EXPRESSION OF GENES ENCODING FUNDAMENTAL COPPER HOMEOSTASIS PROTEINS IN COPPER RESISTANT ESCHERICHIA COLI MUTANTS

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ABSTRACT

EXPRESSION OF GENES ENCODING FUNDAMENTAL COPPER HOMEOSTASIS PROTEINS IN COPPER RESISTANT ESCHERICHIA COLI MUTANTS

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Heavy metals are used as antimicrobial agents for thousands of years. Their areas of usage include water and food disinfection, preservation of food and beverages, prevention of dysentery, treatment of leprosy, tuberculosis, gonorrhea, syphilis, an alternative to laundry detergents and biocontrol agents for crops of fruits in developing countries. Copper is one of the widely used heavy metals for its antimicrobial property. On the other hand, it is an essential heavy metal for the most living systems. Over 30 proteins were shown to contain copper. Copper alternates between reduced Cu(I) and oxidized state Cu(II). Therefore, copper containing enzymes use this alternation in the redox reactions they catalyze. These proteins take part in important metabolic reactions such as respiration, electron transport, and protection against oxidative stress. However, under aerobic conditions when copper is in excess, redox cycling of copper results in the production of highly reactive hydroxyl radicals. These radicals damage proteins, DNA, and lipids. Consequently, copper homeostasis is strictly controlled in the bacterial cells.

In this study, spontaneous and UV-induced copper mutants were generated from *Escherichia coli* ATCC 8739 parent strain. Then, among the known genes that

contribute to copper homeostasis *copA*, *cusS* and *cusF* were selected, and their expression levels were measured. The expression levels of these genes in mutants were compared to the levels in parent strain in the presence or absence of copper in the medium. Expression differences of these genes between control strain (*E.coli* ATCC 8739) and mutant strains (spontaneous and UV-induced, obtained from *E.coli* ATCC 8739) were determined via RT-qPCR technique. The statistical significance of the results was established with one-way ANOVA with Tukey's multiple comparison test. In the end, it has been shown that expressions of *copA*, *cusS*, and *cusF* genes increase significantly both in spontaneous and UV-induced mutants with respect to the parent strain. That indicates that *copA*, *cusF* and *cusS* genes are crucial for the copper-resistance. The analyses of the results indicated that there was no difference between the spontaneously obtained mutants and the UV-induced mutant in terms of copper homeostasis genes' expressions. That implies that the gene expression levels were independent of the processes through which mutants were obtained. This study showed that the copper homeostasis genes' expressions augmented 6 times for *cusS*, 21 times for *copA*, and to 24 times for *cusF* over the parent strain's expressions. These increases in expressions obviously provide a mean for survival under copper stress.

Keywords: Escherichia coli ATCC 8739, spontaneous mutant, UV-induced mutant, RT-qPCR, copper resistance

ÖZ

BAKIRA DİRENÇLİ ESCHERICHIA COLI MUTANTLARINDA ÖNEMLİ HOMEOSTAZ PROTEİNLERİNİ KODLAYAN GENLERİN EKSPRESYONU

Uyanık, Tuğçe
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Ağır metaller binlerce yıldır antimikrobiyal ajan olarak kullanılmaktadır. Su ve gıdaların dezenfekte edilmesi, yiyeceklerin ve içeceklerin muhafaza edilmesi, dizanterinin önlenmesi, cüzam, tüberküloz, bel soğukluğu, frengi gibi hastalıkların tedavisi, gelişen ülkelerde meyve fidelerinde biyokontrol ajanı olarak kullanılması ve alternatif çamaşır deterjanı olarak kullanılabilmesi ağır metallerin kullanım alanları arasındadır. Bakır ise antimikrobiyal özelliğinden dolayı en yaygın kullanılan ağır metallerden biridir. Buna ek olarak, bakır birçok canlı için temel ağır metallerdendir. 30'un üzerinde proteinin bakırı içerdiği görülmüştür. Bakır, indirgenmiş hali olan Cu(I) ve yükseltgenmiş hali olan Cu(II) arasında değişim göstermektedir. Bakır içeren enzimler ise bakırın bu özelliğinden yararlanarak redox tepkimelerini katalize ederler. Bakır içeren enzimlerin yer aldığı önemli metabolik olaylar arasında solunum, elektron taşınması ve oksidatif strese karşı korunma yer almaktadır. Fakat aerobik ortamlarda bakır miktarı çok fazla olduğunda, yüksek derecede reaktif olan hidroksil radikaller üretmeye başlar. Bu radikaller ise DNA'ya, proteinlere ve lipitlere zarar verir. Dolayısı ile, bakır homeostazı, bakteri hücrelerinde sıkı bir şekilde kontrol edilmektedir.

