Measuring Gene Expression of MORN, SANT, and Sig 1, “ ” 4, in *Paramecium caudatum* Over the Course of *Holospora* Infection

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Abstract

Paramecia are single-celled organisms that live in ponds and feed on other single celled organisms like bacteria or algae. They are typically oblong and are covered with short structures called cilia. They have interactions with bacteria as they are infected intranuclearly by *Holospora*. *Paramecium caudatum* is known to express genes such as MORN, SANT, and Sig 1, **“ ”**, 4. *Paramecium* shows a response upon infection by *Holospora* as it navigates through the various stages of infection. The *Holospora* bacteria may cause varying degrees of expression of genes in the single celled organism. The genes were chosen due to their upregulation in *Paramecium* in a previous RNA sequencing study (Kagemann et al, in prep). Through the use of microscopy, imaging and qPCR, gene expression was tracked in the control and in the samples in which MORN, SANT, and Sig 1, **“ ”**, 4 genes were knocked down. The amount of gene expression was measured in each stage of the infection using qPCR. A comparison was made between the results of the control samples, which have no knocked down genes, to the samples which have knocked down genes. The amplitude of expression between Sig 1, Sig 2, Sig 3, and Sig 4 was compared as they function as protein kinases in *Paramecium caudatum.*

Key Words: Cell and Molecular Biology, *Paramecium, Holospora*, MORN, SANT, Sig 1, Sig 2, Sig 3, Sig 4, qPCR, Microscopy and Imaging

Introduction

The family Parameciidae Dujardin is characterized by the members of the genus *Paramecium* (Wichterman, 1986). They are ciliates, which are morphologically complex protozoans. Ciliates are single-celled organisms that possess cilia, short hair like organelles used for food gathering and locomotion (Britannica). *Paramecium* have dorsal and ventral surfaces and a distinct oval groove on the ventral surface. The body and the oral groove are covered in cilia (Wichterman, p. 3). *Paramecium* are free swimming, ovoid, elongated or cigar shaped, and although they are microscopic, they are visible to the naked eye. Their swimming patterns are generally vigorous and rotatory; most spiral to the left while free swimming (Wichterman, p. 6).

Paramecium spp. are easily maintained and cultured during the course of an experiment (MicroBus, 2020). The most common species used in an experimental setting is *Paramecium caudatum.* The species is most commonly characterized by a large macronucleus and a single compact micronucleus (MicroBus, 2020). Both types of nuclei contain the full complement of genes that bear the hereditary information of *Paramecium* spp. (Britannica). The organism cannot survive without the macronucleus and it cannot reproduce without the micronucleus (Britannica).

The protist *Paramecium* spp. are useful as a model organism due to its regular construction and the synchronomy of some processes (Plattner, p. 649). *Paramecium* spp. is also useful as a model organism for experimental investigations into associations with endosymbiotic bacteria (Fujishima, 2009; Görtz and Fokin, 2009; Fujishima and Kodama, 2012). Some protozoans such as *Paramecium* are bacterivorous by nature which results in the possession of endosymbiotic partners. Some of *Paramecium's* prey have evolved ways in which to avoid

digestion by taking up residence in the cytoplasm of the cell, and also in the host nuclei (Preer et al., 1974; Fujishima and Kodama, 2012).

The gram negative bacteria *Holospora* spp. are endonuclear symbionts of *Paramecium* spp. (Fujishima et al., 2011). The bacteria were discovered in the 19th century (Hafkine, 1890), and was extensively researched on its morphology, taxonomic relationships, and infection life cycle (Fokin and Görtz, 2009). Each species of *Holospora* typically infects one single host species, but temporary infection of "non-host" species has been detected (Fujishima et al., 2012; Fokin and Görtz, 2009). The interaction between *Holopsora* and its host seems to be obligate for the bacterium, as the life cycle of the bacterium inside of the host cells, and the comparative analysis of several *Holospora* genomes shows that most amino acid pathways are absent (Garushyants et al., 2018). This means that replication of the bacteria is only possible inside of the host cell.

During host cell division, reproductive forms of the bacteria can be vertically transmitted from the mother to daughter nuclei. After horizontal transmission occurs, the host cells ingest infectious forms while feeding; infectious forms get released during cell division or host death (Fujishima, 2009; Magalon et al., 2010). Inside of the host cells, *Holospora* show parasite features as they disrupt sexual processes and decrease cell survival, motility, and asexual reproduction (Görtz and Fujishima, 1983; Fokin, 1998).

After ingestion, *Holospora* escapes the digestive vacuole and migrates through the cytoplasm toward the nucleus (Fujishima and Fujita 1985; Gortz and Wiemann, 2011). During vegitative growth of *Paramecium, Holospora* forms reproductive short cells (1.5–2 lm in length), which divide by binary fission. When the host cells begin to starve, *Holospora* reproductive forms differentiate into the infectious long form (13–15 lm in length) (Dohra and Fujishima,

1999; Fujishima et al., 1991). During differentiation, the bacterial cell changes in structure (Iwatani, et al., 2005). The infection of *Paramecium* occurs in several successive steps (Fujishima, 2009). *Holospora,* which are from the order *Rickettsiales,* inhabit either the macronucleus or micronucleus of *Paramecium c.* (Garushyants et al., 2018). The nucleus is then filled with many infectious forms which hinders the ability of the host cells to go through meiosis (Fokin 2009); they are eventually killed by the outburst of the infectious forms (Fujishima, 2009). The released infectious forms present outside of the host cell can infect new host cells. The process of infection of the target nucleus is known as "infection" and the stable multiplication of the infected bacteria is known as "maintenance" (Fujishima and Fujita 1985; Fujishima, 2009).

The genes which were chosen for this experiment were Sig 1-4, SANT, and MORN. These genes were chosen because they are known to be upregulated in *Paramecium* (Kagemann, et al, in prep.). Sig 1-4 are kinases which are genes that encode putative serine-threonine kinase proteins (Hanks and Hunter, 1995). Serine-threonine kinase proteins can regulate cell growth, programmed cell death, cell differentiation and development (Bemm, et al., 2009). Sig 1-4 may therefore be involved in signaling pathways in *Paramecium* cells as well. Cells have proteins called receptors which bind to signaling molecules and initiate a physiological response. Different receptors are specific for different molecules. Kinomes have an extensive presence in ciliates (Lemmon and Schlessinger, 2010).

The gene SANT encodes for the putative Myb transcriptional regulator (Klempnauer and Sippel, 1987). Transcriptional regulators are proteins that control the rate of transcription of genetic information from DNA to messenger RNA, by binding to a specific DNA sequence. They help turn specific genes "on" or "off" by binding to nearby DNA (Klempnauer and Sippel,

1987). SANT proteins are common in stress related responses or changes in an intracellular environment. Many Myb-like proteins are shown to bind DNA using the helix-turn-helix motif. The helix-turn-helix (HTH) is a major structural motif capable of binding DNA (Baranowski, et al., 1994). The gene MORN encodes for a putative peptide with six membrane occupational and recognition (MORN) repeats (Li, et al., 2019). It contains two sets of C-terminal repeats and an N-terminal coiled coil domain (Wilson, et al., 2002). MORN is involved in protein-protein interactions and is also known to be a stress response signaling protein (Gubbels, et al., 2006). The infection by *Holospora* may cause varying degrees of expression of these genes.

