# To break or not to break, that is the question: Inducing DNA Damage in the *T. thermophila* gene TTHERM_00459400 results in possible increased mRNA expression

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**Abstract**

The DNA Repair pathway is a part of the central mechanism of the cell, repairing thousands of DNA lesions every day. DNA repair pathways including homologous recombination (HR) and non-homologous end joining (NHEJ) maintain the health and genetic stability of the cells. Many genes in the DNA Repair pathway have yet to be identified, so genes from *Tetrahymena thermophila* have been tested to reach a further conclusion. *T. thermophila* organisms show increased DNA damage and repair during mating, and gene TTHERM_00459400 showed promising gene expression levels during mating. To determine whether the gene expression of candidate gene TTHERM_00459400 increases after being exposed to a DNA damaging agent, custom primers for the gene were designed using Primer3Plus and then verified using a PCR. *T. therm* cells were split; half were incubated with the DNA damaging agent HU and half without. More PCRs were conducted to test the mRNA expression of the gene, and the results were then visualized using a Gel Electrophoresis. After the primers were validated, it was found that the gene expression of TTHERM_00459400 possibly increased after DNA Damage was induced. Gene expression has not been confirmed, so further testing needs to be conducted to conclude whether or not mRNA expression increased. If mRNA expression increases, the gene can be studied further to see if it is a part of the DNA Repair pathway. Answering the many questions regarding the DNA Repair pathway is important to gather an understanding of cellular health and could even lead to breakthroughs in cancer research.

## Introduction

The DNA Repair pathway is a part of the central mechanism of the cell. Every cell in the body “receives tens of thousands of DNA lesions per day” (Jackson & Bartek, 2009). These breaks are caused by “ionizing radiation, reactive oxygen species, DNA replication errors and inadvertent cleavage by nuclear enzymes” (Chang, Pannunzio, Adachi, & Lieber, 2017). To repair these breaks, “at least five major DNA repair pathways—base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ)—are active throughout different stages of the cell cycle” to maintain the health and genetic stability of the cells (Chatterjee & Walker, 2017). DNA Repair is a complicated process with many unknown genes involved that “regulate the recognition of DNA damage” and coordinate “DNA repair pathways” (Li, Pearlman, & Hsieh, 2016). Likewise, many genes in the human genome have unknown functions; some of these could contribute to the DNA Repair Pathway.

This research was done to determine whether candidate gene TTHERM_00459400 with a currently unknown function is part of the DNA Damage Response. In the organism *T. thermophila*, many genes are involved in the DNA repair pathway, only some of which have

known functions. There are about 20+ genes with similar expression patterns to those known to be involved that have unknown functions; some of these genes could be involved in the DNA repair pathway. To answer this question, one gene out of these 20+, TTHERM_00459400 was studied using agents to induce DNA Damage; the mRNA levels were studied before and after to see if an increase occurred. These results were compared with genes known to be involved in the pathway and genes known to not be involved. DNA Repair is very important to the health of the cell, especially in preventing cancers and “other diseases, such as neurodegenerative disorders” that occur when repair fails (Chatterjee & Walker, 2017). Many cancers have been shown to have damaged repair systems “which increases mutagenesis and genomic instability, thereby promoting cancer progression” (Chatterjee & Walker, 2017). Learning more could help in further cancer research. DNA Repair is also an integral part of the CRISPR-Cas9 editing process. To edit a genome, a double stranded break is created by Cas9 which is then repaired by “the error-prone NHEJ or the high-fidelity HDR pathway” (Ran et al., 2013). Learning more about DNA Repair could lead to more information on CRISPR and more advancements in genome editing.

