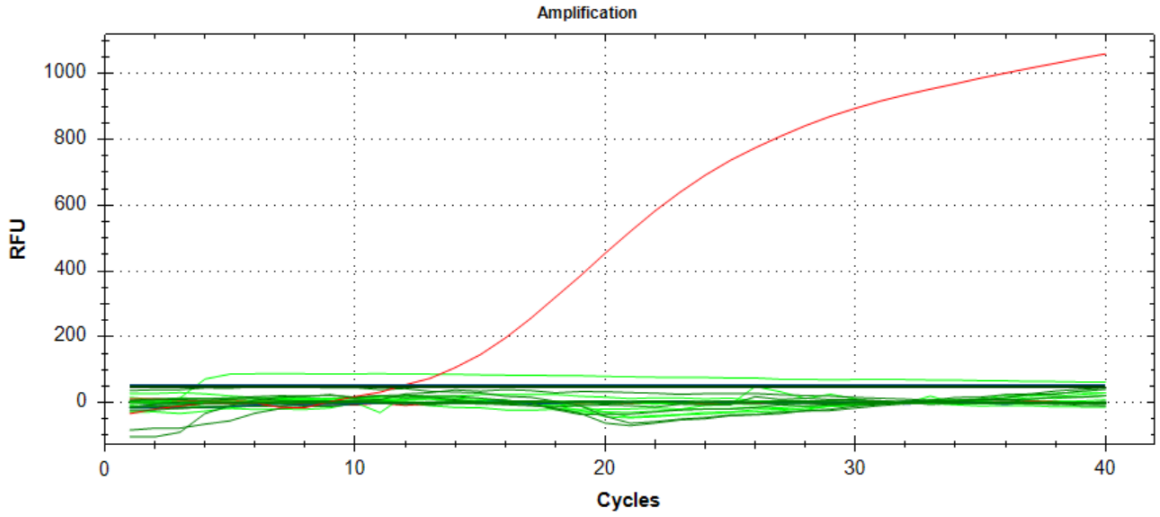


Figure B-16. RT-qPCR analyze of heart 1-10 in different conditions for SAV. From the same H-buffer as last time. 19. May 2022.

Table B-15. Cq values for heart 1-10 from H-buffer.

Well	Fluor	Content	Cq
A06	FAM	Neg Ctrl	N/A
A07	FAM	Unknown	N/A
B06	FAM	Unknown	N/A
B07	FAM	Unknown	N/A
B08	FAM	Pos Ctrl	11.84
C06	FAM	Unknown	N/A
C07	FAM	Unknown	N/A
D06	FAM	Pos Ctrl	N/A
D07	FAM	Unknown	N/A
E07	FAM	Unknown	N/A
F07	FAM	Unknown	N/A
G07	HEX	Neg Ctrl	N/A
A06	HEX	Unknown	N/A
A07	HEX	Unknown	N/A
B06	HEX	Unknown	N/A
B07	HEX	Unknown	N/A
B08	HEX	Pos Ctrl	N/A
C06	HEX	Unknown	N/A
C07	HEX	Unknown	N/A
D06	HEX	Unknown	N/A
D07	HEX	Unknown	3.61