There has not been much research done on tracking gene expression over the course of infection in *Paramecium*. The purpose of this study was to track the expression of the genes MORN, SANT, and Sig 1, **“ ”**, 4 at different time points of infection. By studying gene expression over the course of infection, it can be determined whether the genes were upregulated or downregulated at various time points. The analysis of the expression at different time points was a way to determine *Paramecium* response to *Holospora.* Having significantly differentially expressed genes in the experiment would mean that those genes were more likely to be conserved in the genome (Kagemann, 2021, in prep).

Methods

Infection

The *Paramecium* cells which were used for infectious forms were highly infected due to the 6-8 day starvation period which the cells endured. 14 mls of cells were pipetted into two 15 mL tubes which were then centrifuged. The supernatant was removed to the 1 mL line from each tube. The pellet which was formed was resuspended by pipetting up and down in the tube.

Glass beads of the size 0.5 mm were added to bead beater tubes until they reached right below the middle line of the tubes. The concentrated cultures were added to the two bead beater tubes and each tube was beat for 1.5 min in a bead beater machine. A 200 µL pipette was used to remove as much of the liquid as possible from the bead beater tubes. The liquid was transferred to 15 mL tubes which were checked for cells that were still alive. If live cells were present, the liquid was pipetted into three well dishes so that the living cells could be removed or it was pipetted into bead beater tubes which were run for another 1.5 min. Once live cells were no longer present, the lysate was imaged and IFs counted using a hemocytometer.

RNA Extraction (2/8/21)

RNA was extracted from *Paramecium* cells using Trizol. One milliliter of concentrated *Paramecium* cells were collected and spun down at max speed for 3 minutes at 4 degrees Celsius. The supernatant was removed and cells were resuspended in 1mL of Trizol. The cells were pipetted up and down to be lysed. After vortexing for 15 s, the cells were incubated at room temperature for 5 min. Two hundred µl of CIA (Chloroform:Isoamyl alcohol, 24:1) were added to the sample and vortexed for 15 sec. The sample was incubated for 5 min at room temperature and spun down for 15 min at 4 degrees Celsius at max speed. The aqueous (top) layer was moved to a new tube while it was noted to not aspirate the DNA-containing white interface. One ml of isopropanol and 120 µl of 3M sodium acetate (final concentration of 0.3M) were added. The sample was incubated for 10 min at room temperature and later spun down for 15 min at 4 degrees Celsius at max speed. After the spin, the supernatant was removed.

The pellet was washed with 70% ethanol and inverted. The wash was repeated one time

and so was the inversion. The sample was spun down for 10 min at 4 degrees Celsius. The supernatant was removed and the sample was air dried for about 10 min. Leaving the sample to dry for too long would cause the pellet to overdry and not be able to be redissolved. The pellet was resuspended in 50 µL of water and 1.25 µL of Ribolock. The RNA samples were treated with DNase using the Ambion turbo kit. Five µL of 10X DNase buffer and 1 µL of DNase were added to the sample. The sample was incubated at 37 degrees Celsius for 20-30 min.

The DNase was inactivated by resuspending the DNase inactivation agent by flicking the tube. Five µL of 0.1X inactivation reagent were added to the sample which was incubated for 5 min at room temperature and flicked 2-3 times during the incubation. The sample was centrifuged at 10,000 xg for 1.5 min and transferred to a new tube. To check RNA quality and quantity, the sample was nano dropped.

RNA Extraction (2/24/21) - Filter Tube

This was conducted using the same protocol as previous extraction up to the addition of 200 µL of CIA and a 15 second vortex. The aqueous top layer was added to a new tube; solution added to a RNeasy Mini spin column instead of a microcentrifuge tube.. Five hundred µL of isopropanol and 50 µL of 3M sodium acetate (final concentration of 0.3M) were added to the liquid at the bottom of the filter tube. The solution was pipetted up and down and let sit for 5 min. The solution was added back to the collection tube and left to sit for 5 min.

700 µL of Buffer RW1 were added to the RNeasy Mini spin column. The sample was centrifuged for 15 sec at 8,000 xq and the flow through was discarded. 500 µL of Buffer RPE were added to the spin column and the sample was centrifuged for 15 sec at 8,000 xq and the flow through was discarded. 500 µL of Buffer RPE were added to the spin column and the

sample was centrifuged for two minutes at 8,000 xq. The RNeasy spin column was placed into a new collection tube and 30 µL of RNase-free water was added to the spin column membrane. The tube was centrifuged for 1 minute at 8,000 xq to elute the RNA.

After the tube was centrifuged, 0.75 µL of Ribolock were added to the tube. The sample went through a DNase treatment by adding 3 µL of 10X DNase buffer and 0.6 µL of DNase. The sample was incubated at 37 degrees Celsius for 20-30 minutes. The same steps were followed for the sample as were in the previous extraction.

RNA Extraction (3/8/21) - Syringe

The same protocol was followed for the extraction as was used in the first extraction. However, after the cells were resuspended in Trizol, the solution was pipetted and then vortexed for 15 sec. A syringe needle was used to break up the cells even further. The protocol continued to follow the same steps, but some changes were made. The first extraction required an addition of 1 mL of isopropanol, but the following extractions used 500 µL of isopropanol. Also, instead of adding 50 µL of 3M sodium acetate, 70 µL were added.

There was a change in the amount of Ribolock added to the sample while resuspending the pellet. The sample had 0.5 µL of Ribolock and 20 µL of water added to the tube. The DNase treatment also changed as 2 µL of 10X DNase buffer and 0.4 µL of DNase were added to the sample. The rest of the protocol was followed as it was in the previous extractions.

Conversion of RNA to cDNA

The cDNA was made from the extracted RNA from the time course samples. The samples which were used had the highest RNA concentrations when they were nano dropped. Three samples were chosen from each time point and transformed into cDNA. Each of the samples was diluted in a mix. There were two mixes per sample as one had RT and the other mix had no RT. Mixes were made in 0.2 µl PCR tubes and to each tube 4 µL of mix, 11 µL of water, and 5 µL of RNA were added. The RNA which was added to each mix, corresponded with the time course sample being used. The tubes were placed into a BIO-RAD MJ MiniTM Personal Thermal Cycler for a 40 min run. The run consisted of a 5 min portion at 25 degrees Celsius, a 30 min portion at 42 degrees Celsius, and a final 5 min portion at 85 degrees Celsius. The samples remained at 4 degrees Celsius until they were used.

