EXPERIMENT 5-6

cDNA GENERATION FROM ISOLATED RNAs

&

qPCR by USING GENERATED cDNAs

Çerağ Oğuztüzün
21704147

Experiment dates: 17.03.2021 – 24.03.2021
Submission date: 01.04.2021

Introduction

One of the aims of proliferating the RNAs from the previous experiment was to proliferate the DNA segments the RNA transcribed for. This is done by conversion of RNA to cDNA using the reverse transcriptase enzyme. cDNA ain't a genomic DNA regarding the reverse transcriptase generated it from an mRNA which is processed [1]. cDNA can be used to clone eukaryotic genes. Additionally, viruses use a naturally occurring cDNA mechanism in retroviruses where their genome is merged to the host organism's genome [1].

qPCR is the quantitative polymerase chain reaction that is used to observe DNA proliferation in real-life quantitated by using fluorescent dye. The fluorometer and the fluorescent dye aids in the quantization and labeling of the DNA where the fluorometer detects the fluorescent labels in real-time. This way, If an error is made in the experiment, the experiment can see a failure in the proliferation of dsDNA and do changes as soon as possible [2]. The initial steps of doing qPCR are using reverse transcriptase on RNAs and producing cDNAs.

Facilitators include GAPDH and EGFR. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) is used in cancer research due to the fact that the GAPDH gene's upregulation has resulted in tumor formation [3]. EGFR is linked with kidney diseases and is involved in growth factor activity. This as well as GAPDH should be kept at a certain level in the body. GAPDH was the control group where EGFR was the experimental group.

The purpose of the cDNA experiment was to convert mRNAs that were purified into cDNAs. The purpose of the qPCR experiment was to observe the levels of gene expression in control and experiment groups. GAPDH was the control group where EGFR was the experimental group.

Materials & Methods

For generating cDNA, the 4 ul of 5x iScript reaction mix was used to optimize the reverse transcriptase activity. 1ul of iScript reverse transcriptase was used for the conversion of mRNA into cDNA. 14.73 ul of DNAase-free water is used for providing an optimum environment for chemical processes to occur. This amount was calculated from experiment 4's RNA purity value (3649*10^-6 ug/ul) to complete a volume of 15 ul, 0.27 ul of the RNA template sample from the previous experiment was used for the generation of cDNA. These components which add up to 20 ul were mixed in an Eppendorf. Then, the Eppendorf was put into a thermal cycler where priming is done for 5 minutes at 25 0C, reverse transcription is done for 20 mins at 46 0C, RT inactivation was done for 1 minute at 95 0C, and finally, the tube was held at 4 0C under optimum conditions.

A mastermix was prepared using CYBR Green I Mix which is a dsDNA binding protein that is used to label synthesized dsDNA in real-time, Nuclease free water in order to eliminate contamination, forward primer, and reverse primer for generating dsDNA are used. The mastermix is then vortexed and ran into a thermal cycler. GAPDH cDNA sample was provided from the cDNA experiment and the EGFR was provided from tumors.

Results

A mastermix to be loaded in each well was prepared as follows. The mastermix was prepared to be loaded to 7 wells for preparing extra for erroneous situations. But in fact, the mixture was to be loaded into 12 wells, where 6 wells were treated with EGFR cDNA mixture, and 6 were with GAPDH cDNA mixture as housekeeping group. For each of the 6 wells, 3 wells were treated with the cDNA which was prepared in the previous experiment, and 3 wells were treated with cDNA HCC which is tumorogenesis. The mixture for 1 well was prepared by: 5 ul of CYBR Green I Mix, 2 ul of Nuclease free water, 0,5 ul of forwarding primer, and 0,5 ul reverse primer. The reason for using more than 3 wells as triplets were to take an average of them and create a more statistically significant value from the experiment. For each well, 8 ul mix and 2 ul cDNA are loaded.

Samples	**GAPDH**	**EGFR**
Control	40	33.62
Control	40	33.34
Control	40	33.44
Treated	32.04	25
Treated	20.62	28.56
Treated	20.44	29.57

Figure 1: Raw Data from qPCR machine with Ct values

Calculating 2^(CTcontrol – CTgene) for each row where CTcontrol is the Ct value of the housekeeping gene which is GAPDH. CTgene is the Ct value of the gene of interest which is EGFR. Results of this calculation can be seen in Figure 2.

Samples	EGFR
Control	2^(40-33.62) = **83.2858787483**
Control	2^(40-33.34) = **101.125287919**
Control	2^(40-33.44) = **94.3532299066**
Treated	2^(32.04-25) = **131.598569812**
Treated	2^(20.62-28.56) = **0.00407213187**
Treated	2^(20.44-29.57) = **0.00178482705**

Figure 2

Then, the arithmetic average of replicated of control and treated values are calculated in Figure 3.

