Republic of Iraq
Ministry of Higher Education and
Scientific Research
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The Probable Effect of Some Chemical Inhibitors on Gene Identification in Forensic Samples

A Research

Submitted to Council of the College of Science / University of Babylon in Partial Fulfillment to the Requirements for the Degree of High Diploma in Science /Forensic Evidence

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2022 A.D **1443 H.D**

بسم الله الرحمن الرحيم

((فَلْيَنظُرِ الْإِنسَانُ مِمَّ خُلِقَ))

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Dedication

To my mother and father

To my wife and my children

To all my extended family and friends

With loyalty

Khaldun

Acknowledgment

First of all, I would like to thank Allah for his merciful and his continuous help in accomplishing my study and his helping me over all the periods of my life.

I would like to express my sincere gratitude to my supervisor Assist. Prof. Dr. Bassam M. Al-Yaseen for suggesting this work, his patience, great support, help and continuous guidance to complete this study.

My thanks go to all my professors at the University of Babylon for their help and guidance.

I owe special appreciation to the Department of Biology, College of Science, University of Babylon for giving the opportunity of joining their higher studies.

Finally, I am very grateful for all the support I have received from all my friends over the study year.

Khaldun

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List of Abbreviation

Ct	**Cycle threshold**
DNA	**Deoxyribonucleic acid**
Tm	**Melting temperature**
PCR	**Polymerase chain reaction**
qPCR	**Quantitative Polymerase Chain Reaction**
RNA	**Ribonucleic acid**
mRNA	**Messenger Ribonucleic acid**
RT-PCR	**Reverse Transcription Polymerase Chain Reaction**
KCl	**Potassium chloride**
NaCl	**Sodium chloride**
GAPDH	**Glycrideraldehyde-3-phosphate dehydrogenase**
dF	**Delta fluorescentation**
dT	**Delta Temperature**
RMSD	**Root-Mean-Square-Deviation**
SDS	**Sodium dodecyl sulphate**
dF/dT	**First derivative of signal**
HBB	**Hemoglobin beta**
rt-PCR	**Real time Polymerase Chain Reaction**

Summary

The aim of this study is to investigate the effect of inhibitors that can affect the detection of various body fluids at crime scenes, especially those found naturally in the environment or from the action of other factors. Urea and tannic acid are both chemical environmentally present factors have been recorded to be relatively found in crime scene, thus being useful for forensic applications. One of the following parameters is the cycle threshold (Ct) which is the point at which the fluorescence intensity exceeds that of the background fluorescence. As a result, the greater the amount of target DNA in the starting material, the better. The other parameter was the melting temperature(Tm), the slope of amplification and the plateau intensity. Therefore, the effects of these parameters were studied on the real time-PCR amplification to determine the inhibitory effect of these two factors with the different concentrations of 10µM, 100µM, and 500µM when add to blood specimens and compared with control group, the results indicated that urea did not work as a stressor or inhibitor.as for the mean of Ct there decrease compared with control and no significant difference for all the concentrations at $p<0.05$,the amplification slope mean showed increase compared with control group but no significant difference for all the concentrations at $p<0.05$, for the plateau intensity mean the results showed that the mean of the plateau intensity for the control group and 10 µM are differ from 100 µM and 500 µM groups and there significant difference for the concentration of 100 µM and 500 µM compared with control and the concentration 10 µM at $p<0.05$,the result for all concentrations showed no significant difference in mean of melting temperature compared with control at $p<0.05$. However the Ct value for tannic acid was less than 2.5 at all concentrations because it worked as a inhibitor.

CHAPTER 1

Introduction

1.1 General Introduction

Polymerase chain reaction (PCR) inhibitors are the factors that prevent the amplification of the nucleic acids through the PCR. When there are enough copies of DNA, PCR inhibition is the most prevalent reason for amplification failure. PCR inhibitors usually affect PCR through interference with the DNA polymerase or interaction with DNA. Through binding directly to single-stranded or double-stranded DNA, inhibitors can avoid being removed during the DNA purification process. Instead, by reducing the availability of cofactors (like Mg^{2+}) or else interfering with their interaction with the DNA polymerase, PCR is inhibited (Alaeddini 2012).

The real-time reverse transcription polymerase series response (RT-qPCR) has appeared as the technique of select for messenger Ribonucleic acid (mRNA) quantification in microbiology molecular medicine, biotechnology and diagnostics. In spite of the detail that it is regularly appeared as gold procedure, being a standard assay it is far. Changeability in Ribonucleic acid RNA designs, assay, templates and methods, similar to uneven data clarification and improper data normalization can make substantial problems. It is significant to check that RT-qPCR outcomes are merely a picture of data about the quantity of a convinced record in tissue or cell. Extra information must be comprised in any valuation of the biological impacts of varying mRNA stages about protein levels, regulatory RNAs, and protein activity (Kim *et al*. 2019).

1.2 Aim of study

This study aims to investigate the impact of PCR inhibitors on different parameters for gene identification using real-time PCR.

1.3 Objects of study

1. Using Real time amplification curve comparative quantitation (first derivative).
2. Perform threshold cycle value (Ct: cycle number at which the fluorescent signal passes the threshold of detection).
3. Estimate the correlation between each of these parameters and inhibitor concentration.

CHAPTER 2

LITERATURE REVIEW

2.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Several researchers have recently published a series of studies focusing on the practical aspects of mRNA profiling utilizing crime scene stains. The subjects of these pieces are the difficulty of isolating high-quality mRNA and DNA a large number of stains from little amounts (Bauer et al. 2003).

In one bodily fluid, there are high levels of expression and very low levels of expression a different level as a result, a qualitative study, such as one based on endpoints is required (Zubakov et al. 2008).

Referring to (Lynch and Fleming 2021) the detection of bodily fluids contained in a dye be able to help with the reconstruction scenes of crime . RNA-based approaches for identification of body fluids overcome some of the drawbacks of traditional testing, such as sample degradation and lack of specificity. Due to the lack of capillary electrophoresis instruments, several forensic laboratories are now using mRNA endpoint profiling reverse transcription polymerase chain reaction (RT-PCR) as a confirming RNA approach in casework. When the RNA input is within the ideal range, this approach is highly specific and dependable. When RNA entry is below the detection limit or too high, false positives and false negative \cross reaction might occur. Quantitative real-time quantitative PCR (qPCR) is quantitative and more sensitive.

As a result of enormous range of dynamic, real-time PCR has become one of the most popular methods for amplification because of its sensitivity, capacity to be very sequence-specific, little to no post-amplification processing, and ability to enhance sample throughput extensively used approaches of gene quantification. Real-time PCR can be used to measure mRNA in one of two ways: as a one-step reaction, in which the entire reaction from cDNA synthesis to PCR amplification takes place in one tube, or as a two-step reaction, in which reverse transcription and PCR amplification take place in tubes were separated (Wong and Medrano 2005).

Initial screening for the presence of biological material is usually the first step in the analysis of biological evidence. Chemical analysis, immunological assays, protein catalytic activity testing, spectroscopic approaches, and microscopy are all used to identify body fluids. The (mRNA) profiling, tissue-specific DNA methylation, and microbiological analysis are some of the new technologies. Biological stains that remained at the scene where the crime happen, such as saliva, semen, urine, and blood are presently submitted toward bodily identification of fluids using traditional procedures such as serological and enzymatic examinations. There have been a few reports of mRNA such a valuable instrument in forensic investigations (Matsumura *et al.* 2018)

Using recently proposed approach such as a molecular genetics-based and using RNA profiling or DNA methylation detection to supplant conventional body fluid identification method. Several RNA markers which are particular to forensically relevant body fluids have been identified, and their specificities and sensitivities have been tested using various samples (An *et al.* 2012).

The samples that contain of qPCR inhibitors ,on the other hand, limits the full potential of qPCR (Hedman *et al.* 2013).

2.2 Inhibitors of PCR

Inhibitors of PCR are a diverse collection of chemical compounds. Many distinct inhibitory compounds can be found in a single matrix, and the same inhibitors can be found in multiple matrices. PCR inhibitors can be organic or inorganic compounds that are either liquid or solid. Inorganic compounds that impede the PCR include calcium ions, for example. The majority of known inhibitors, however, are organic compounds, such as bile salts, urea, phenol, polysaccharides, ethanol, sodium dodecyl sulphate (SDS), tannic acid, melanin, and various proteins, such as collagen, myoglobin, hemoglobin, immunoglobin G (IgG), and proteinases (Schrader et al. 2012)from the chemical class, the compound's concentration is crucial for its inhibitory properties.

Environmental samples can derive from a variety of sources, including water, soil, and the atmosphere. As a result of this wide range, many distinct PCR inhibitors exist, including those already mentioned. Furthermore, humic and fulminic acids, which block PCR even at low doses, may be found in dead biomass and soil(Mohammad 2018). Fats, proteins, polyphenols, and heavy metals can be detected in sewage sludge, while waste water contains polysaccharides, metal ions (such as iron and aluminum), and RNases - all of which are frequent PCR inhibitors in environmental samples(Schrader et al. 2012). In addition, some inhibitors have been discovered in animal feed. For the detection of pathogens, a wide range of water samples is usually employed.

Excess salts including sodium chloride (NaCl) ,potassium chloride(KCl) and ionic detergents for example SDS, sodium deocycholate,

ethanol, sarkosyl , isopropanol and phenol and Urea in addition Tannic acid and others, all contribute via various inhibitory mechanisms, to the reduction of PCR efficiency(Schrader et al. 2012) .

The PCR sensitivity and accuracy can be harmed by a number of inhibitors found in clinical and environmental matrices. Interfering with the lysis of cells, polymerase activity and nucleic acid sequestration or degradation suppression are all frequent PCR Inhibition Mechanisms (Moehling et al. 2021). To minimize PCR inhibition, bovine serum albumin betaine and proteinase inhibitors have been utilized. Other inhibitor alleviation strategies depend on DNA polymerase modification, for example removal of the N-terminal region of the polymerase's enzyme (Kermekchiev et al. 2009).

The target template's concentration, the elimination of inhibiting components, the purified target nucleic acid's release is one of the goals of processing of samples prior to PCR detection. However, because the efficiency of extraction technologies is not 100 percent, sample processing outputs have always been lower than the input of nucleic acid. (Shipley et al. 2012). If the target nucleic acids are present in low amounts, as they are in best clinical and environmental samples, this is a significant problem. Additionally, some inhibitors might co-elute after purification, resulting in false negatives. For example, false-negative tests for hepatitis B virus were seen after a variety of purification procedures followed by PCR detection False negatives, particularly for very pathogenic pathogens, have serious implications for treatment and quick containment in the field (Shipley et al. 2012).