Determining Primer Efficiency - qPCR

Each well had 5 µL of iTAq mix, 0.5 µL of the forward primer, 0.5 µL of the reverse primer, and 3 µL of water. Individual wells in the plate contained 9 µL of the master mix and 1 µL of cDNA. The genes were pipetted horizontally across the plate and each gene occupied one row. The cDNA which was added to each well had various dilutions and three columns were used per dilution. The first three columns contained dilution A which had 30 µLof cDNA. Three µL were taken out and added to 27 µL of water; this became dilution B. Dilution C had 3 µL of sample from B along with 27 µL of water. Dilution D had 3 µL of sample from C along with 27 µL of water. The samples were vortexed for 15s and added to the wells. Once the process was complete, the plate was run on a qPCR machine. The run consisted of 6 steps which varied in time and temperature. Step 1 was 5 min long at 95 degrees Celsius, step 2 was 5 s long at 95

degrees Celsius, step 3 was 30 minutes long at 60 degrees Celsius, step 4 was the 39 time repetition of steps 2 and 3, step 5 was 10 min long at 95 degrees Celsius, and step 6 was was 0.05 s long followed by a 5 sec section at 95 degrees Celsius. Results were produced and analyzed.

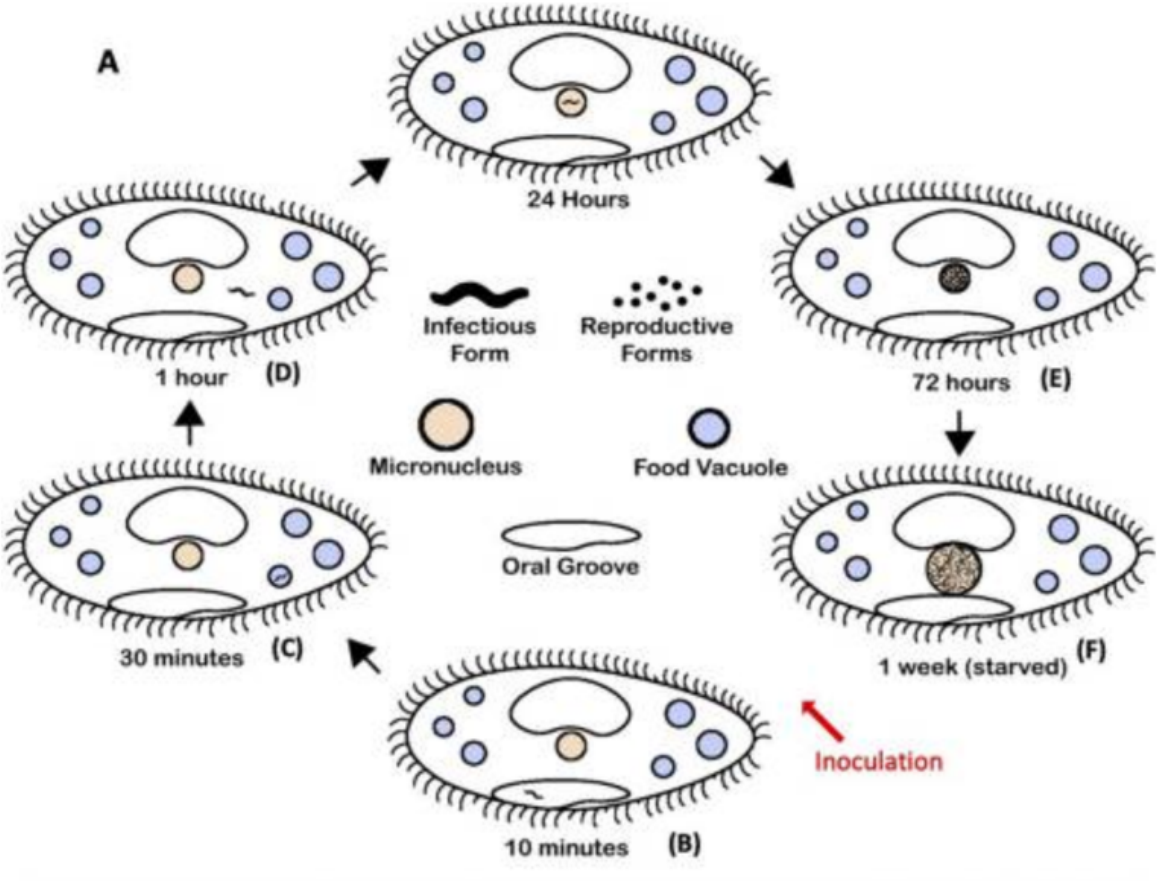
Time Course Gene Expression - qPCR

Three samples were chosen from the RNA extractions for the 0 hour, 24 hour, 7 day, and 14 day time points. Three samples were chosen for each time point based on their ng/µL values. The cDNA was diluted in a 1:5 dilution with water; 10 µL of cDNA and 40 µL of water were combined. The dilutions were conducted for the RT and No RT samples. A mix was made which included 3µL of water, 0.5 µL of the reverse primer, 0.5 µL of the forward primer, and 5 µL of iTaq Universal SYBR Green Supermix. The values were multiplied by the number of wells present to make a larger amount of the mix. The mix was made for each gene which was being loaded onto the plate; each time course qPCR plate contained one gene of interest and a housekeeping gene Tubulin. The No RT mix consisted of 5 µL of iTaq Universal SYBR Green Supermix, 1 µL of No RT cDNA, 1 µL of the forward primer, 1 µL of the reverse primer, and 2 µL of water.

To load the plate, 9 µL of the mix and 1µL of cDNA were added to each well in the rows A-F. The 0 hour samples were loaded into columns 1-3, the 24 hour samples into 4-6, the 7 day samples into 7-9, and the 14 day samples into 10-12. Rows A and B, C and D, and E and F contained cDNA from three different extractions. The wells with No RT were loaded individually with the row G containing the housekeeping gene and row H containing the gene of interest. The gene of interest was different with every plate that was run on a qPCR machine.