Samples	EGFR
Control	(83.2858787483 + 101.125287919 + 94.3532299066) / 3 = 278.764396574 / 3 = **92.9214655246**
Treated	(131.598569812 + 0.00407213187 + 0.00178482705) / 3 = 131.604426771 / 3 = **43.868142257**

Figure 3: Calculated Average

Then, relative expressions are calculated so that the tumor tissue and normal liver tissue can be compared. The values from Figure 2 will be divided by the values in Figure 3 for each row. The results of calculations can be seen in Figure 4.

Samples	EGFR
Control	83.2858787483 / 92.9214655246 = **0.89630397323**
Control	101.125287919 / 92.9214655246 = **1.08828769917**
Control	94.3532299066 / 92.9214655246 = **1.0154083276**
Treated	131.598569812 / 43.868142257 = **2.99986648719**
Treated	0.00407213187 / 43.868142257 = **0.00009282663**
Treated	0.00178482705 / 43.868142257 = **0.00004068617**

Figure 4: Calculated Relative Expressions

Later, the averages are taken for control and treated values.The resulting values can be seen in the final table of Figure 5.

Samples	**EGFR**
Control	(0.89630397323 + 1.08828769917 + 1.0154083276) / 3 = 3 / 3 = **1**
Treated	(2.99986648719 + 0.00009282663 + 0.00004068617) / 3 = 2.99999999999 / 3 = **0.99999999999**

Figure 5: Final Results

The melting curves for each gene is provided below as Figure 6 for GAPDH and Figure 7 for EGFR. Their discussion will be provided in the Discussion part of the report.