Detection of direct PCR from complicated samples could be a solution for losing yield, having the additional advantage of a shorter time to identify the etiology agent. The use of inhibitor-resistant PCR reagents, as well as

direct amplification of nucleic acid targets at semi-matrix inhibitor concentrations, could improve the sensitivity of traditional real-time PCR investigations. (Margraf et al. 2006)

2.2.1 Urea

Urea, sometimes identified as carbamide, is a diamide of carbonic acid. The formula is H2NCONH2, as shown in the figure2.1 below. Urea is used as a fertilizer, a feed additive, and a raw material in the manufacture of polymers and medicines. Urea is a colorless, crystalline material that melts at 132.7°C (271°F) and decomposes prior to boiling (De boo et al. 2005).

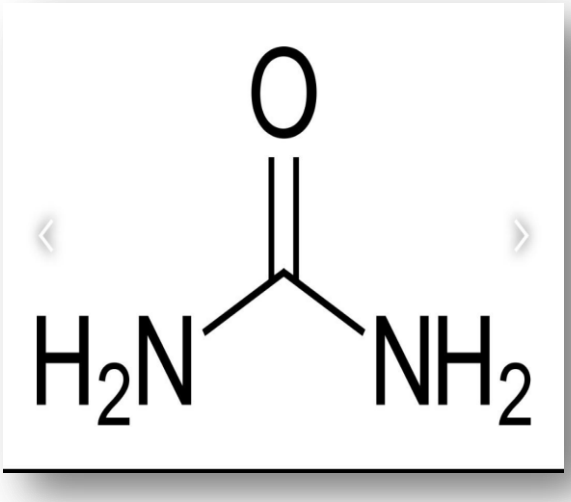

Figure 2.1: Urea Structure
(Urea-American Chemical Society, 2021).

Urea produces methylene–urea fertilizers with formaldehyde, which emit nitrogen in slow rate and consistently, allowing for a full year's supply to be applied at once. While urea nitrogen is in a no protein form, it can be used by animals of ruminant (sheep, cattle), and it can be used to meet a considerable portion of these animals' protein requirements. Urea is use to create urea–formaldehyde resin is second only to its usage as a fertilizer in terms of importance(De boo et al. 2005) .

Artificial nitrogen fertilizers are a vital component in intensive agricultural and a requirement for international food creation, making them essential in achieving food security around the world. Because accessible nitrogen is often the limiting component that prevents soils from supporting intensive crop development, urea is the most significant nitrogenous fertilizer. The most common source of urea is ammonia (NH3) combined with carbon dioxide (CO2). Approximately 80% of the NH3 is used in this process. In this regard, the famous Haber–Bosch process, which is recognized as one of the greatest innovations of the twentieth century, plays a crucial role in ammonia synthesis from molecular nitrogen and hydrogen (Liu et al. 2021).

Ammonia and carbon dioxide are used to make urea. Ammonia and carbon dioxide are produced the urea is generated in two steps after being injected into the reactor at high pressure and temperature;

$CO_2 + 2NH_3 \longrightarrow NH_2COONH_4$ reaction (ammonium carbamate)

$NH_2COONH_4 \longrightarrow NH_2CONH_2$(urea) $+ H_2O$

CO2 and unreacted NH3 as well as ammonium carbamate are found in urea. The pressure is increasing when NH2COONH4 is reduced and heated, it decomposes into NH3 and CO2. The ammonia is a poisonous gas Carbon dioxide is recycled, as well The solution of urea is subsequently concentrated to produce Molten urea, 99.6% by weight(w/w), which is then powdered for utilize usage as a fertilizer as a source of chemical feedstock(Sigurdarson, Svane, and Karring 2018).

In this study (Baer-Dubowska et al. 2020) The enzyme activity is inhibited by the presence of Urea, whereas the Taq DNA polymerase enzyme is degraded by certain detergents Tannins are polyphenolic

compounds with carbohydrate backbones found in a variety of plant species.

Urea is the most abundant organic waste product and the major inhibitory component in urine. The results obtained by (Thompson, Duncan, and McCord 2014)shown that when increasing concentrations of urea were added to the PCR mixture included a four cycle increase in CT values that was more related to changes in the slope of the amplification curve than loss of template. There was also a gradual loss of efficiency, especially those at higher concentrations of inhibitor. The melt curve showed a decrease in Tm of 1.5°C, indicating some binding of urea to DNA. Urea is known to be a potent disruptor of noncovalent bonding and therefore may act directly on the polymerase, or it may prevent primer annealing. Thus, the mechanism of inhibition for urea is likely to be mixed mode inhibition.

(Schrader et al. 2012) stated that the most critical component in urine samples is urea, which may lead to the degradation of the polymerase. However, the effect is dependant on the concentration of urea in the sample and initiates with a concentration of about 50 mmol l^{-1}. As the urine excretion in humans is – dependent on diet and age – in the range between 340 and 580 mmol urea per day, inhibitory concentrations are not unusual.

2.2.2Tannic acid

Tannic acid is a kind of tannin which is consist of a glucose core is surrounded by ten galloyl (3,4,5) trihydroxyphenyl) units. Tannic acid sold in stores. Tannic acid has no groups of carboxyl on the other hand is slightly acidic due to the many phenolic hydroxyls. It is additionally particularly water soluble due to the hydroxyls. It is classified as a

nonhazardous substance by all regulatory bodies. As shown in the figure 2.2 below.

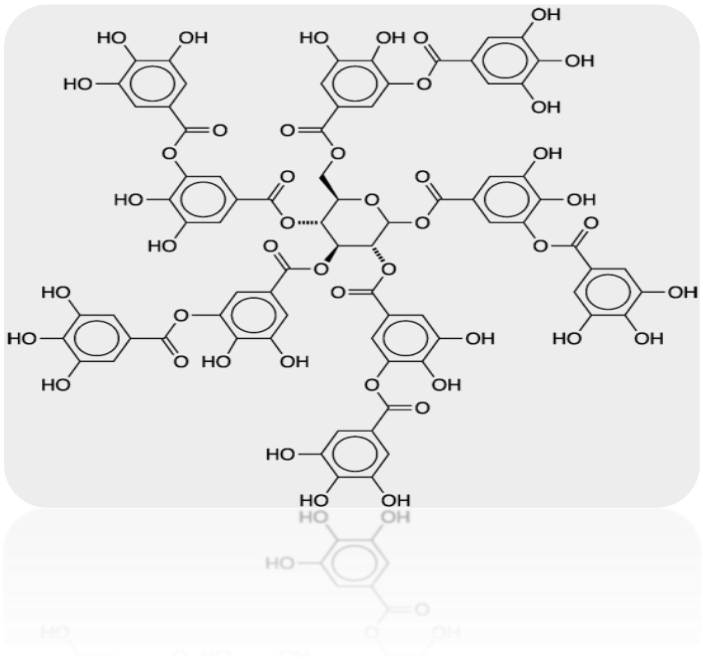

Figure 2.2: Tannic Acid

Reference to (L'Energie Homo-Hydrogne, 2004).

Tannins are employed in the tanning of leather, as the name suggests. Dyeing, ink manufacturing, paper sizing, food and wine processing, and the generation of gallic acid and pyrogallol are some of the other commercial uses (Park et al. 2018).

Tannic acid is a kind of polyphenol and form of tannin that is available for purchase. It has the properties an acid with a low pH. It is can discovered in the nutgalls produced by insects on the branches of some oak trees (Quercus species and other Quercus infectoria) as shown in the figure 2.5 below. It is taken out and used for medical purposes. It was once employed as an antidote for a variety of toxins. Tannic acid is now used to treat fever blisters, diaper rash cold ulcer and poison ivy on a topical basis. Tannic acid can also be used orally and administered topically to treat bleeding, prolonged diarrhea, urine with blood in it , Joints pain , chronic coughs, in addition malignancy(Park et al. 2018) .

Referring to this study (Huggett et al. 2005) to avoid variations from measurement techniques like as variation in executions, disparities in mRNA amount and quality between samples due to pipetting mistakes, and variances in the effectiveness of enzymes employed for reverse transcription and amplification steps, mRNA levels are often standardized. Even with its widespread usage in biological samples to quantifying mRNA, real-time PCR has numerous drawbacks, including variability in extraction of RNA techniques and It is vital to choose a regulation strategy that can reduce PCR performance and control errors. Various data normalizing techniques have been developed for real-time PCR. (Banda et al. 2007).

According to this study (Tichopad et al. 2003)the real-time PCR normalization the reference genes, also known as housekeeping genes, are expressed in a variety of tissues and cell types and show no or only small expression changes across samples and conditions. Prior to real-time PCR comparisons between samples, these genes are used to normalize the mRNA levels of genes of interest. The best option is crucial for effectively assessing the findings of qRT-PCR. To effectively assess the results of qRT-PCR, the optimal choice is critical. These reference genes, like normal reference genes, are responsible for assessing and decreasing errors caused by differences in sample quality, extracting, and RNA effectiveness in cDNA synthesis, as well as internal controls and different testing materials, such as healthy cells and sick tissue (Huggett et al. 2005).

A study carried out by (Kontanis and Reed 2006) demonstrates that rt PCR efficiency analysis is a sensitive inhibitor detection strategy when assays are contaminated with tannic acid. Tannic acid is a known PCR inhibitor and is a recognized template DNA contaminant requiring

specialized purification protocols. The results of another study performed by (Matsumura et al. 2018)where the tannic acid was used as PCR inhibitors showed that Ct values for hemoglobin beta (HBB) in samples containing tannic acid in real-time PCR mix increased in a concentration-dependent manner, and were undetectable at higher concentrations. Moreover, Ct values for HBB in tannic acid-spiked samples reached a maximum once, and inhibition decreased at higher concentrations. dCt values decreased in tannic acid-spiked samples. Tm values decreased following the addition of tannic acid. The observed inhibitory effects were weaker than those when inhibitors were added to the PCR cocktail. PCR inhibition was effectively reduced by repurification of complimentary DNA with DNA extraction kits.

2.3 Housekeeping gene

Housekeeping genes are genes that are required for the maintenance of basal cellular functions that are essential for the existence of a cell, regardless of its specific role in the tissue or organism. Thus, they are expected to be expressed in all cells of an organism under normal conditions, irrespective of tissue type, developmental stage, cell cycle state, or external signal. From a fundamental point of view, full characterization of the minimal set of genes required to sustain life is of special interest(Eisenberg and Levanon 2013) . Housekeeping genes are also commonly utilized as internal controls in both experimental and computational research (Robinson and Oshlack 2010). Furthermore, numerous studies have emphasized the unique genomic and evolutionary characteristics of this particular gene family. Housekeeping genes, for example, were found to have shorter introns and exons (Carmel and Koonin 2009), a distinct repeating sequence environment [short interspersed elements (SINEs) enriched, long interspersed elements

(LINEs) depleted] (LINEs) (Eller et al. 2007), In the 50 untranslated region, there are more basic sequence repeats (UTR)(Farré et al. 2007), lower conservation of the promoter sequence (Lawson and Zhang 2008), and lower potential for nucleosome formation in the 50 region of these genes (Ganapathi et al. 2005). Some domain families have an abundance of housekeeping gene protein products (Lehner and Fraser 2004).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an enzyme catalyzes the 6th step in the biosynthesis of glyceraldehyde. Glycolysis is a conversion of glucose to energy by a metabolic process. Its involvement in glycolysis isn't its sole one; it also helps with transcriptional control and DNA repair (Sirover 2005)GAPDH is a frequent reference gene that is used to standardize data on gene expression and in the quantitative analysis as an endogenous control of RT-PCR until its expression is extremely constant in several experimental setups GAPDH concentrations, on the other hand, can fluctuate between people for example, during pregnancy development stage and cell cycle(Klie and Debener 2011).