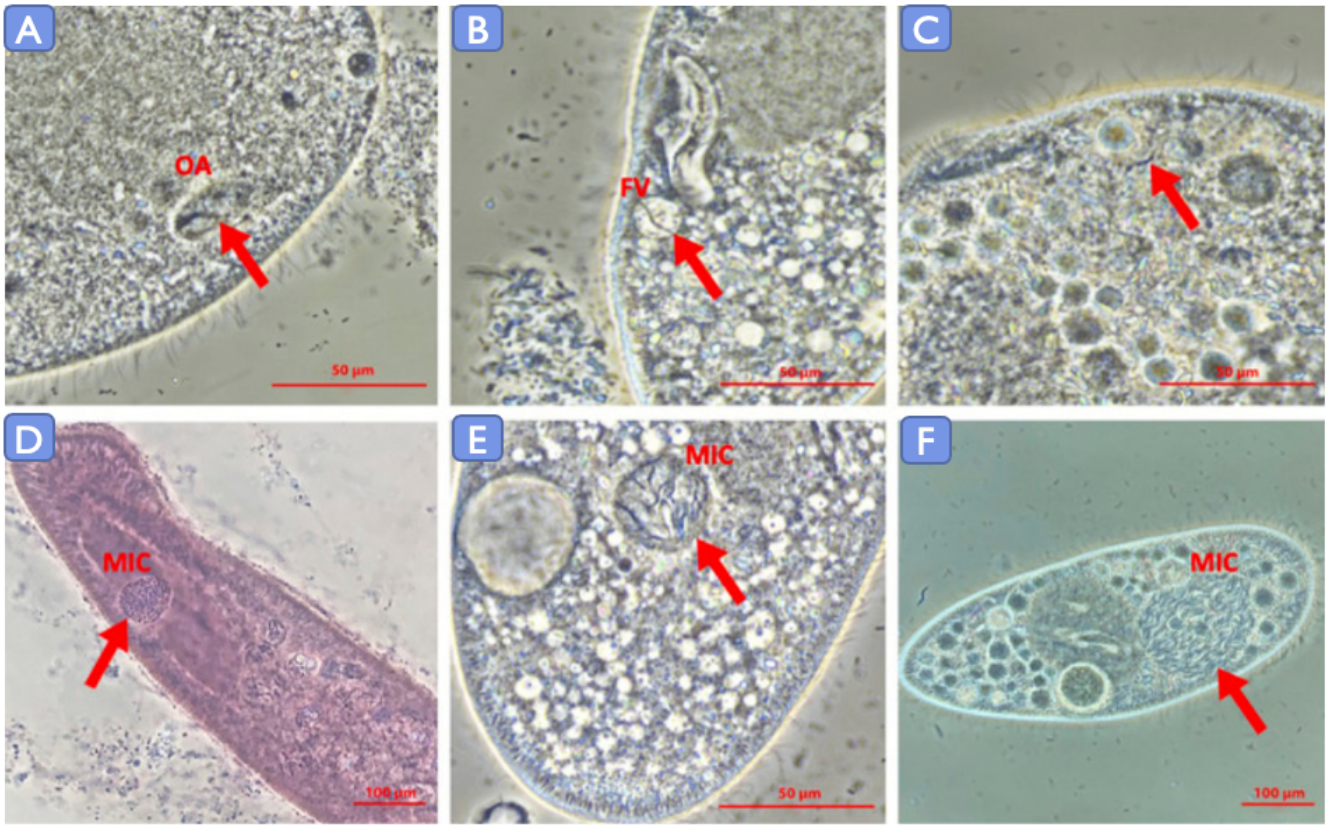
The results from the qPCR machine were analyzed through the Ct values. The average was taken for the three technical replicates of Tubulin and the gene of choice for each time point in the time course. The averages were conducted by sample tube. The ΔCTC was determined by subtracting the Average Ct value for the Housekeeping gene from the Average Ct value for the test gene. The ΔΔCt was determined by subtracting the control, which was the 0 Hour time point, from the experimental values. The fold change values were produced by 2^-ΔΔCt. The values from each sample tube, per each time point, were averaged to get the average fold change of each gene over the course of infection.

Results



(Weiler, 2021)

Figure 1. Holospora Life Cycle. A system of diverse interactions that occurs between *Paramecium* and *Holospora* both at the stage of infection and during infection maintenance. *Holospora* form reproductive short cells during vegitative growth of *Paramecium*. The short cells differentiate into infectious long forms and infect host cells. Some of the time points present differ from the experiment. The time points used for this experiment were 0 Hours, 24 Hours, 7 Days and 14 Days. Only the 24 Hour and 7 Day time points are present on the diagram.



(Weiler, 2021)

Figure 2. Images from different time points in infection of *Paramecium caudatum* by *Holospora undulata*. (A) IF within the oral groove at 10 min. (B) IF in a food vacuole at 30 min. (C) IF in the cytoplasm at 1h. (D) RFs in the micronucleus at 3 days. (E and F) IFs and RFs in the micronucleus at 7 day.

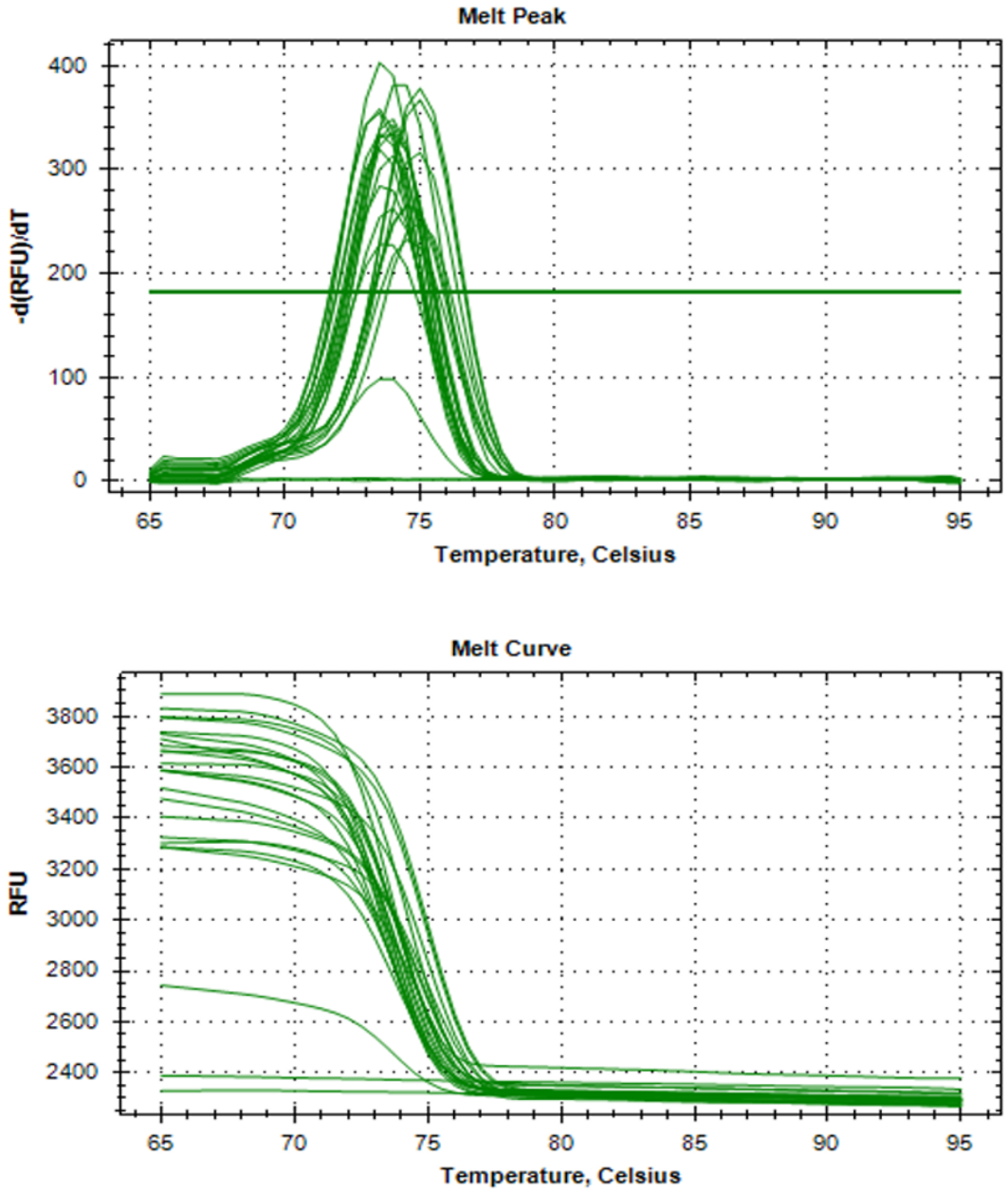


Figure 3. Assay Validation through qPCR Melt Curve Analysis. Testing specificity of primers of the chosen genes Sig 1-4, SANT, and MORN. Four technical replicates present for each gene. Single peaks represent a pure, single amplicon while multiple peaks are interpreted as separate amplicons. Fluorescence which is associated with amplicons was measured.