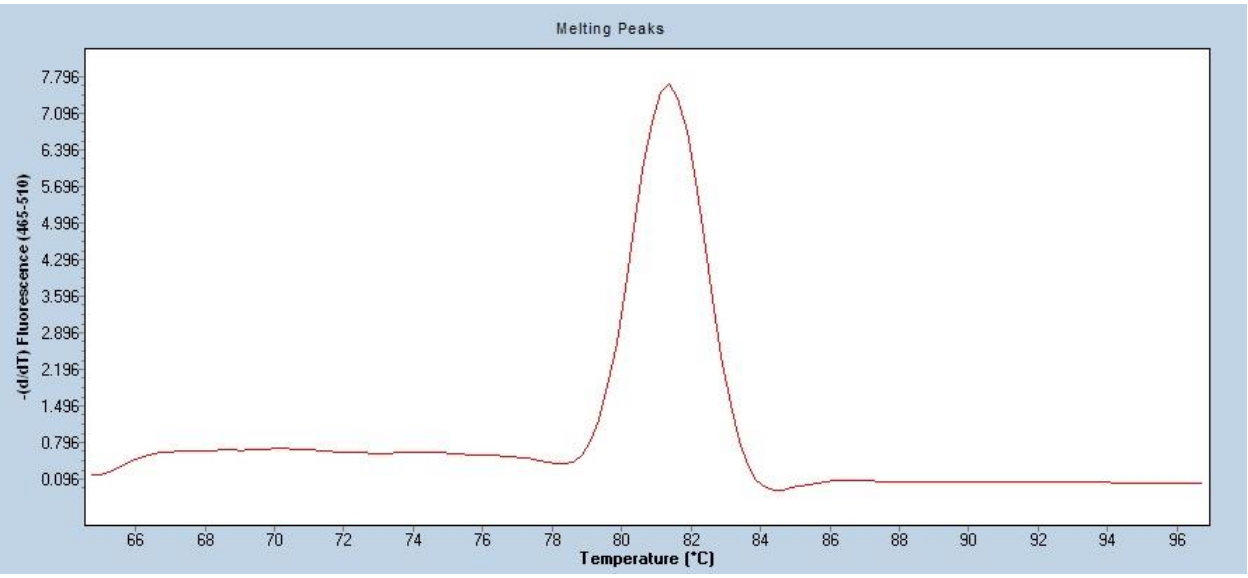

Figure 6: Melting Curve of GAPDH

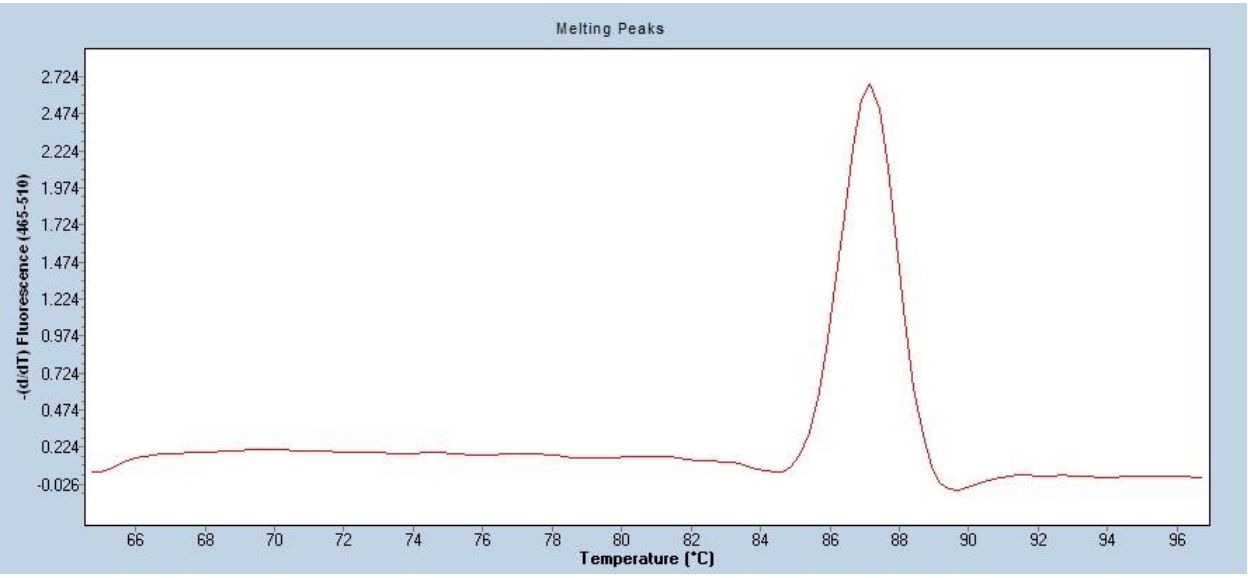

Figure 7: Melting Curve of EGFR

Discussion

As the replicates are the same: 1 for Control in EGFR and 0.99 for Treated in EGFR, this suggests the gene of interest is not depleted in the treated group. We can see from the relative expressions table that, the expression of both control and treated genes are the same for the triplets. This observation is done by averaging the expressions as their individual relative expression fails to give a direct conclusion. 2 control rows have 1 and one treated row has 2, where the other 2 treated rows are 0.

As EGFR is involved in cell growth, It is normal for EGFR to have activity on normal cells [4]. However, It is also found on cancer cells to increase the proliferation of cancer cells. It can be understood that the proliferation of the treated group is similar to the control groups. It can also be inferred that the treated gene group is expressed at normal liver cells as the relative expression values are positive. As we see the expression of treated cells, which can cause cancer, It can be concluded that the liver cell could use a tumor-suppressing mechanism to downregulate the oncogene activity for tissue health in the long run.

Looking at the melting curve figures for primer control, It can be seen that for both figures there is a single curve, which suggests that the primers worked. If we saw a more fluctuating curve or multiple curves of different amplitudes, this would mean that primers did not work properly. This finding is in favor of the experiment and suggests the findings are more reliable for both EGFR and GAPDH.

The reason relative expressions of control and treated groups differ by 0.000000000001 can be due to contamination while working with micropipettes, or change in temperature when taking Eppendorf from the freezer might have degraded samples by a small percent.

References

[1] "Complementary DNA," *Complementary DNA - an overview | ScienceDirect Topics*. [Online]. Available: https://www.sciencedirect.com/topics/medicine-and-dentistry/complementary-dna. [Accessed: 01-Apr-2021].

[2] A. A. S. Staff, "What is qPCR?," *Ask a Scientist*, 07-Feb-2020. [Online]. Available: https://www.thermofisher.com/blog/ask-a-scientist/what-is-qpcr/#:~:text=qPCR%20stands%20for%20quantitative%20polymerase,for%20measuring%20DNA%20using%20PCR. [Accessed: 01-Apr-2021].

[3] K. Liu, Z. Tang, A. Huang, P. Chen, P. Liu, J. Yang, W. Lu, J. Liao, Y. Sun, S. Wen, Y. Hu, and P. Huang, "Glyceraldehyde-3-phosphate dehydrogenase promotes cancer growth and metastasis through upregulation of SNAIL expression," *International Journal of Oncology*, 01-Jan-2017. [Online]. Available: https://www.spandidos-publications.com/10.3892/ijo.2016.3774#:~:text=GAPDH%20is%20an%20important%20glycolytic,patients%20(25%E2%80%9327). [Accessed: 01-Apr-2021].

[4] "NCI Dictionary of Cancer Terms," *National Cancer Institute*. [Online]. Available: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/egfr-inhibitor. [Accessed: 01-Apr-2021].