GAPDH was selected as reference gene because its expression showed stability(van Tol et al. 2008) ;in zebrafish embryos, the GAPDH gene was considered less stable (Lin et al. 2009). In fibroblast cell lineages of mice under different treatment conditions with antidepressants, the levels of GAPDH were unstable (Sugden et al. 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Samples Collection

A blood sample was taken from a proposed crime scene from nature in EDTA tube.

3.1.2 Kit

The GENEzolTM TriRNA Pure Kit with phenol and guanidine isothiocyanate, as well as a spin column was used to purify high-quality total RNA from a range of sources.

This kit is manufactured by Taiwanese company Geneaid.

3.2 Study design

The study subjects a blood sample was taken from a hypothetical crime scene from nature in EDTA tube and divided into 40 subsample after extraction of RNA, 10 subsample without inhibitors as control and then was added urea as a inhibitor with different concentrations (10,100,500) μM to 15 subsample and to 15 subsample was added Tannic acid with different concentrations (10,100,500) μM and was compared the results with control group as shown in Figure 3-1.

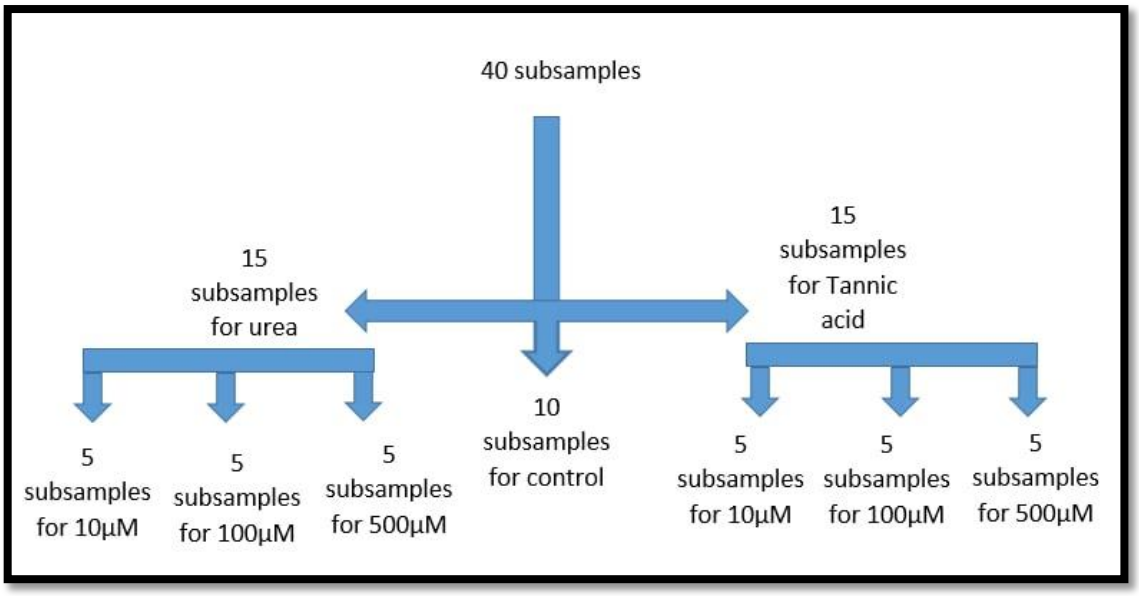

Figure (3-1): Experimental design.

3.3 Extraction of RNA

1. Blood specimen were divided into two fragments and allowed to mix blood for 10 minutes with roller.

2. A mount of 0.2 ml of each specimen was transferred and placed in 1.5 ml Eppendorf tube (free from RNase).
3. Three volumes of GENEzo Reagent were added per one volume of specimen (3:1) then was mixed well by vortex for 1 minute.
4. At room temperature, the specimen mixture was incubated for five minutes.
5. The specimen was centrifuged for 1 minute at 12000 x g to remove cell debris, then the clear supernatant was transferred to another 1.5 ml eppendorf tube (RNase-free)..
6. One volume of absolute ethanol alcohol was added directly to one volume of specimen mixture (1:1) in GENEzo Reagent, then was mixed thoroughly by vortex, and an RB Column was placed in a 2 ml Collection Tube.

7. A mount of 0.7 ml of the specimen mixture was transferred to the RB Column. Centrifuged at 12000 x g for one minute then was discarded the flow-through.
8. The RNA Binding Step was repeated by transferring the remaining specimen mixture to the RB Column.
9. The specimen was Centrifuged at 12000 x g for 1 minute then was discarded the flow-through. Placed the RB Column in a new 2 ml Collection Tube.
10. A mount of 0.4 ml of Washing Buffer was added to the RB Column then was centrifuged at 12000 x g for 30 seconds.
11. The flow-through was discarded and was placed the RB Column back in the 2 ml Collection Tube.
12. DNase I solution was prepared in a 1.5 ml eppendorf tube (RNase-free) as follows:

    Transferred 5 µl of DNase (2 U/µl) + DNase / Reaction Buffer 45 µl = total volume 50 µl
13. The DNase I solution was pipetted gently to mix (without vortex) then was added DNase I solution (50 µl) into the center of the RB column matrix.
14. The column was incubated for fifteen minutes at room temperature (20-30ºC) then was proceed with RNA Wash.
15. Amount of 0.4 ml of Pre-Wash Buffer was added to the RB Column then centrifuged at 12000 x g for thirty seconds.
16. The flow-through was discarded then was placed the RB Column back in the two ml Collection Tube.
17. A mount of 0.6 ml of Wash Buffer was added to the RB Column. And was centrifuged at 12000 x g for thirty seconds then discarded the flow-through. then was placed the RB Column back in the two ml Collection Tube.

18. The steps 16 and 17 were repeated
19. The RB Column was placed back in the two ml Collection tube and was centrifuged at 12000 x g for three minutes for drying the column matrix.
20. The dried RB Column was placed in a clean 1.5 ml eppendorf tube (RNase-free), for RNA elution
21. A mount of 0.1 ml of RNase-free water was added into the center of the column matrix and was standing for at least 5 minutes to ensure the RNase-free water is completely absorbed by the matrix. Then was centrifuged at 12000 x g for one minute to eluted the purified RNA

3.4 PCR inhibitors Preparation

3.4.1. Urea

To prepare 5 mM stock solution of urea preparation, 0.03 g of urea powder was weighted and dissolved in 10 ml doubled deionized water (DDW) then was added 90 ml of DDW to complete final volume to 100ml.

3.4.2. tannic acid

To prepare 5 mM stock solution of tannic acid, 0.85 g of tannic acid powder was weighted and dissolved in 100 ml of (DDW) with stirring.

The final concentration needed were 10,100 and 500 µM. Therefore, two more stocks were prepared for each one:

First stock 5 mM.

Second stock 1 mM (prepared by take1 ml first stock and completed to 5 ml DDW)

Third stock 0.1 mM (prepared by take1 ml 2ed stock and was completed to 10 ml DDW).

3.5 Addition of Inhibitors to forensic subsamples

TransScript® Green One-Step qRT-PCR SuperMix is a one-step qRT-PCR supermix. This kit is manufactured by TransGen Biotech Co., Ltd in China.

Housekeeping gene (primer) GLYCERALDEHYDE_3-PHOSPHATE (GAPDH) DEHYDROGENASE has been used as housekeeping gene as primer:

gapdf GAAGGTGAAGGTCGGAGTCA

gapdr GAAGATGGTGATGGGATTTC

1. the mixture was prepetrated for the RT-real time PCR as a control in the first step as shown in table 3-1 includes:

Table 3-1 mixture of a control for urea RT-real time PCR

Component	Volume
Enzyme	0.4 µl
master mix	10 µl
RNA extracting	2 µl
Primer	1 µl
DDW	6.5 µl
Total volume	20 µl

For RT-real time PCR preparation (20 µl), 5 subsamples control without inhibitors were prepared for run of urea, 5 subsamples control without inhibitors were prepared for run of tannic acid.

2. A.

Preparation the mixture for the device RT-real time PCR as an inhibtor1 urea concertation 500µM as shown in table 3-2 was included:

Table 3-2: Mixture of inhibtor1 urea concertation 500µM for RT-real time PCR

Component	Volume
Enzyme	0.4 µl
master mix	10 µl
RNA extracting	2 µl
Primer	1 µl
DDW	4.5 µl
stock 1 concentration 5 mM	2 µl
Total volume	20 µl

Five subsamples of stock 1 were Prepared

2. B.

Five subsamples were Prepared of stock 2 urea concentration 100µM as shown in table 3-3 included:

Table 3-3: Mixture of inhibtor1 urea concertation 100µM for RT-real time PCR

Component	Volume
Enzyme	0.4 µl
master mix	10 µl
RNA extracting	2 µl
Primer	1µl
DDW	4.5 µl
stock 2 concentration 1mM	2 µl
Total volume	20µl

2.C.

Five subsamples were Prepared of stock 3 by add stock 3 concentration 10µM as shown in table 3-4 include:

Table 3-4: Mixture of inhibtor1 urea concertation 10µM for RT-real time PCR

Component	Volume
Enzyme	0.4 µl
master mix	10 µl
RNA extracting	2 µl
Primer	1µl
DDW	4.5 µl
stock 3 concentration 0.1 mM	2 µl
Total volume	20µl

3. A.

Preparation the mixture for the RT-real time PCR as an inhibtor2 tannic acid as shown in table 3-5 was included:

Table 3-5: Mixture of inhibtor1 tannic acid concertation 500 µM for RT-real time PCR

Component	Volume
Enzyme	0.4 µl
master mix	10 µl
RNA extracting	2 µl
Primer	1µl
DDW	4.5 µl
stock 1 tannic acid concentration 5 mM	2 µl
Total volume	20µl

3. B& C

Five subsamples were prepared of stock 1 and five subsample of stock 2 by added stock 2 instead stock 1 and five subsample of stock 3 by added stock 3 instead stock 1.