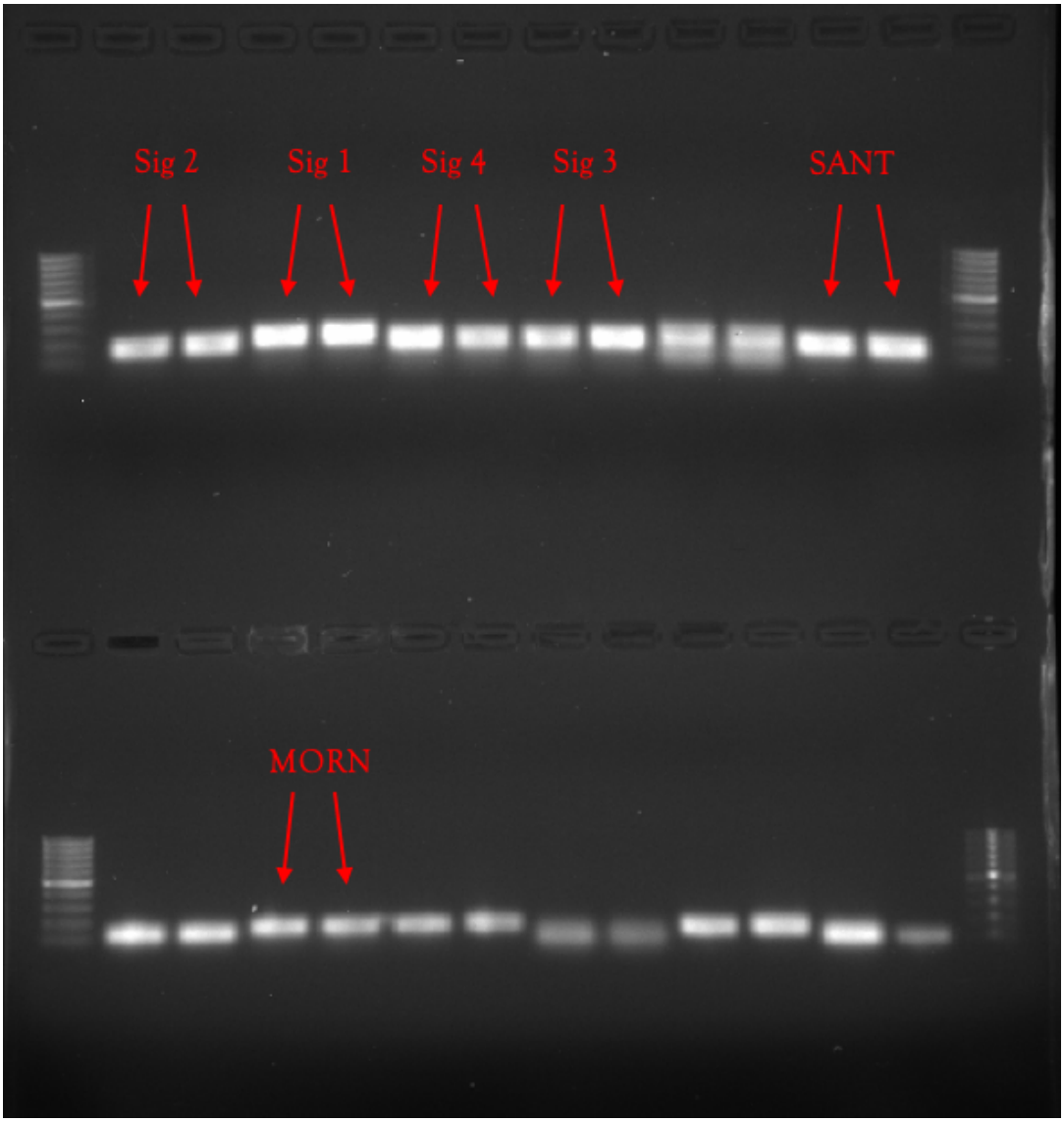


Figure 4. Assay Validation through Gel Electrophoresis. Primer specificity results of qPCR checked by running a gel to determine if bands were present for each primer. Gel was run to ensure that the samples from the qPCR and the samples from the gel shared the same characteristics when the same product was generated. Gel electrophoresis identified the number and size of the PCR products.