*T. thermophila* is an organism that has mastered DNA Repair; during their mating process, they undergo significant DNA damage and repair to renew the genetic information. In this process, they may use some proteins that have not been discovered yet; there is a lot of bioinformatic data on *T. therm* that could help to predict which genes may play a role in the DNA repair pathways in humans. *T. therm* is also a great organism for this research because it is cheap and easy to grow and work with, which makes it an ideal lab organism. The *T. therm* gene TTHERM_00459400 is one of the genes that has yet to be researched for this question. There has not been much information gathered on TTHERM_00459400, but this gene is a good candidate because the gene expression profile shows a similar pattern to genes known to be involved in the DNA Repair pathway, in that it is active during the mating of *T. therm*. If the gene can be identified as a part of *T. therm*’s DNA Repair Pathway, it can help to find similar genes in the human genome that have the same role. To accomplish this, primers were designed for the gene using Primer3Plus, verified with a PCR, and imaged with a Gel Electrophoresis. Various PCRs with the DNA damaging agent HU were conducted to test the expression of the gene TTHERM_00459400 with DNA Damage. After performing these experiments, it was found that the gene expression of TTHERM_00459400 possibly increased after DNA Damage was applied to the gene, though the results were unclear.

**Materials and Methods**

*Primer Design:* To find unique primers that matched the target gene TTHERM 00459400, Primer3Plus was used for both the forward and reverse primers. The parameters were set to specific values: Product Size Range was set between 17 and 28nt, Primer Size set to 20nt, Primer Tm set to 55C, Primer GC% set to 50%, Max Tm Difference value set to 5, Max Poly-X and Max #N' set to 4, CG Clamp set to 1, and TH: Max Hairpin set to 40. 3 possible primer pairs were picked out of 5 options based on factors such as the chance of the primer to bind elsewhere in the DNA sequence and the likelihood of homodimer formation in the primer. Primers were sent to Primer3Manager and loaded into the Blast interface. The primer sequences were compared to all other possible sequences that could be found in Tetrahymena thermophila(taxid:5911). Possible primers were evaluated against each other by comparing the E-values and Graphic Summaries of each sequence. The best primer set was selected under the conditions that the sequences did not align with any other sequence, particularly at the 3' end. For gene TTHERM 00459400, the primers that fit best were: (forward) 5'TGAAAATTGGCAGTCAAACC3' (reverse) 5'GGCTGTATATGTTCCTCCTC3'. Both the forward and reverse primer sequences were highlighted and primer annotations were created and saved under the benchling file of the DNA sequence. The size of the PCR product using the primers was calculated and recorded.

*PCR:* For every PCR conducted, the PCR mixes were composed of 2X GoTaq Green Master Mix 12.5 µl, 10 µM upstream and downstream primers, and target DNA for amplification. A PCR was performed to test the primers using the designed primers with cDNA, gDNA, and no DNA, as well as the RPP0 primers with cDNA, gDNA, and no DNA, with six total PCR products made. PCR tubes were briefly vortexed and centrifuged before being placed in the thermocycler at 95°C for 5 minutes. 35 PCR cycles rotated through denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes. PCR tubes sat at 72°C for 5 minutes and were stored at 12°C. Another PCR was performed to test the mRNA expression of the gene with DNA Damaging Unit HU. To prepare the cDNA samples, *T. thermophila* cells were isolated and split into two samples. One sample was left alone, and the other was treated with the HU Damaging Agent. Nine total PCR products were made using the Rad51 primers with cDNA without HU, cDNA with HU, and no DNA, the RPP0 primers with cDNA without HU, cDNA with HU, and no DNA, as well as the designed primers with cDNA without HU, cDNA with HU, and no DNA. The products were put through the same PCR cycles as with the first PCR with a shorter extension time of 45 seconds.