Note: since the sizes are small, a mixture for each repeater was made

- from the mixture 18 µl +2 µl RNA (extracted) were taken as a control for 5 repeaters.
- from the mixture 16 µl +2 µl RNA(extracted) +2 µl stock1 were taken for 5 repeaters
- from the mixture 16 µl +2 µl RNA (extracted) +2 µl stock2 (urea) as an inhibitor (100 µM) were taken for 5 repeaters.
- from the mixture 16 µl +2 µl RNA(extracted) +2 µl stock3(urea) as an inhibitor (500 µM) were taken for 5 repeaters, Thus, the total number of subsamples were became 20 subsamples. The same for inhibitor 2(tannic acid) .

  Then were putting it in qRT-PCR and circumstances were proved

  45º C 10min

  94º C 30 sec

  95º C 10sec (denaturation) for 40 cycle

  60º C 30sec (extension+elongation) for 40 cycle

  Acquire on green channel

  Dissociation Stage or melting at 60-95º C

  The same steps were repeated with the other inhibitor (tannic acid).

3.6 Statistical analysis

Statistical analysis was performed by the aid of SPSS software IBM SPSS Statistics Version21 .

CHAPTER 4

RESULTS AND DISCUSSION

The results of our study showed that take off points (ct value) for the samples without urea are higher than ct values for the samples that contain urea as it illustrated in figure (4.1A and 4.1B) below. Which indicates that samples with urea worked as a promotor and had an activation impact on the amplification and did not work as an inhibitor or a stressor(Khan et al. 1991).

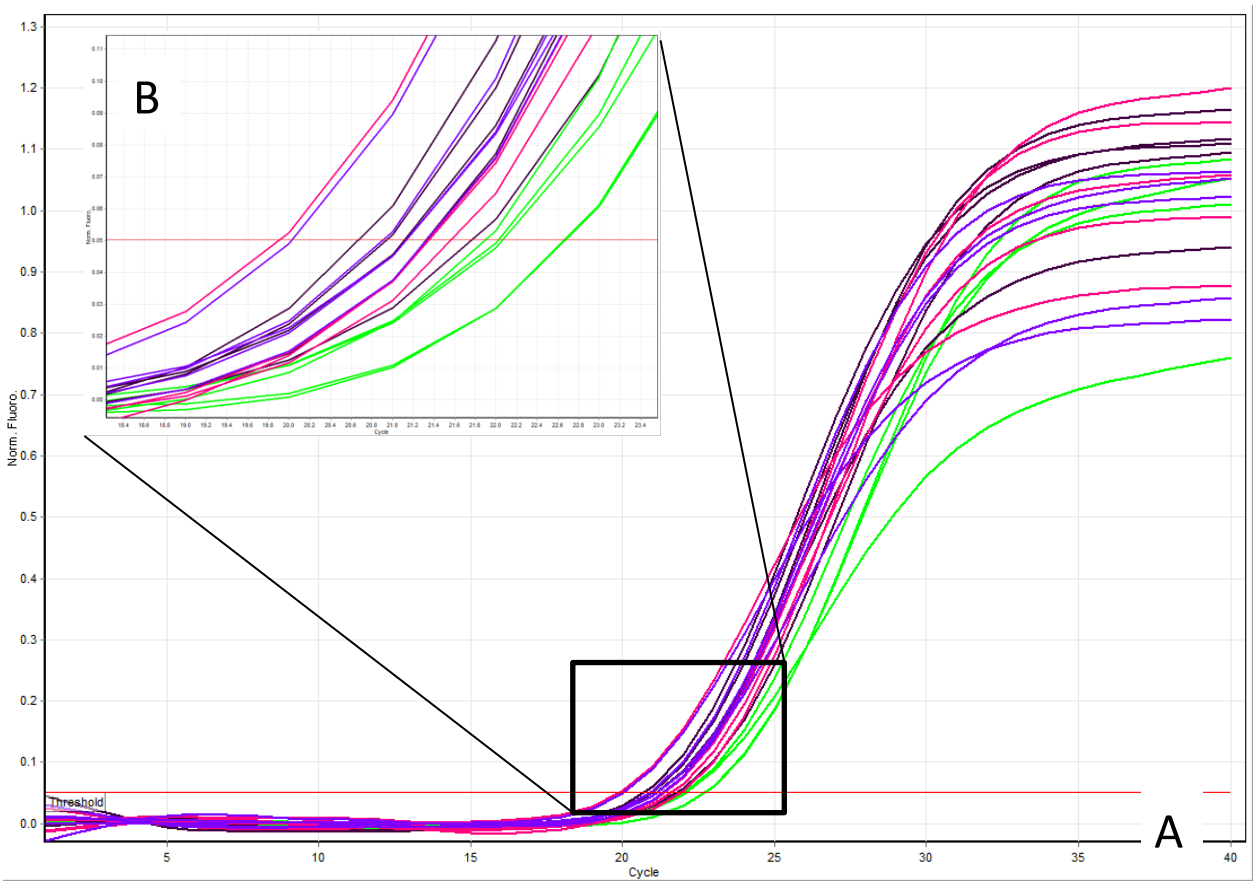

Figure (4.1) A- Rt-Real time PCR amplification of mRNA using different concentration of urea as contaminant . B- is zoomed view on take off point (cycle of threshold).

Where grean cureves are control without urea, black curves are 500µM urea, red curves are 100 µM urea, violet curves are10 µM urea.

The Table 4.1 and figure 4.2 below explain the statistics for the ct values, and it can be seen that the ct values mean for the control (22.3925) which is higher than the mean of ct values for the samples with urea as explained earlier.

Table 4.1: **Cycle of threshold mean, standard error and other parameters for urea experiments groups.**

	N	**Ct Mean**	**Mean ± Std. Error**
Control	5	22.3925	0.19215
10 µM	5	20.9360	0.23551
100 µM	5	21.2540	0.34448
500 µM	5	21.2040	0.18527

The two-way Anova test results are listed in table 4.1 which shows that there is a significant difference at ($P<0.05$) between control group and other groups and that means that urea in 10 µM caused activation to the amplification and from above 10 µM to 500 µM concentration caused increase in amplification.

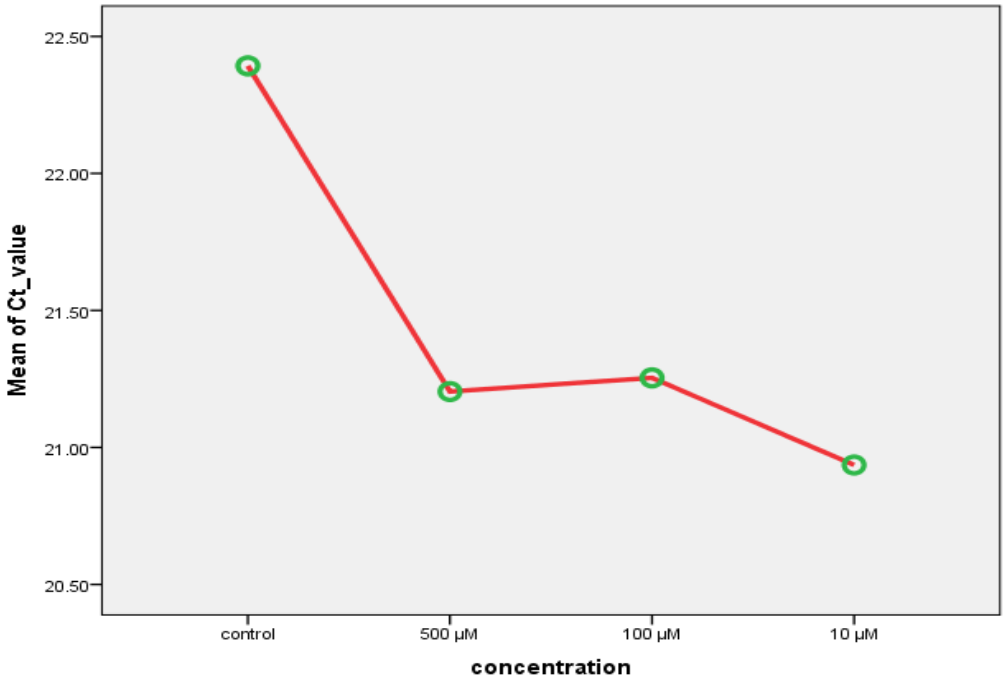

Figure4.2 Mean of ct value of control and different group of urea elements.

Table 4.2 Statistical analysis for the Urea experiment of cycle of threshold mean

Concentration	Concentration	Mean Difference (I-J)	Mean ± Std. Error	P. value
Control (0 µM)	500 µM	1.18850*	0.37198	**0.006**
	100 µM	1.13850*	0.37198	**0.008**
	10 µM	1.45650*	0.37198	**0.001**
10 µM	Control	-1.45650*	0.37198	**0.001**
	500 µM	-0.26800	0.35070	0.457
	100 µM	-0.31800	0.35070	0.379
100 µM	Control	-1.13850*	0.37198	**0.008**
	500 µM	0.05000	0.35070	0.889
	10 µM	0.31800	0.35070	0.379
500 µM	Control	-1.18850*	0.37198	**0.006**
	100 µM	-0.05000	0.35070	0.889
	10 µM	0.26800	0.35070	0.457
*. The mean difference is significant at the 0.05 level.				

The result of the current study regarding urea disagreed with the result of Khan *et al* (1991) who found that urea was an inhibitor for the PCR and this could be a result to the type of the PCR used in our study which rt-PCR not PCR therefore, urea could activate the reverse transcriptase or activate the bond between the primer with RNA or decrease the degradations of RNA.

The other studied parameter in our study was the effect of urea on amplification slope as can be seen in figure 4.3 A and 4.3 B below which is an indicator of the efficiency of the amplification so as the slope is higher the amplification is better.