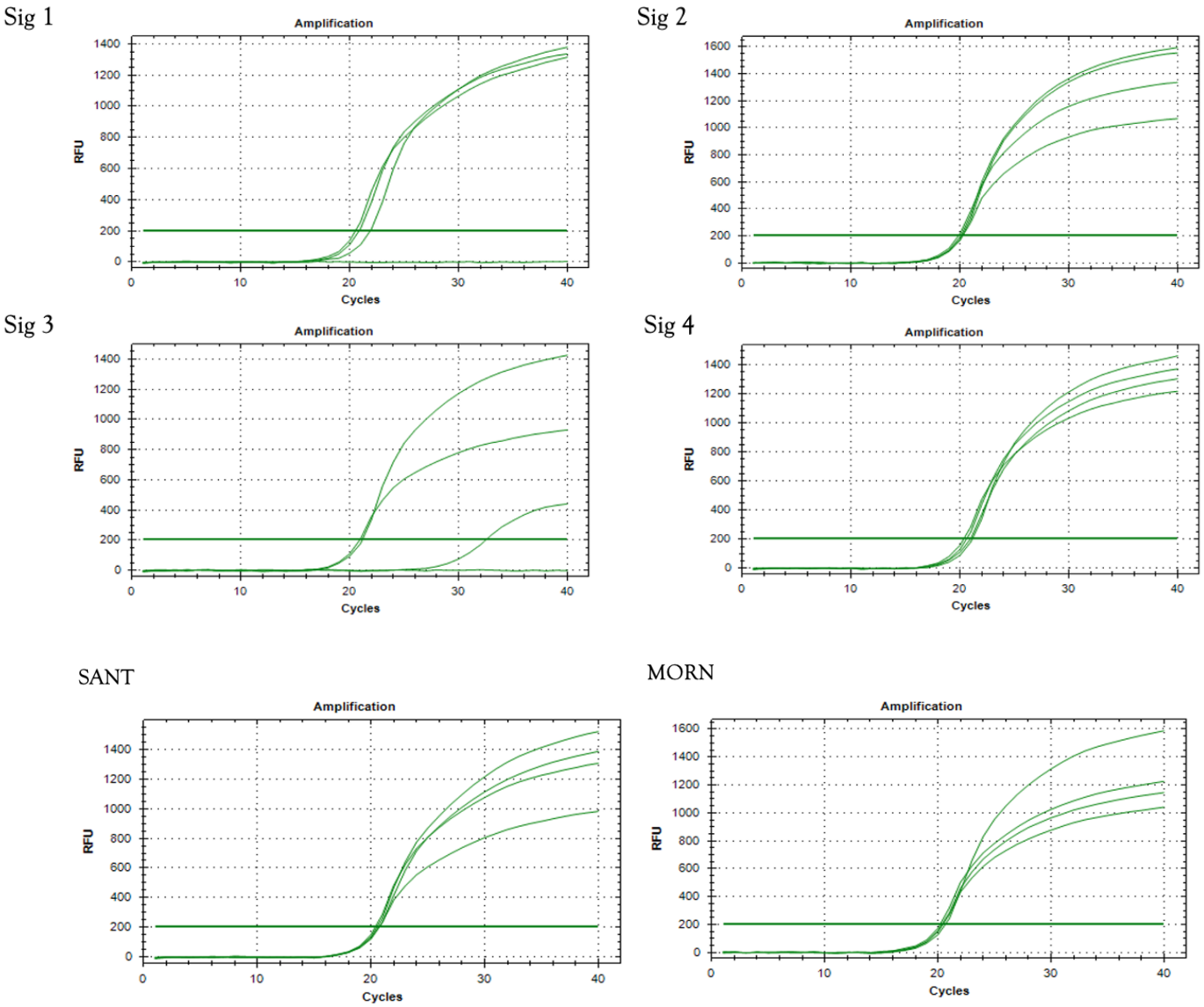


Figure 5. Determining Efficiency of Primers through qPCR Amplification Curves. Tests conducted to determine the efficiency of the primers of the chosen genes. Cq values indicated at which PCR cycle a certain amount of PCR product was reached. The Amplification curves showed how efficient a primer would be at producing a product.

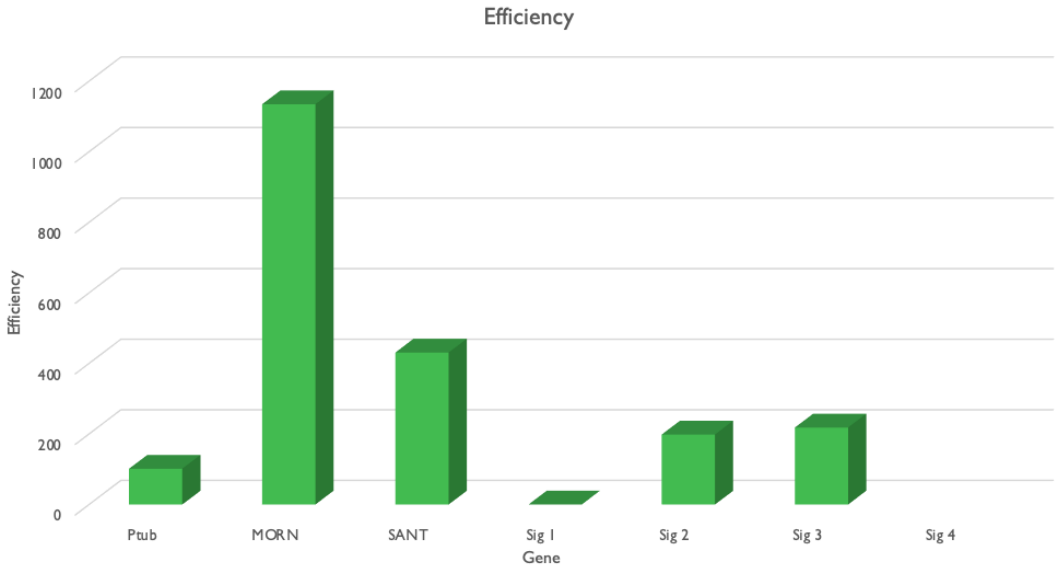


Figure 6. Primer Efficiency in Comparison to the Housekeeping Gene Tubulin. Efficiency of the primers analyzed in comparison to the housekeeping gene Tubulin. The efficiency of Tubulin was 101.4%. Desired range of efficiency for primers was in the 90%-110% range.

A

Sample	ng/µL
0 Hr #2	41.7
0 Hr #4	38
0 Hr #5	25.6
24 Hr #2	41.3
24 Hr #4	31.7
24 Hr #6	73.5
7 Day #4	38.2
7 Day #5	76.4
7 Day #6	46.3
14 Day #1	43.7
14 Day #3	53.8
14 Day #6	42.9

B

Sample	ng/µL
0 Hr #2	41.7
0 Hr #4	38
0 Hr #5	19.9
24 Hr #2	41.3
24 Hr #4	31.7
24 Hr #6	73.5
7 Day #4	28.2
7 Day #5	38.2
7 Day #6	46.3
14 Day #1	16.7
14 Day #3	32.8
14 Day #6	42.9

Figure 7. Nanodrop RNA Values for Time Course. A. Samples for Sig 3. B. Samples for Sig 1, 2, and 4, MORN, and SANT. Quantity and quality of the RNA checked by being nano dropped.

Table 1. Samples Chosen for the Experiment. RNA samples collected at various time points. The samples represent the 12 biological replicates chosen for the experiment.

Sample Group	0 Hour	24 Hour	7 Day	14 Day
1	#2 (2/8/21)	#2 (2/8/21)	#2 (2/8/21)	#2 (2/8/21)
2	#4 (2/24/21)	#4 (2/24/21)	#4 (2/24/21)	#4 (2/24/21)
3	#6 (3/8/21)	#6 (3/8/21)	#6 (3/8/21)	#6 (3/8/21)