*Gel Electrophoresis:* The results of each PCR were visualized using gel electrophoresis. To visualize the first PCR set, a 1.5% agarose gel was made with 1X TAE buffer mix. After the agarose solution was microwaved for a total of 75 seconds and cooled, 1XSybrSafe was added. The gel was poured into a mold with a 10-prong comb and left to solidify for 20 minutes. Once set, the gel apparatus was set up with used and fresh 1X TAE buffer covering the gel. 1Kb GeneRuler DNA ladder was loaded into the first well, and each PCR sample was loaded into the other wells. The gel was run at 120V for 45 minutes. After the gel was removed, a picture of the gel was taken using the gel imager and the results were visualized. To visualize the second PCR set, the same procedure was followed, with a 1.7% agarose gel run for 25 minutes.

**Results**

To determine if the designed primers RF could properly anneal to the TTHERM_00459400 gene in the *T. thermophila* cells, a PCR was run using the RF primers as well as RPP0 primers. The results from the PCR were visualized using a 1.5% gel run at 120V for 45 minutes. A band at 350bp in the cDNA+RF lane and a faint band at 500bp in the gDNA+RF lane were observed along with a bright band at 500bp in the cDNA+RPP0 lane. No bands were observed in either lane with water as the DNA template or in the lane with gDNA+RPP0 (Figure 1). This suggested the primers annealed correctly to the DNA given the presence of a band at the correct length for both the cDNA and gDNA of *T. thermophila*.

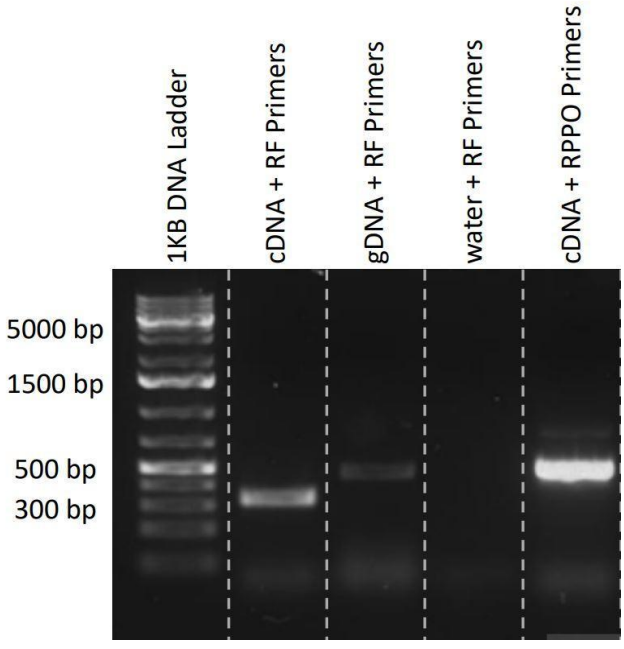


**Figure 1: The gel image of a PCR run to validate the designed primers RF for gene TTHERM_00459400 shows expression of both the cDNA and gDNA.** Three PCR products were made using the designed primers RF with cDNA, gDNA, and water as the DNA template in lanes 2-4. Three PCR products were made using the RPP0 primers with cDNA, gDNA, and water as the DNA template in lanes 5-7. The target cDNA gene of TTHERM_00459400 was

expected to be 351 bp long using the RF primers, and the target gDNA gene was expected to be 1118bp long. The target cDNA was expected to be 490 bp long using the RPP0 primers, and the target gDNA gene was expected to be 1990bp long. A Gel Electrophoresis was run at 120V for 45 min using a 1.5% agarose gel with a 1KB DNA Ladder . The resulting image was taken using a gel imager. A visible band was seen in the cDNA+RF lane at approximately 350bp, and a faint band was seen in the gDNA+RF lane at approximately 500bp. The primer set RPP0 was used as a positive control, with a bright band in the cDNA+RPP0 lane at approximately 500bp, and no band in the gDNA+RPP0 lane. The water as the DNA template was used as a negative control, with no bands shown in either lane water+RF or lane water+RPP0.