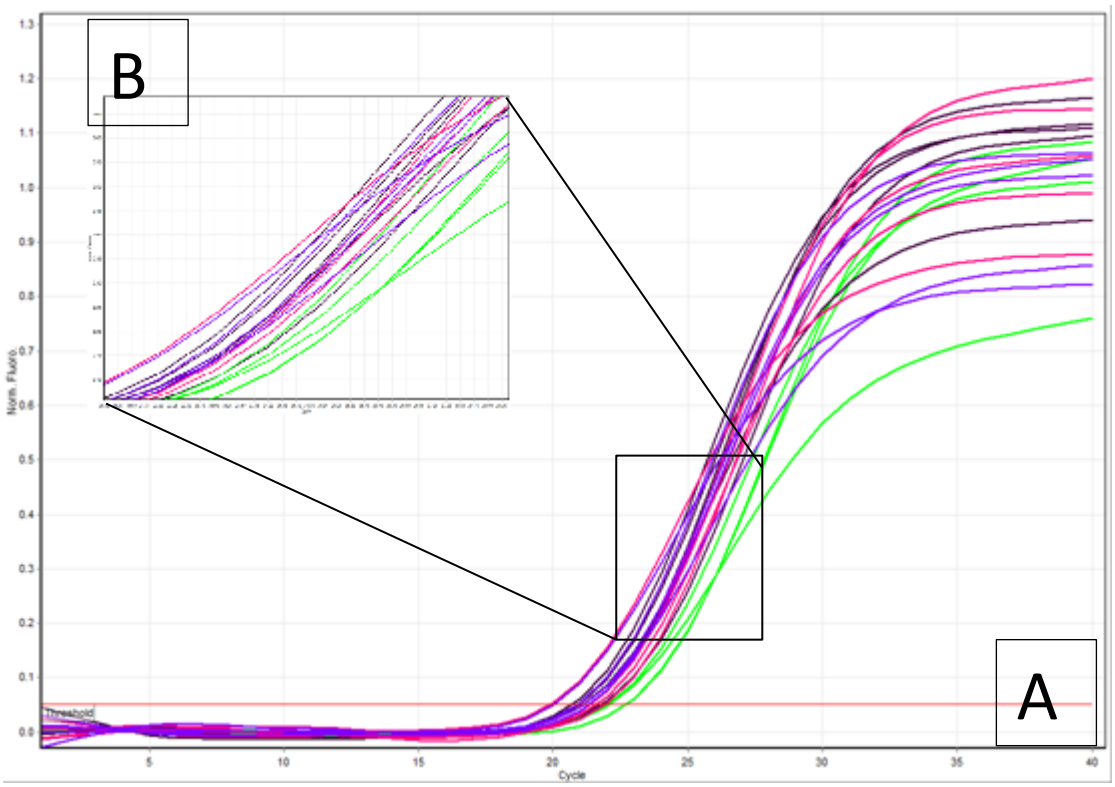

Figure 4.3 A- Rt-Real time PCR amplification of mRNA using different concentration of urea as contaminant. B- is zoomed view on The amplification slope for the urea samples.

The results showed that the mean of the slope for the control group is differ from the other treatment groups as can be seen in table 4.3 below

which means that urea presents a better amplification as for the ct value or for the slope of amplification.

Table 4.3 Amplification slope mean, standard error and other parameters for urea experiments groups.

	N	Mean of slope	Mean ± Std. Error
Control	5	0.0719	0.00373
10 µM	5	0.0899	0.00503
100 µM	5	0.0927	0.00259
500 µM	5	0.0971	0.00427

The statistical analysis in table 4.4 shows that the control group is significantly differ at ($P<0.05$) from other groups and the other treatment group did not differ from each other

Table 4.4 The slope of amplification statistical analysis for the Urea experiment

(I) concentration	(J) concentration	Mean Difference (I-J)	Mean ± Std. Error	P. value.
Control	500 µM	-0.02515*	0.00593	**0.001**
	100 µM	-0.02080*	0.00593	**0.003**
	10 µM	-0.01798*	0.00593	**0.008**
10 µM	Control	0.01798*	0.00593	**0.008**
	500 µM	-0.00717	0.00559	0.219
	100 µM	-0.00282	0.00559	0.622
100 µM	Control	0.02080*	0.00593	**0.003**
	500 µM	-0.00435	0.00559	0.448
	10 µM	0.00282	0.00559	0.622
500 µM	Control	0.02515*	0.00593	**0.001**
	100 µM	0.00435	0.00559	0.448
	10 µM	0.00717	0.00559	0.219
*. The mean difference is significant at the 0.05 level.				

And it can be easily seen in figure 4.4 that the mean of slope for the control 0.07 while other treatment means in the range of 0.09.

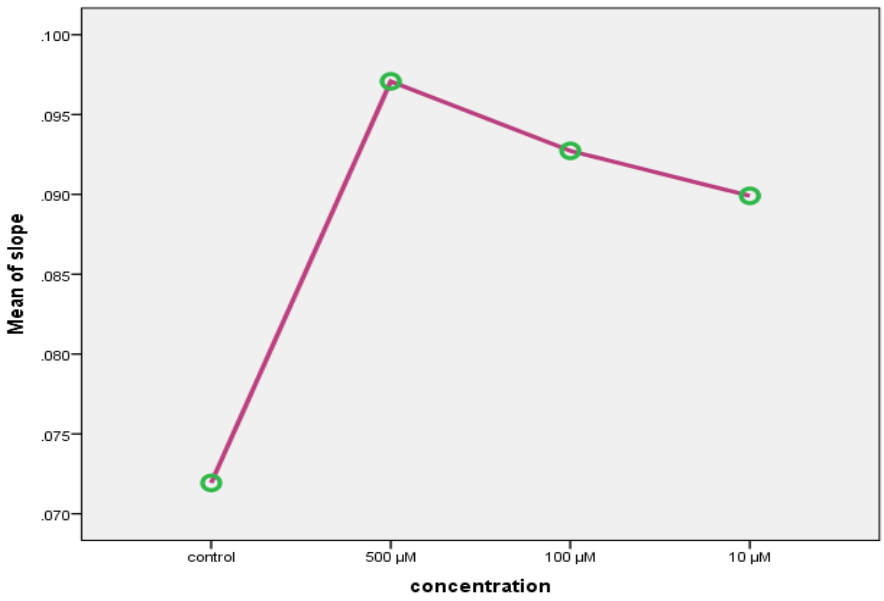

Figure 4.4 Mean of slope of amplification of control and different group of urea treatments.

The other studied parameter in this study was the effect of urea on plateau intensity which is an indicator of the strength of the total amplification power. So, where the amplification is better it gives a better signal. This was calculated by measuring the intensity of the last 5 cycle for each sample as it can be seen in Figure 4.5 A and B.

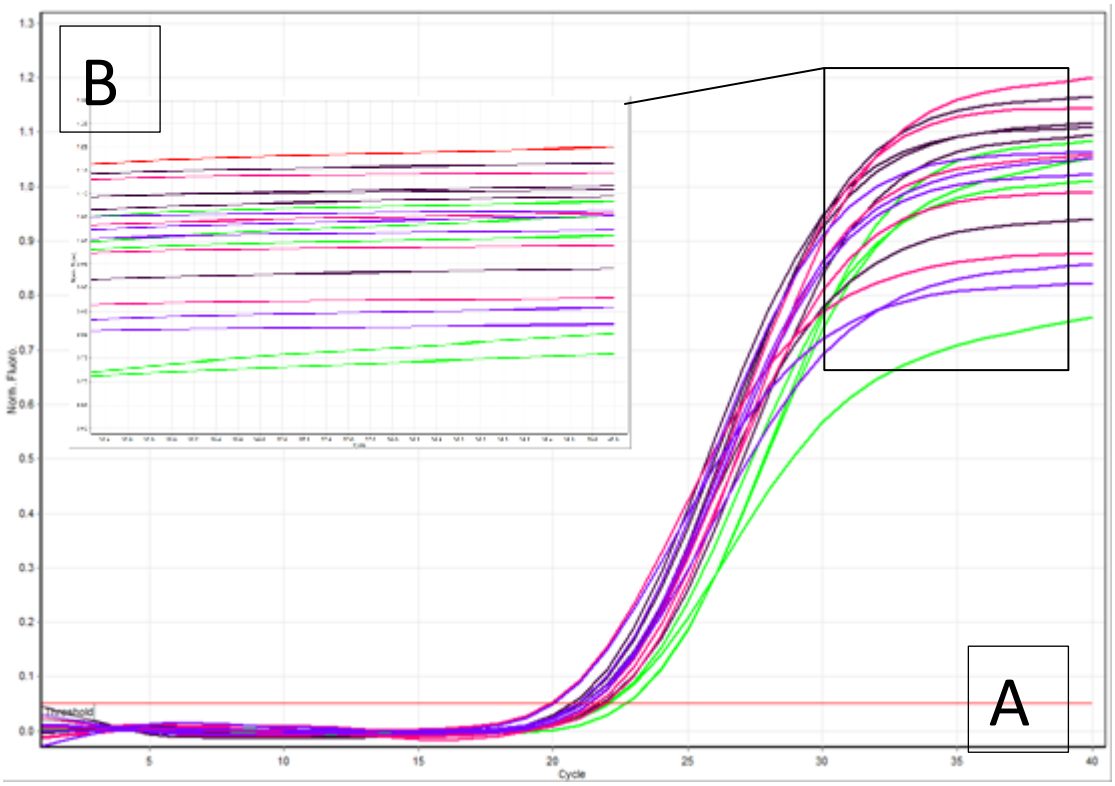

Figure 4.5 A- Rt-Real time PCR amplification of mRNA using different concentration of urea as contaminant. B- is zoomed view on The plateau intensity phase for the urea samples

Table 4.5 Plateau intensity mean, standard error and other parameters for urea experiments groups

	N	Mean	Mean ± Std. Error
control	20	0.9289	0.03174
10 µM	20	0.9592	0.02356
100 µM	20	1.0497	0.02578
500 µM	20	1.0802	0.01753

The results showed that the mean of the plateau intensity for the control group and 10 µM are differ from 100 µM and 500 µM groups as can be seen in table 4.5 and figure 4.6 which means that the plateau intensity is

in direct proportion to the concentration till the concentration of 500 µM. this could also mean the urea is interacting with the dyes that have been using in the rt real-time pcr amplification as cyber green dye that was used in this study, and the urea might increase the bond between the cyber green dye and DNA or increase the fluorescentation of DNA.

Statistically, there was significant difference at ($P<0.05$) between the control group and 10 µM group (Table 4.6) although it is very clear visually (Figure 4.6).

Table 4.6 The plateau intensity of amplification statistical analysis for the Urea.

(I) concentration	(J) concentration	Mean Difference (I-J)	Mean ± Std. Error	P. value
Control	500 µM	-0.15131*	0.03559	**0.000**
	100 µM	-0.12087*	0.03559	**0.001**
	10 µM	-0.03036	0.03559	0.396
10 µM	Control	0.03036	0.03559	0.396
	500 µM	-0.12095*	0.03559	**0.001**
	100 µM	-0.09051*	0.03559	**0.013**
100 µM	Control	0.12087*	0.03559	**0.001**
	500 µM	-0.03045	0.03559	0.395
	10 µM	0.09051*	0.03559	**0.013**
500 µM	Control	.015131*	0.03559	**0.000**
	100 µM	0.03045	0.03559	0.395
	10 µM	0.12095*	0.03559	**0.001**
*. The mean difference is significant at the 0.05 level.				