Bu çalışmada, spontane ve UV'ye maruz bırakılarak elde edilen bakıra dirençli mutantlar *Escherichia coli* ATCC 8739 suşundan elde edilmiştir. Bakır homeostazında görev aldığı bilinen genler arasından ise *copA, cusS* ve *cusF* genleri seçilmiş ve ekspresyon seviyeleri ölçülmüştür. Ölçülen ekpresyon seviyeleri orijinal *Escherichia coli* ATCC 8739 suşu ve mutant suşlar kullanılarak bakır varlığında ve yokluğunda incelenmiştir. Ekspresyon farklılığının tespiti için RT-qPCR tekniği kullanılmıştır. Sonuçların istatistiksel önemi ise tek faktörlü varyans analizi (one-way ANOVA) ve Tukey çoklu karşılaştırma testi ile gösterilmiştir. Çalışma sonucunda, belirlenen *copA, cusS* ve *cusF* genlerinin ekspresyonunun hem spontane hem de UV ile elde edilmiş mutantlarda orijinal suşa göre belirgin bir şekilde arttığı tespit edilmiştir. Bu sonuç, belirlenen 3 genin bakır homeostazı için önemli genler olduğunu göstermiştir. Buna ek olarak, istatistiksel analizler göstermiştir ki spontane ve UV ile elde edilmiş mutantlar birbiri ile karşılaştırıldığında belirlenen genlerin ekspresyonu açısından herhangi bir fark bulunmamaktadır. Bu sonuç mutantların elde edildiği mutagenez yönteminin bakır homeostaz genlerinin ekspresyonu için bir önem taşımadığı anlamına gelir. Bu çalışma aynı zamanda, yüksek bakırlı ortamda, orijinal suşa oranla, bakır homeostaz genlerinin *cusS* geni için en az 6, *copA* geni için en az 21, *cusF* geni içinse 24 kat arttığını göstermiştir. Bu durum belirlenen genlerin yüksek bakır içeren ortamlarda yaşayabilmek için hayati önem taşıdığını ifade etmektedir.

Anahtar Kelimeler: Escherichia coli ATCC 8739, spontane mutant, UV ile uyarılmış mutant, RT-qPCR, bakır direnci

To my family…

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ABBREVIATIONS	
Ag	Silver
ATCC	American Type Culture Collection
Bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CFU	Colony Forming Unit
Cq	Cycle quantification
Cu	Copper
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
EDTA	Ethylenediamine tetraacetic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GC	Guanine-Cytosine Content
GTE	Glucose Tris EDTA
MFP	Membrane fusion protein
$MgCl_2$	Magnesium chloride
MIC	Minimal Inhibitory Concentration
OMF	Outer membrane factor
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid

RNase	Ribonuclease
RND	Resistance nodulation cell division
Rpm	Revolution per minute
RT-Qpcr	Quantitative Real Time Polymerase Chain Reaction
TAE	Tris-acetate-EDTA
Tm	Melting Temperature
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1. Heavy Metal Resistance Mechanisms

Microorganisms are exposed to many different heavy metals in their natural environments. Some of the heavy metals do not have any function in cells; on the other hand, some are necessary for biological processes. The need of metals in biological processes calls for the use of uptake systems. Bacterial cells use two systems for the metal ion uptake. The first uptake system depends on the gradient difference between two sides of the cytoplasmic membrane. This system is fast but unspecific, which is used by many metal ions when there is a concentration difference. The second uptake system is substrate-specific and slower compared to the first system. Those active transporters use ATP for uptake against concentration gradient. Therefore, the second system is used only when there is a starvation or an extraordinary metabolic circumstance. Under normal conditions, heavy metal ion uptake is done via nonspecific ion uptake systems (Nies & Silver, 1995).