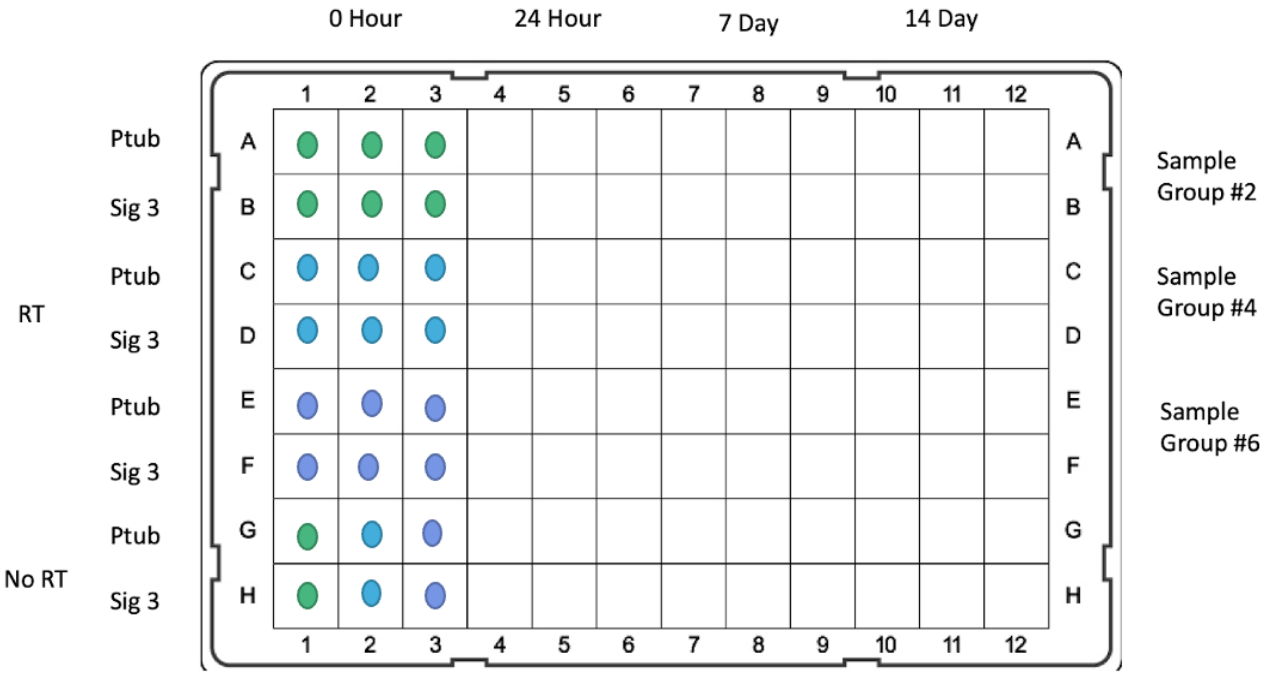
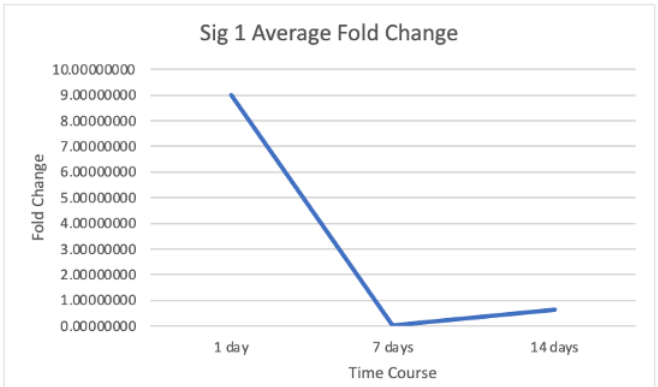
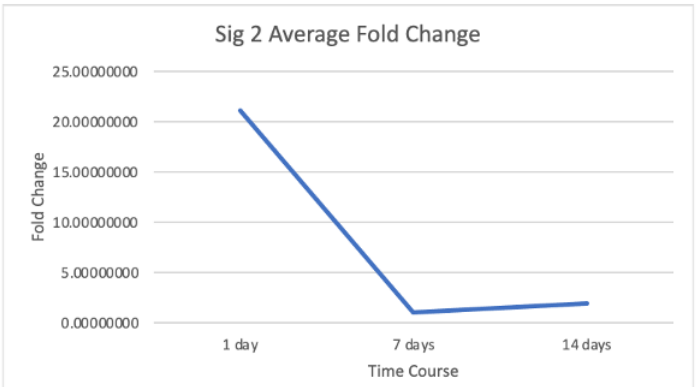
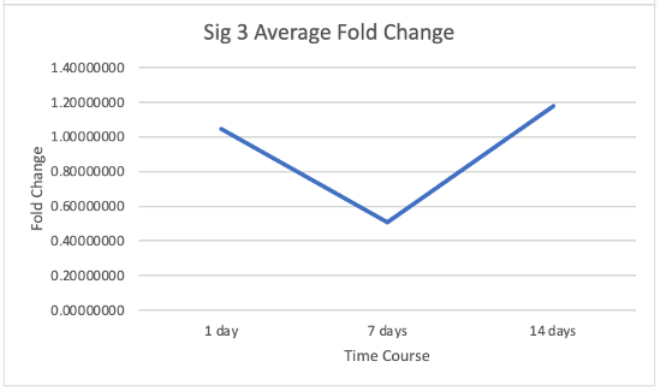
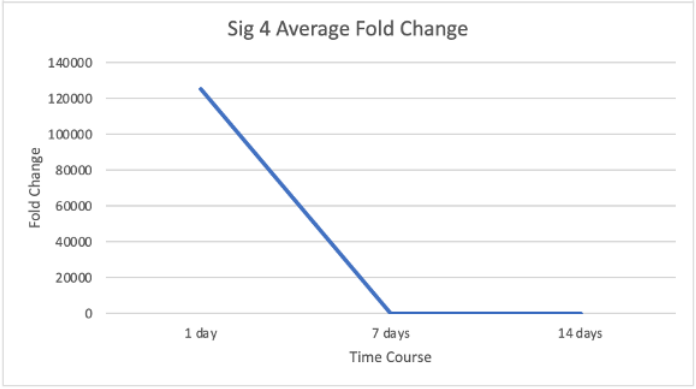


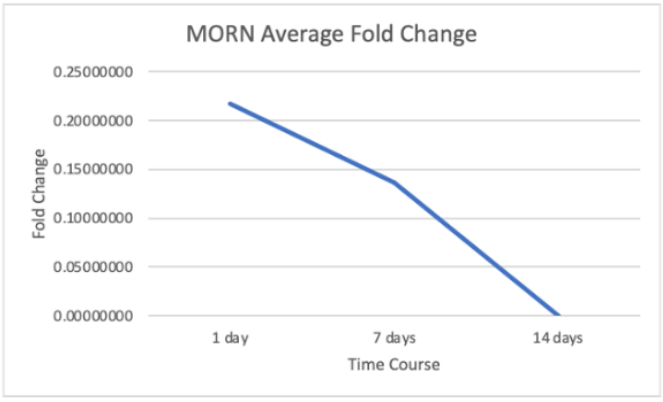
Figure 8. qPCR Plate for the Time Course Experiments. The plates were set up with the housekeeping gene Tubulin and a gene of interest alternating from row to row. The cDNA which was used in the rows A-F contained RT while rows G and H had no RT. RT is a reverse transcriptase which is an enzyme used to generate complementary DNA (cDNA) from an RNA template. Each time point spanned across three columns of the plate. The sample groups which were previously chosen occupied two columns each. The no RT rows contained all the replicates from each time point in a chronological order.











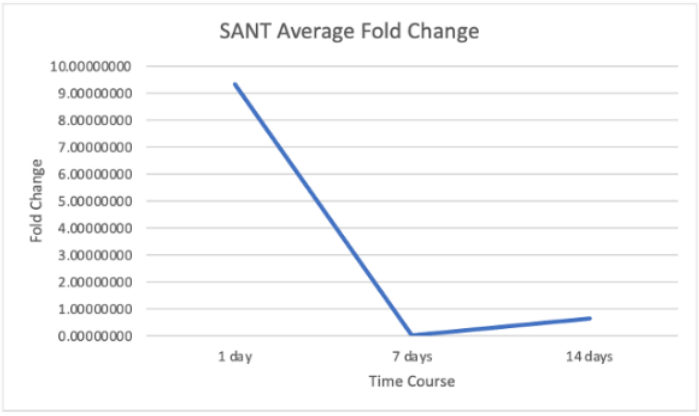


Figure 9. Time Course Gene Expression through Average Fold Change. The measure of how much the expression of the chosen genes changed between the 0 Hour, 24 Hour, 7 Day, and 14 Day time points. The 0 Hour time point is not presented on the graphs as it has 0 average fold change.