To determine if the mRNA expression of the TTHERM_00459400 gene in the *T. thermophila* cells increased after DNA Damage was induced, a PCR was run using cDNA from *T. therm* induced with DNA damage. The PCR used the Rad51 primers, RPP0 primers, and RF primers. The results from the PCR were visualized using a 1.7% gel run at 120V for 25 minutes. A band at 700bp in the -cDNA+Rad5 lane was observed along with a brighter band at 700bp in the +HU cDNA+Rad51 lane. There were bands of equal brightness observed at 500bp in both the -cDNA+RPP0 and +HU cDNA+RPP0 lanes. There was a faint band observed at 350bp in the +HU cDNA+RF lane, and no visible band was observed at 350bp in the -cDNA+RF lane. A faint band was observed at 500bp in the water+RPP0 lane, and no bands were observed in the other two lanes with water as the DNA template and primers Rad51 and RF (Figure 2a). This suggested a possible increase in mRNA expression of TTHERM_00459400 after DNA damage was induced, but the results are inconclusive.

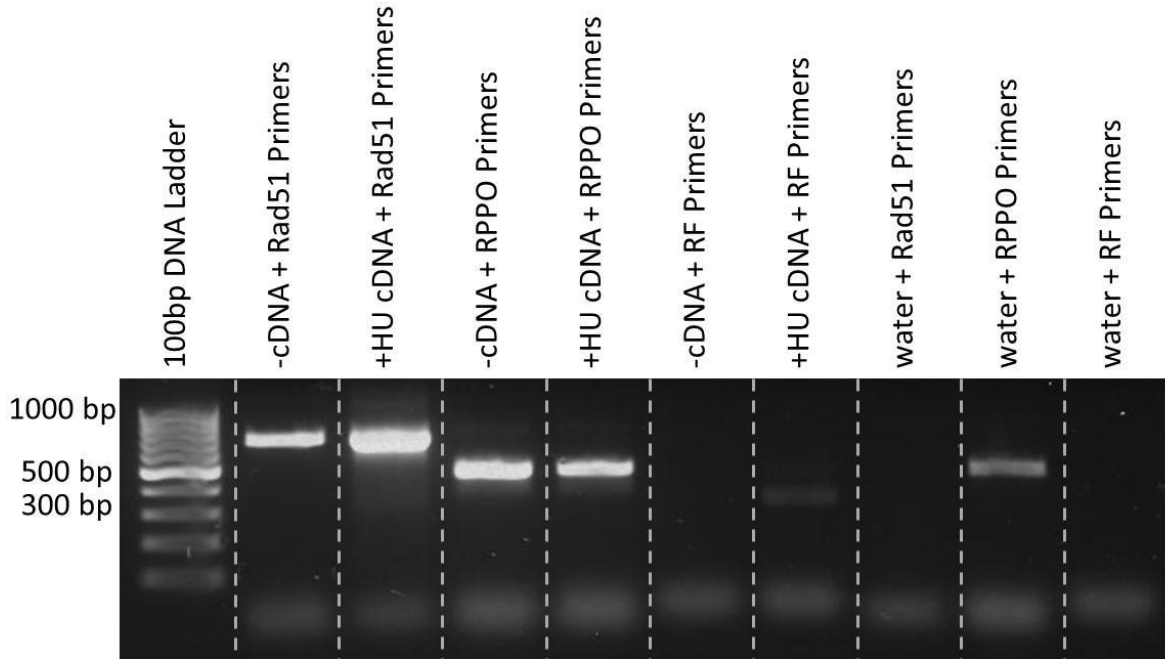

**Figure 2a: The gel image of a PCR run with the DNA damaging agent HU shows a possible increase of mRNA expression of gene TTHERM_00459400.** Two PCR products were made using the Rad51 primers with cDNA without HU in lane 2, and cDNA with HU in lane 3. Two PCR products were made using the RPP0 primers with cDNA without HU in lane 4, and cDNA with HU in lane 5. Two PCR products were made using the RF primers with cDNA without HU in lane 6, and cDNA with HU in lane 7. Three PCR products were made with water as the DNA template using the Rad51 primers, RPP0 primers, and RF primers in lanes 8-10. The target cDNA was expected to be 722 bp long using the Rad51 primers, 490 bp long using the RPP0 primers, and 351 bp long using the RF primers. A Gel Electrophoresis was run at 120V for 25 min using a 1.7% agarose gel with a 100bp DNA Ladder. The resulting image was taken using a gel imager. The primer set Rad51 was used as a positive control, with a band in the -cDNA+Rad51 lane at approximately 