E07	HEX	Unknown	N/A
F07	HEX	Unknown	N/A
G07	HEX	Unknown	N/A

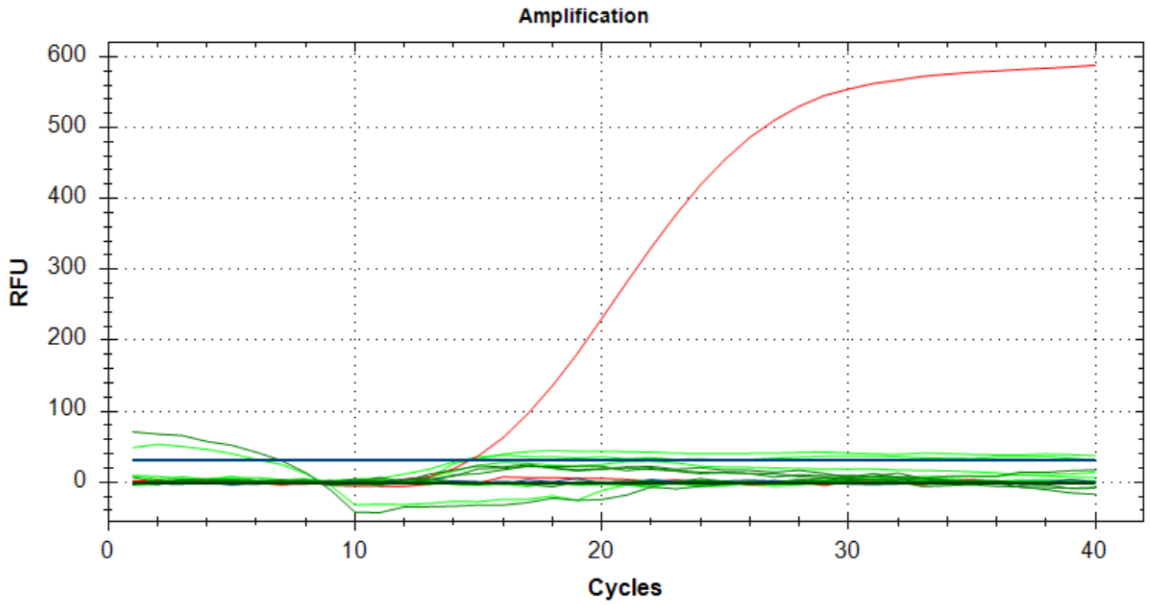


Figure B-17. Gills (1) and 16 (put in H-buffer and freezed) with sieve. 20. May 2022.

Table B-16. Cq values for gills and heart 16 stored in H-buffer, both filtered.

Well	**Fluor**	**Content**	**Cq**
A06	FAM	Neg Ctrl	N/A
B06	FAM	Unknown	N/A
C06	FAM	Unknown	N/A
D06	FAM	Unknown	N/A
E06	FAM	Unknown	N/A
F06	FAM	Unknown	N/A
G06	FAM	Unknown	N/A
H06	FAM	Pos Ctrl	14.63
A06	HEX	Neg Ctrl	N/A
B06	HEX	Unknown	12.85
C06	HEX	Unknown	10.46
D06	HEX	Unknown	12.78
E06	HEX	Unknown	4.09
F06	HEX	Unknown	32.73
G06	HEX	Unknown	31.6
H06	HEX	Pos Ctrl	15.74

Fish hearts, liver and positive control from the veterinary institute

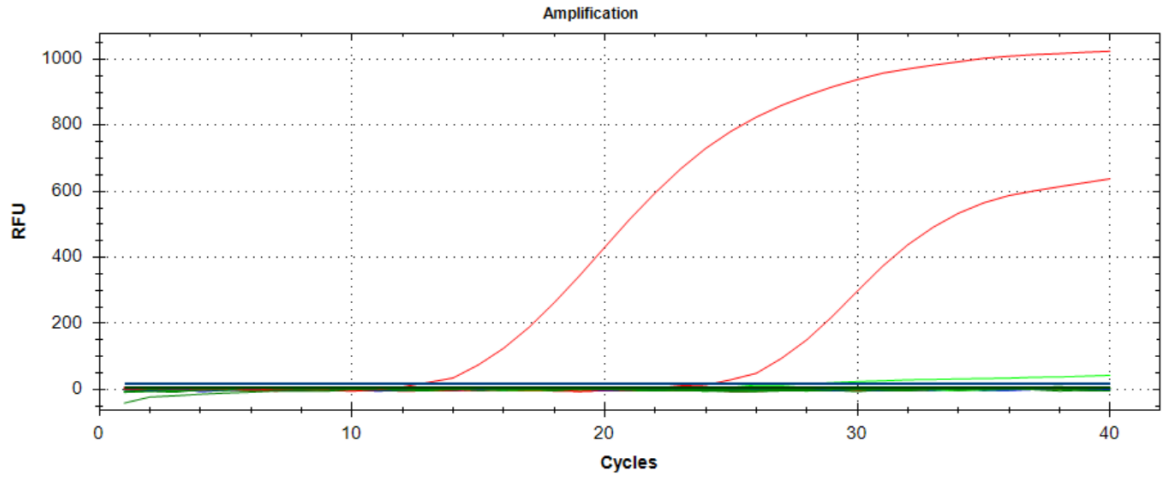


Figure B-18. RT-qPCR analysis for SAV in four salmon hearts, one liver and one positive control from the Veterinary Institute.

Table B-17. Cq vakyes for four salmon hearts, one liver and one positive control.

Well	**Fluor**	**Content**	**Cq**
A06	FAM	Neg Ctrl	N/A
B06	FAM	Unknown	N/A
C06	FAM	Unknown	N/A
D06	FAM	Unknown	N/A
E06	FAM	Unknown	N/A
F06	FAM	Unknown	N/A
G06	FAM	Pos Ctrl	12.64
H06	FAM	Pos Ctrl	24.19
A06	HEX	Neg Ctrl	N/A
B06	HEX	Unknown	N/A
C06	HEX	Unknown	N/A
D06	HEX	Unknown	N/A
E06	HEX	Unknown	25.09
F06	HEX	Unknown	N/A
G06	HEX	Pos Ctrl	15.86
H06	HEX	Pos Ctrl	N/A