Results and Discussion

Imaging

The genes SANT, MORN, and Sig 1-4 were upregulated in *Paramecium.* Measuring their expression over the course of infection by *Holospora* provides insight on what timepoints the genes are upregulated or downregulated. In order to conduct the experiment, the cells were infected and then imaged at various time points in infection. The cells (Figure 2) were imaged at 10 minutes, 30 minutes, 1 Hour, 3 Days, and 7 Days. The cells which were used in the experiment had time points which slightly differed. The time points used were 0 Hour, 24 Hour, 7 Days, and 14 Days. Imagining cells ensured that cells were infected at various time points.

RNA Samples and Values

RNA samples were collected at various time points. To check for the quantity and quality of the RNA which was extracted from the samples, the samples were nanodropped (Figure 7). Initially, the samples with the highest concentrations were chosen to be turned into cDNA which was used for the time course qPCR of the Sig 3 gene. The samples which were chosen later were from the same sample group. The same sample groups were used to get results which would be from the same tube. Sample group 1 represented the #2 tubes, sample group 2 represented the #4 tubes, and sample group 3 represented the #6 tubes (Table 1). Three samples from each time point were turned into cDNA. These samples were the 12 biological replicates which were chosen for the time course experiment. Biological replicates are defined as measurements of biologically distinct samples that show variation. The different samples gave different results even though they were from the same time points. The samples for Sig 3 were

slightly different as they were chosen before a decision was made to have the same sample tube for each sample group.

qPCR - Primer Specificity

Once cells were confirmed to be infected, qPCR was used to measure the starting concentration of a specific DNA fragment by monitoring the increase of fluorescence during the cyclic amplification of a fragment. It was used to determine how much genetic material was present at the beginning of the experiment. The qPCR machine tracked the amplification of the products in real time. qPCR tests were conducted to test the specificity of the primers of the genes which were chosen. Each gene had four technical replicates which were loaded into the qPCR plate and run on the qPCR machine. Melt curves were used to assess whether a single, specific product was produced (Figure 3). A single peak is known to represent a pure, single amplicon while multiple peaks are interpreted as separate amplicons. The peaks for each gene were analyzed to determine if all of the peaks fell under the main one or if some fell outside of the main peak. Having multiple peaks indicated that the product which was desired was not being produced. Having a different product may suggest contamination of the sample. The machine measured fluorescence which was associated with the amplicon. The dyes used in qPCR fluoresce when they are bound to double stranded DNA and do not fluoresce when in the presence of single-stranded DNA. The thermal cycler continued to measure fluorescence until double stranded DNA denatured and became single stranded. This caused the dye to dissociate and the fluorescence to decrease.

Gel - Primer Specificity

The qPCR results were checked by running a gel to determine if bands were present for each primer (Figure 4). The melt curves had to be checked to make sure that the samples shared the same characteristics when the same product was generated on a gel. Gel electrophoresis aided in identifying the number and size of the PCR products. The samples which were run on the gel, were the two technical replicates of the chosen genes Sig 1-4, SANT, and MORN, which had poor results on the Melt Curves. The bands on the gel determined that the product which was desired was being produced.

qPCR - Primer Efficiency

The qPCR results were analyzed by looking at the Cq values which indicated at which PCR cycle a certain amount of PCR product was reached (Figure 5). The Cq values represented the points at which the fluorescent signal of the reaction crossed the threshold. The threshold is the level of signal that reflects a statistically significant increase over the baseline signal and can be seen as the single horizontal line at the bottom of the images. The threshold allowed for the separation of the relevant amplification signal from the background. The samples were diluted in a series of ten-fold dilutions which were used to establish a curve that aided in assessing the efficiencies of the primers. Since each additional dilution contained an appropriately lower starting amount of DNA, differences occurred between Ct values in the serially diluted samples. Ideally, the curves of each primer should have been closer together as their Cq values should have been relatively close. The Amplification curves showed how efficient a primer would be at producing a product. Based on the Amplification curves, the gene which was the most efficient was Sig 4. The curves were equally separated as they reached the top of the graph. However, to

have the best efficiency, the curves should not cross at the bottom of the graph. The curves for Sig 4 crossed through each other at the bottom of the graph. The gene which had the poorest results was Sig 3. The curves had too much separation between each other and only three out of four curves were present on the graph.

The efficiency of the primers was analyzed in comparison to the housekeeping gene Tubulin (Figure 6). The efficiency of Tubulin was 101.4% which indicated that the efficiencies of the primers should have been in the 90%-110% range. However, the efficiencies of the chosen primers were all higher than the top end of the range. The gene MORN had the poorest efficiency as its percentage reached above a 1000%. The genes Sig 1 and Sig 4 also had poor efficiencies as their percentages were close or at 0%. The genes which were the closest to the housekeeping gene were Sig 2 and Sig 3. The poor efficiencies meant that the primers were not as efficient as expected. The reason for lower efficiencies may have been bad primer design or non-optimal reagent concentrations. Having efficiencies which are over 100% could be a sign of contamination.

qPCR - Gene Expression of Time Course

Studying the average fold expression of each gene showed the change of expression over the time course. The results from the time course qPCR plates showed the level of expression for each gene at various time points (Figure 8).. The graphs showed an average fold change in the expression of the genes starting with the 0 Hour time point which had a value of 0 (Figure 9). The data showed downregulation post infection and upregulation after the 7 Day time point. The control for this experiment was the housekeeping gene Tubulin. Calculations conducted with Ct values from the amplification curves provided relative gene expression. The

values which were over 0 were upregulated and the values that were under one represented downregulation. The expression which resembled the expected results the closest was the expression of Sig 3. The genes Sig 1, Sig 2, Sig 4, and SANT also followed the pattern of downregulation from the 24 Hour time point to the 7 Day time point, and upregulation to the 14 Day time point. The expression of the gene MORN was unexpected as the gene continued to be downregulated over the course of infection. It was expected that the genes would eventually become upregulated. The reason for the continued downregulation of MORN could be contamination of the sample.

Conclusion and Future Directions

The experiment was conducted by looking at genes which were expected to be upregulated in Paramecium. The genes Sig 1-4, SANT, and MORN were shown to be upregulated over the course of infection through RNA sequencing. The genes were expected to be downregulated at the 7 day time point and upregulated as the time course continued to 14 days. The gene which had the results which matched the expected results the closest was Sig 3. The gene which did not follow the expected pattern was MORN. The gene also had the poorest efficiency which means that it was the least efficient at producing the desired product.

A focus should be placed on primer development in which the primers with the low efficiencies are re-designed. RNA extraction can also be a focus as the nanodrop values for some of the samples were not close enough to 100 ng/µL. The samples which had poor values, should be tested for contamination. Not all of the genes which are present in *Paramecium* were tested. The research should extend to other genes to analyze their expression over the course of infection.

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