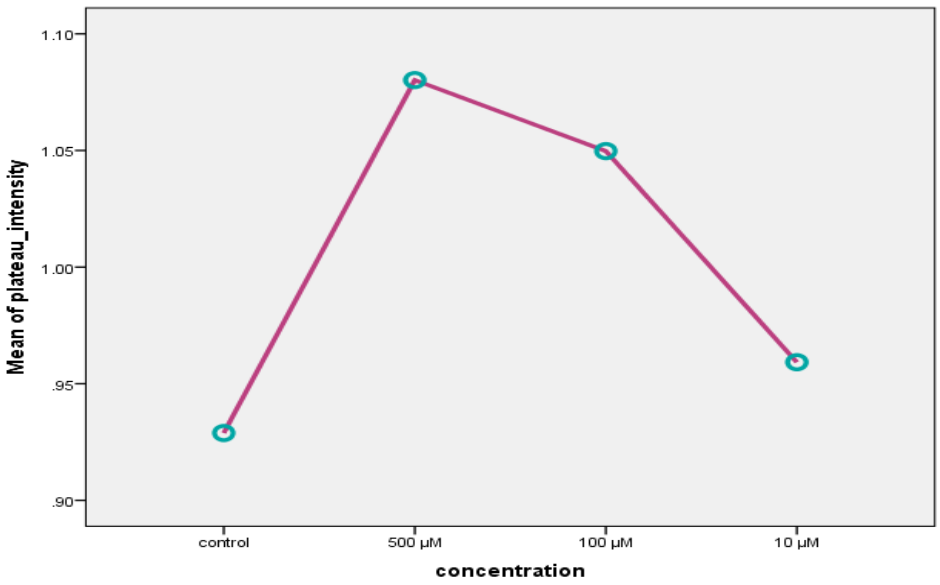

Figure 4.6 The effect of different concentration of urea on the intensity plateau intensity of amplification on RT real-time PCR.

One parameter for the quality of amplification is the melting curve. The effect of urea on melting temperature was recorded in this study. This melting curve is called raw data because after the amplification was carried out, the amplicon was exposed into different temperature and in this study the temperatures were ranged from 73 ^{o}C to 92 ^{o}C and was recorded after each degree till it reach to the point where the double strand starts to dissociate, and a drop happen in the fluorescentation. This point is called the melting point of template as illustrated in Figure 4.8.

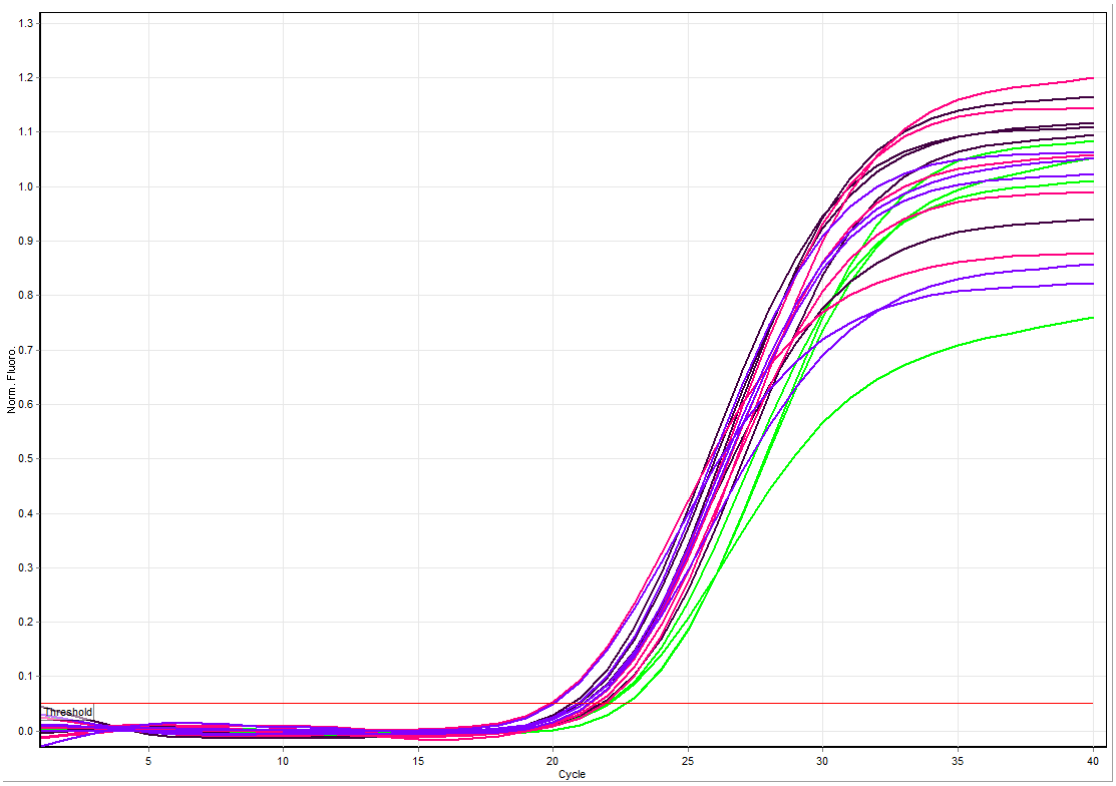

Figure 4.7 Rt-Real time PCR amplification of mRNA using different concentration of tanic acid as contaminant

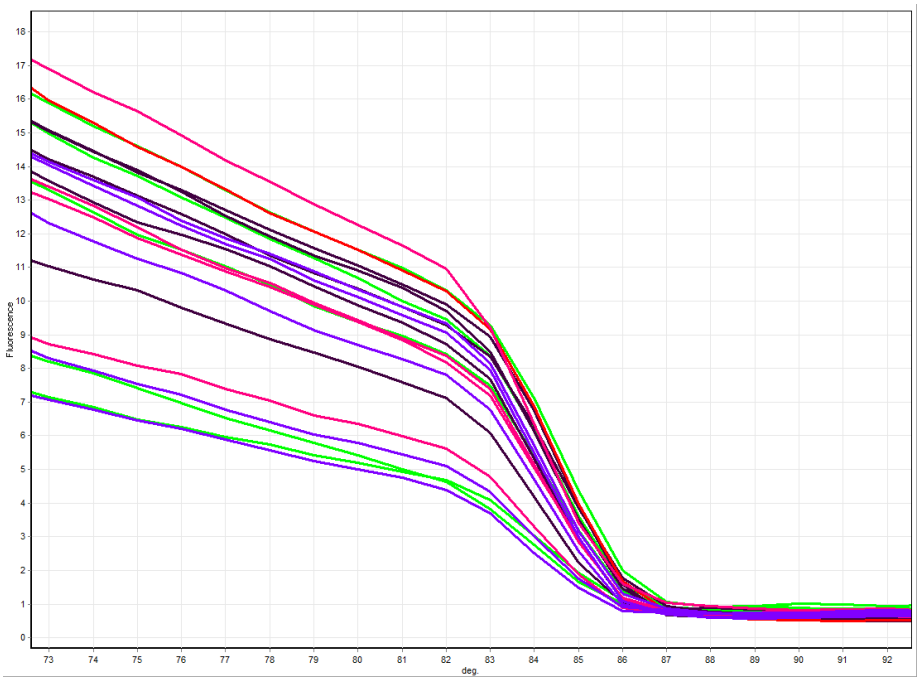

Figure 4.8 melting curve analysis of different urea concentrations

Then the raw data was analyzed and the first derivative of signal (dF/dT) was measured in the figure 4.9. The first derivative determined the exact melting point

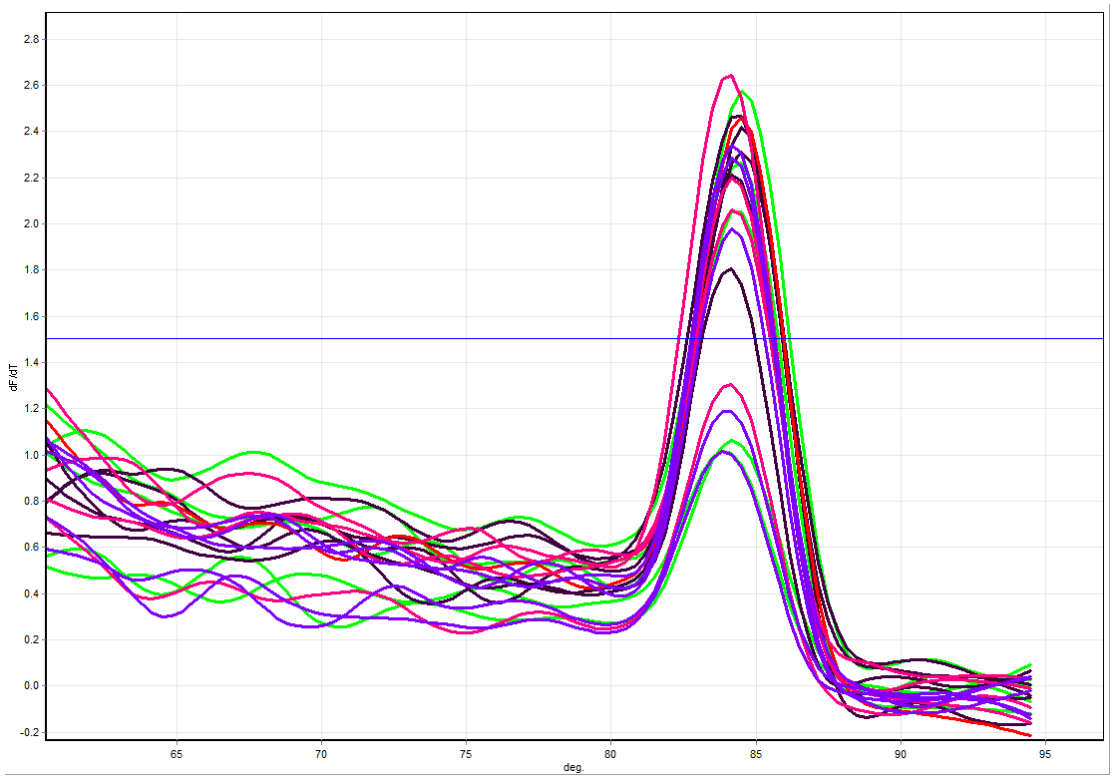

Figure 4. 9 First derivative of signal (dF/dT)

Where dF is delta fluorescentation and dT is delta Temperature. The results showed that there were no significant difference between the different groups of treatment as can be seen in tables 4.7 and 4.8 and figure 4.10 and that means that there was no effect of urea on the melting point of the amplicon.

Table 4.7 Melting curve mean, standard error and other parameters for urea experiments groups

	N	Mean Tm	Mean ± Std. Error
Control	5	84.4600	0.04000
10 µM	5	84.4200	0.04899
100 µM	5	84.3600	0.09798
500 µM	5	84.4600	0.04000

Table 4.8 Statistical analysis for the melting curve .