700bp, and a brighter band in the +HU cDNA+Rad51 lane at approximately 700bp. The primer set RPP0 was used as a negative control, with a band in the -cDNA+RPP0 lane at approximately 500bp, and a similar sized band in the +HU cDNA+RPP0 lane at approximately 500bp. No visible band was seen in the -cDNA+RF lane, and a faint band was seen in the +HU cDNA+RF lane at approximately 350bp. The water as the DNA template was used as a negative control, with no bands shown in either lane water+Rad51 or lane water+RF, and a faint band shown at 500bp in lane water+RPP0.

To determine if the mRNA expression of the TTHERM_00459400 gene in the *T. thermophila* cells increased after DNA Damage was induced, the data from the gel in Fig 2a was quantified using Fiji for software analysis. The data for Rad51 and RF primers were normalized to the negative controls for RPP0 and water as the DNA template. It was observed that the mRNA expression of TTHERM_00459400 using RF primers increased by 300% after DNA damage was induced, and the mRNA expression using Rad51 primers increased by 150% after DNA damage was induced (Figure 2b). This suggested a confirmed increase in mRNA expression of TTHERM_00459400 after DNA damage was induced.

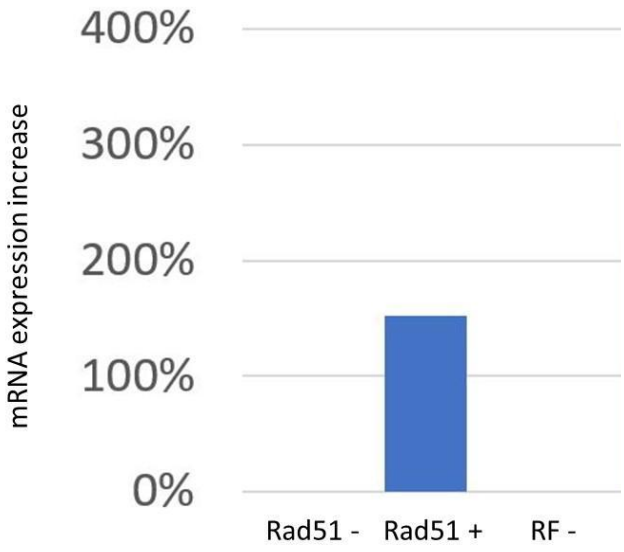


**Figure 2b: The gel image of the PCR run with the DNA damaging agent HU was quantified, showing an increase of mRNA expression of gene TTHERM_00459400.** The data from the gel electrophoresis in Fig 2 was quantified using Fiji. The background data from the gel was subtracted from the Rad51, RPP0, and RF positive and negative results, then the results from the Rad51 and RF were normalized to the RPP0 control. The Rad51 and RF positive results were then normalized to the negative results and graphed as

percentages. The cDNA with the Rad51 primers as the positive control showed a 150% increase in mRNA expression after being exposed to DNA Damage from HU. The cDNA with the RF primers showed a 300% increase in mRNA expression after being exposed to DNA Damage from HU.

**Discussion**

In the PCR experiment to validate the RF gene-specific primers (Figure 1), the results showed a band at 350bp in the cDNA+RF lane and a faint band at 500bp in the gDNA+RF lane. Due to the presence of a band of the correct size with both the cDNA and gDNA of *T. thermophila*, it was concluded that the primers annealed correctly to the DNA. The RPP0 primers were used as a positive control; there was a band observed at 500bp in the cDNA+RPP0 lane but no band observed in the gDNA+RPP0 lane. The lack of a band with the gDNA did not matter for this experiment, and the future experiments were only conducted with the cDNA of *T. thermophila*. The presence of the band at the correct length in the cDNA lane indicated that the cDNA could be annealed to by a primer, and that the PCR worked. Water as the DNA template was the negative control, and there were no bands observed in either lane. The lack of any band indicated that there was no contamination in the results from the PCR ingredients.

The PCR run using cDNA from *T. therm* induced with DNA damage to measure mRNA expression (Figure 2a) resulted in a faint band observed at 350bp in the +HU cDNA+RF lane, and no visible band at 350bp in the -cDNA+RF lane. The lack of a band in the -cDNA lane suggested that the PCR did not work, but the presence of the band in the +HU cDNA lane confirmed the PCR did work. Due to the presence of a band of the correct size in the +HU cDNA lane as compared to the -cDNA lane, mRNA expression could have increased from almost nothing (as seen in the -cDNA lane) to a miniscule amount after DNA damage, which would still be an increase. The Rad51 primers were used as a positive control; there was a band at 700bp in the -cDNA+Rad5 lane with a brighter band at 700bp in the +HU cDNA+Rad51 lane. The brighter band in the +HU lane indicated an increase in mRNA expression when DNA damage was induced, which was the expected result for Rad51. This suggested that the HU damaging agent worked to induce damage, and that an increase in mRNA expression levels can be seen on the gel. The RPP0 primers were used as a negative control; there were bands of equal brightness observed at 500bp in both the -cDNA+RPP0 and +HU cDNA+RPP0 lanes. The equal brightness of the two bands indicated no increase in mRNA expression when DNA damage was induced, which was the expected result for RPP0. This showed the base amount of mRNA that

was added to the PCR and could be seen, which helped make a conclusion for the RF primers. Water as the DNA template was another negative control. A faint band was observed at 500bp in the water+RPP0 lane, and no bands were observed in the other two lanes with water as the DNA template and primers Rad51 and RF. The lack of any band in the Rad51 and RF lanes indicated that there was no contamination in the results from the PCR ingredients, but the band in the RPP0 lane suggested possible contamination of the RPP0 PCRs. However, there was no contamination in the RF lane, so conclusions can still be made from the gel.

The data from Fig 2a was quantified to provide further conclusions if the mRNA expression increased after DNA Damage was induced, and the results showed that there was an increase of 300% in expression after damage (Figure 2b). This suggested that there was a significant increase in mRNa expression as compared to the expression increase of 150% for Rad51. However, it cannot be confirmed that mRNA expression increased, because the gel and the quantification did not support each other.

There are many genes in the DNA repair mechanism that are still unknown, and this gene could possibly be one of them. This research provides a stepping stone to unlock the possible involvement of this gene in the DNA repair mechanism, which can provide answers to many questions regarding the mechanism. It is important to understand the DNA repair mechanism because the cells in the human body are constantly undergoing damage and repair in the genes. Some damage induced by the body itself is important to cell function and genetic evolution through homologous recombination, so understanding a piece of the DNA repair mechanism can allow the scientific community to understand other cellular mechanisms and cell health as a whole.

Before the gene can move forward in research with the DNA repair system, further tests need to be run on the gene. The results from the mRNA expression PCR were not conclusive, so the same experiment would need to be run again. The band for the cDNA without HU was not visible on the gel, but the quantitative data showed expression increase due to the presence of a band for cDNA with HU; the conflicting results would need to be sorted out first. If the results from that experiment show conclusively that the mRNA expression increased after DNA Damage was applied, the gene can then undergo further testing to see if it is a part of the DNA repair pathway for *T. thermophila*.

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