(I) concentration	(J) concentration	Mean Difference (I-J)	Mean ± Std. Error	P. value
Control	500 µM	0.00000	0.08718	1.000
	100 µM	0.10000	0.08718	0.268
	10 µM	0.04000	0.08718	0.653
10 µM	Control	-.04000	0.08718	0.653
	500 µM	-.04000	0.08718	0.653
	100 µM	0.06000	0.08718	0.501
100 µM	Control	-.10000	0.08718	0.268
	500 µM	-.10000	0.08718	0.268
	10 µM	-.06000	0.08718	0.501
500 µM	Control	0.00000	0.08718	1.000
	100 µM	0.10000	0.08718	0.268
	10 µM	0.04000	0.08718	0.653

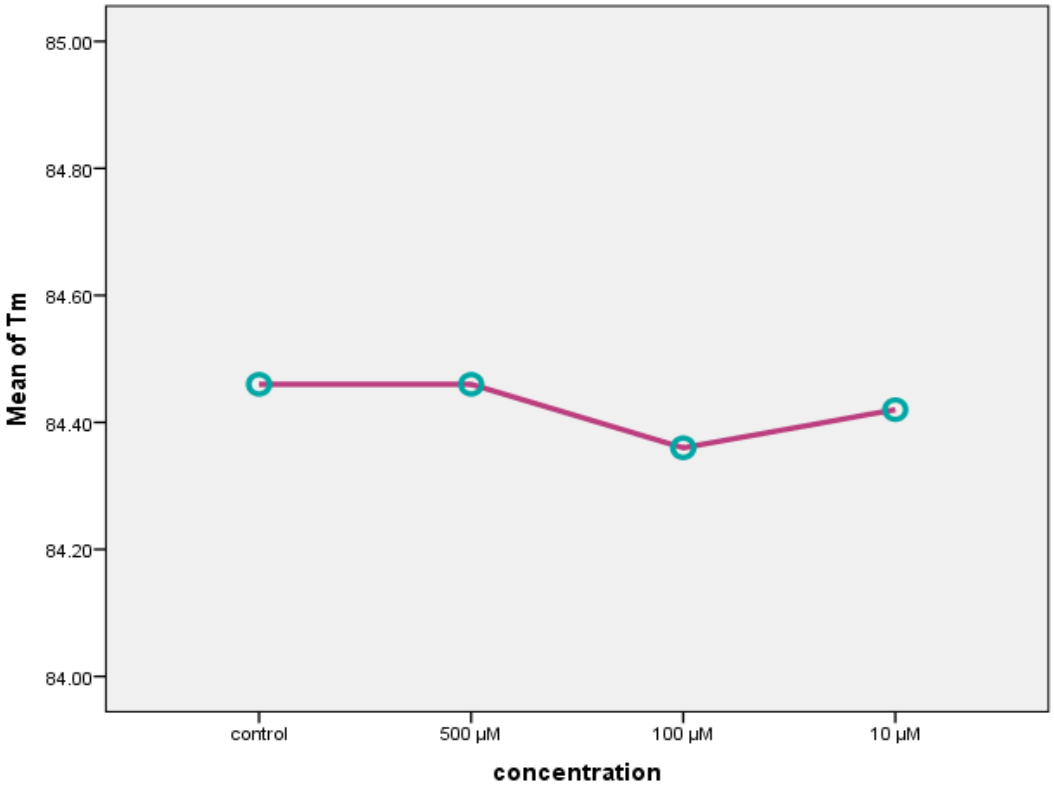

Figure 4.10 The mean of melting temperature (Tm) of different concentrations of urea treatment.

Other important parameter studied is the amplification specificity and signal stability. The specificity of amplicon and signal stability was determined by calculating the RMSD of the melting curve first derivatives (df/dt) for the range of temperature 63-80 C^{o} as shown in figure 4.11.

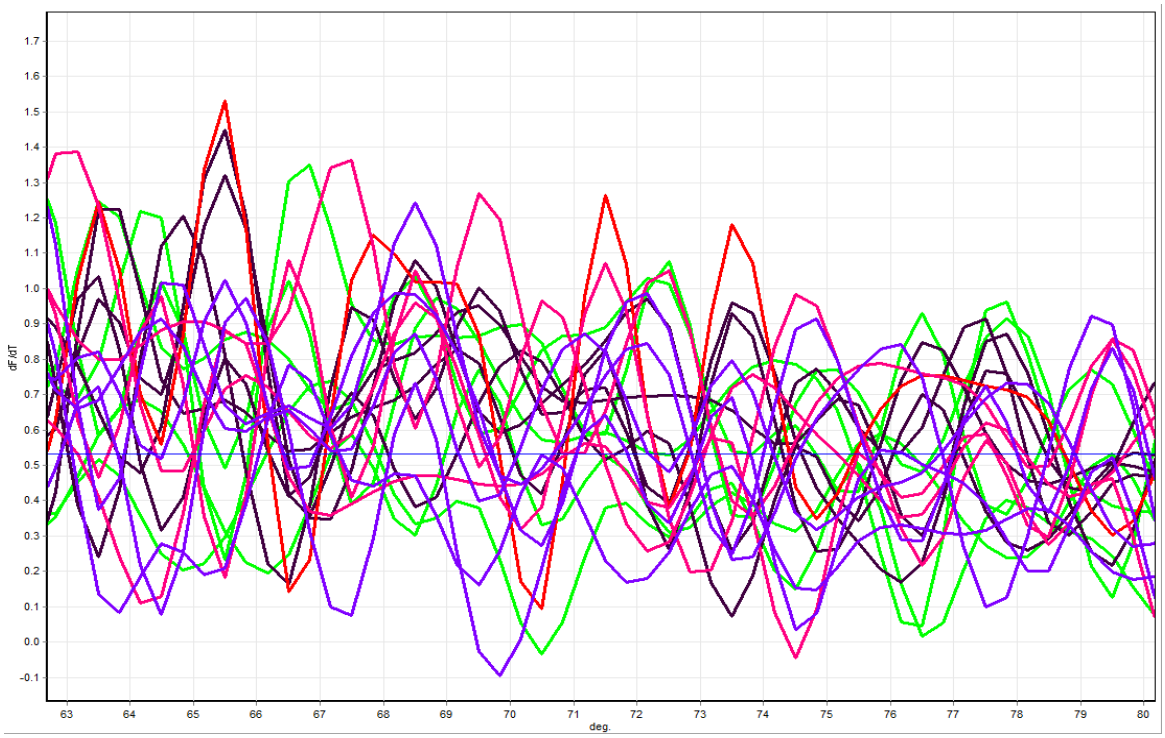

Figure 4.11 The signal stability.

In order to check if the urea had an impact on the specificity of amplicon and signal stability statistics was carried out and it showed that there is no significant differences as it can be seen in tables 4.9 and 4.10 and figure 4. 12.

Table 4.9 Specificity of amplicon and signal stability mean, standard error and other parameters for urea experiments groups

	N	RMSD Mean	Mean ± Std. Error
Control	5	0.2641	0.02933
10 µM	5	0.2503	0.01780
100 µM	5	0.2903	0.03271
500 µM	5	0.2516	0.01858
Total	20	0.2641	0.01225

Table 4.10 Statistical analysis for the specificity of amplicon and signal stability

(I) concentration	(J) concentration	Mean Difference (I-J)	Mean ± Std. Error	P. value.
Control	500 µM	.01249	0.03600	0.733
	100 µM	-.02621	0.03600	0.477
	10 µM	.01382	0.03600	0.706
10 µM	Control	-.01382	0.03600	0.706
	500 µM	-.00134	0.03600	0.971
	100 µM	-.04004	0.03600	0.283
100 µM	Control	.02621	0.03600	0.477
	500 µM	.03870	0.03600	0.298
	10 µM	.04004	0.03600	0.283
500 µM	Control	-.01249	0.03600	0.733
	100 µM	-.03870	0.03600	0.298
	10 µM	.00134	0.03600	0.971

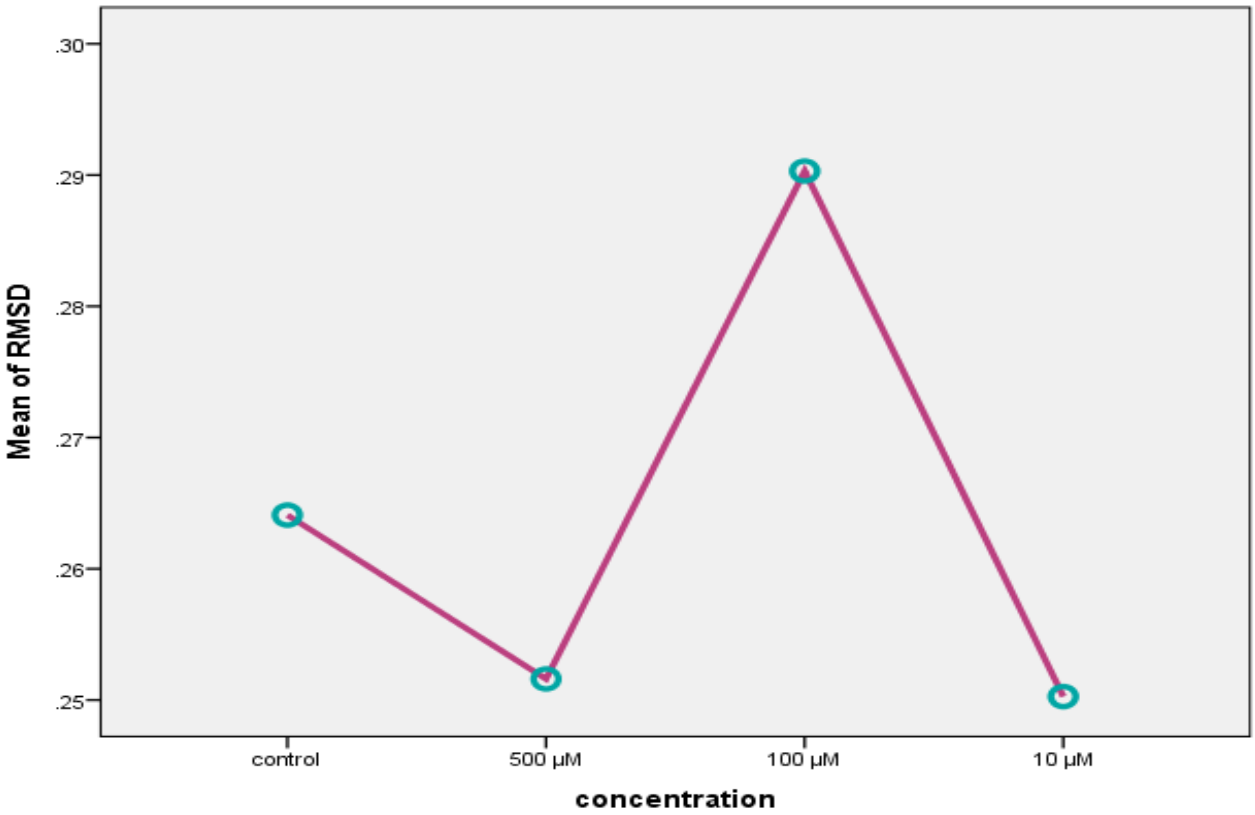

Figure 4.12 the mean of amplicon and signal stability of different concentrations of urea treatment.

For the tannic acid, the amplification worked as a stressor as it can be seen in figure (4.13 A and 4.13 B). The amplification revealed that even a trace quantity of tannic acid (10µM) able to inhibited the total amplification of RT- real time PCR this is may be due to the effect of tanic acid on Reverse transcriptase enzyme, or Taq polymerase enzyme, or may be effect on RNA or DNA

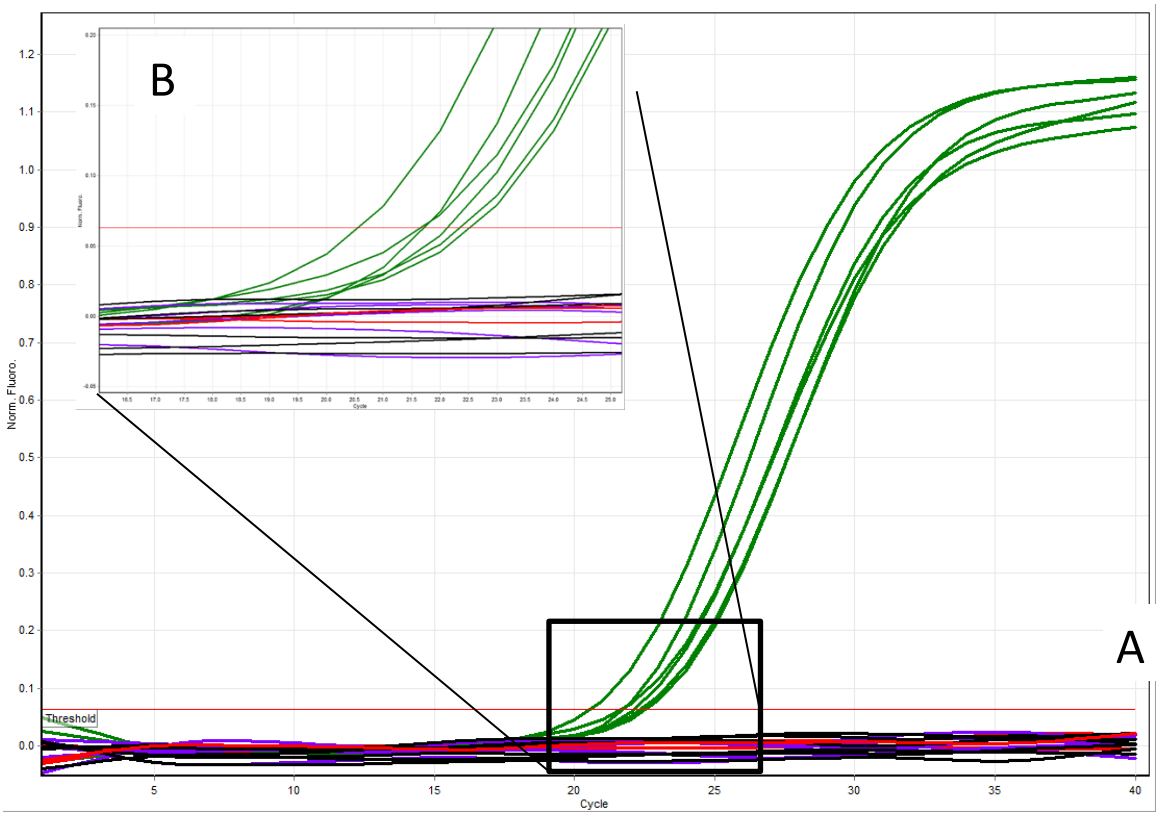

Figure 4.13 A- Rt-Real time PCR amplification of mRNA using different concentration of tannic acid as contaminant, and B- is Amplification Zoomed take off region.

Where grean cureves are control without tanic acid , black curves are 500µM , red curves are 100 µM , and violet curves are 10 µM . Melting curve for the tannic acid is illustrated in figure 4.14, the figure showed that tannic acid inhibited the formation of RT-PCR product completely.

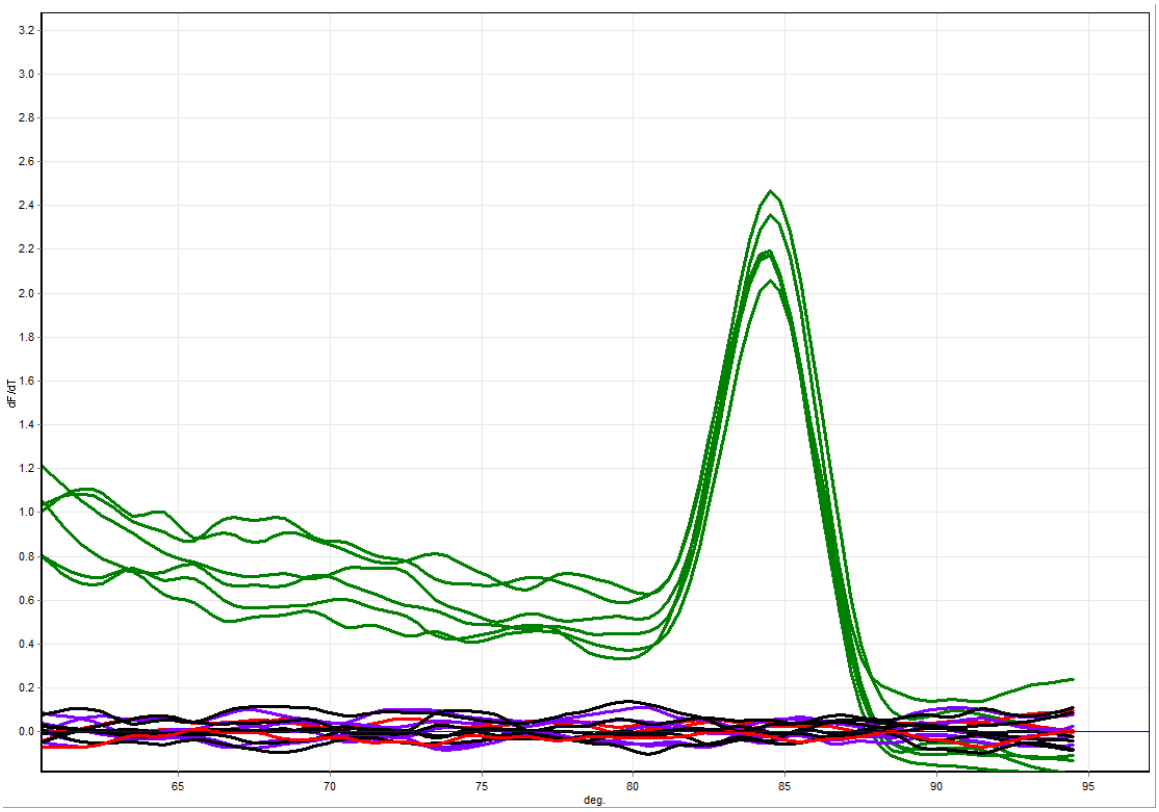

Figure 4.14 Melting curve analysis of different tannic acid concentrations.

Conclusion

- Urea promoted the amplification and did not work as an inhibitor.
- Urea interacted with the dye that used in this study, and the urea might increase the bond between the cyber green dye.
- Urea had no impact on the specificity of amplicon
- Tannic acid inhibits the amplification even trace quantity of 10µM.

Recommendations

1. The stressor impact of urea and tannic acid should be evaluate before forensic investigations.
2. More extensive studies on the effects of urea and tannic acid are needed.
3. Future exploration into the effect of other chemical inhibiters could be useful to solve the common forensic problems worldwide.

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الخلاصة

الهدف من هذه الدراسة هو التحقيق في تأثير المثبطات التي يمكن أن تؤثر على الكشف عن سوائل الجسم المختلفة في مسرح الجريمة ، وخاصة تلك الموجودة بشكل طبيعي في البيئة أو من تأثير العوامل الأخرى. اليوريا وحمض التانيك كلاهما من العوامل الكيميائية الموجودة بيئيًا والتي تم تسجيلها نسبيًا في مسرح الجريمة ، وبالتالي فهي مفيدة لتطبيقات الطب الشرعي. إحدى المعلمات التالية هي عتبة الدورة (Ct) وهي النقطة التي تتجاوز فيها شدة التألق كثافة التألق الخلفية. نتيجة لذلك ، كلما زادت كمية الحمض النووي المستهدف في مادة البداية ، كان ذلك أفضل. المعلمة الأخرى كانت درجة حرارة الانصهار (Tm) ، ومنحدر التضخيم وشدة الهضبة. لذلك ، تمت دراسة تأثير هذه المعلمات على تضخيم تفاعل البوليميراز المتسلسل في الوقت الحقيقي لتحديد التأثير المثبط لهذين العاملين بتركيزات مختلفة من 10 مايكرومولاري و 100 مايكرومولاري و 500 مايكرومولاري عند إضافتها إلى عينات الدم ومقارنتها مع المجموعة الضابطة ، أوضحت النتائج أن اليوريا لم تعمل كعامل إجهاد أو مثبط. أما بالنسبة لمتوسط Ct ، فقد انخفض مقارنة مع التحكم ولا يوجد فرق معنوي لجميع التركيزات عند $p < 0.05$ ، أظهر متوسط منحدر التضخيم زيادة مقارنة بمجموعة التحكم ولكن لا يوجد فرق معنوي لـ جميع التركيزات عند $p < 0.05$ ، بالنسبة لشدة الهضبة ، أظهرت النتائج أن متوسط كثافة الهضبة لمجموعة التحكم و 10 مايكرومولاري يختلف عن مجموعات 100 مايكرومولاري و 500 مايكرومولاري وهناك فرق معنوي لتركيز 100 مايكرومولاري و 500 مايكرومولاري مقارنة مع السيطرة والتركيز 10 مايكرومولاري عند $p < 0.05$ ، لم تظهر النتيجة لجميع التراكيز أي فرق معنوي في متوسط درجة الانصهار مقارنة بالتحكم عند $p < 0.05$. ومع ذلك، كانت قيمة عتبة الدورة(Ct) لحمض التانيك أقل من 2.5 في جميع التركيزات لأنه يعمل كمثبط.

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التأثير المحتمل لبعض المثبطات الكيمياوية على تحديد الجين في عينة دم جنائية

بحث مقدم الى
مجلس كلية العلوم – جامعة بابل
كجزء من متطلبات نيل درجة الدبلوم العالي في العلوم/ أدلة جنائية
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1443هـ 2022م