Nevertheless, when the metal ions are in excess in the environment, cells need to develop resistance mechanisms in order to prevent damage to the cellular components. There are six accepted resistance mechanisms for the bacterial cells (Rouch et al., 1995a; Silver, 1992). The first one is excluding the metal ions by altering the membrane permeability. This can be done by the changes in the cell wall, cell membrane or the envelope of the cell (Bruins et al., 2000). For instance, altering the production of outer membrane porins to exclude Cu^{2+} ions (Rouch et al., 1995a). The second mechanism is active transport or efflux of heavy metals which already entered into the cells. In this mechanism, the excess metal ions are exported from the cell before they associate with metal-sensitive components of the cell (Bruins et al., 2000).

This mechanism is highly specific for the ion and the efflux can be ATPase dependent or independent (Nies & Silver, 1995; Silver, et al., 1989). The third resistance mechanism is intracellular isolation of metal ion with the help of protein-binding which is called intracellular-sequestration. By this mechanism, the metal ions accumulates within the cytoplasm by binding to specific proteins, which result in the prevention of the heavy metal ion interaction with the vital cellular components (Bruins et al., 2000). The fourth mechanism is extracellular sequestration of the metal ion. This is done by secreting a specific protein outside the cell which binds to heavy metals with a high affinity. This way, toxic metals form a large complex structure and cannot enter the cell (Bruins et al., 2000). The fifth resistance mechanism is detoxifying metals by enzymatic reactions to less toxic forms. In this system, less toxic forms of heavy metal cation are obtained generally by sequential enzymatic reactions and toxic effects of heavy metal ions diminish intracellularly (Bruins et al., 2000). The sixth and the last resistance mechanism is decreasing the sensitivity of cellular components to heavy metals. This system is seen in some microorganisms for the adaptation to heavy metal presence in the environment. This is a natural way of cells to protect themselves and that is achieved mainly in two ways. The first is gaining mutations which cause decrease in the sensitivity of the vital cellular components without affecting their basic functions. The second is over-producing the particular cellular components so that, some components do not encounter the metals (Bruins et al., 2000).

Microorganisms may have one or more of these resistance mechanisms in several different combinations to survive (Rouch et al., 1995a). The number of mechanisms they acquire determines their resistance range (Bruins et al., 2000). The acquired resistance may be chromosomal or plasmid-mediated (Rouch et al., 1995b). However, the resistance to essential heavy metals such as copper is usually chromosomal. Because the presence of essential heavy metals is obligatory for the biochemical reactions to occur, if the metal is at toxic concentrations in the environment, chromosome-based resistance is necessary. Those types of mechanisms are dependent

and driven through homeostatic regulation systems. They appear to be more complex than those of the plasmid-based resistance mechanisms. Generally, plasmid-based resistance mechanisms depend on empowering efflux activity which is one constitutive action (Bruins et al., 2000).

1.2. Copper Homeostasis

Copper is an essential heavy metal for the most living systems. The reactions, these enzymes catalyze, include respiration, electron transport and protection against oxidative stress (Grass et al., 2011). By alternating between reduced Cu(I) and oxidized Cu(II) states, copper provides metallo-enzymes an appropriate center for redox reactions (Lippard & Berg, 1994). However, under aerobic conditions when in excess, redox cycling of copper results in production of highly reactive hydroxyl radicals (Equation 1)(Grass et al., 2011; Halliwell & Gutteridge, 1984).

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + OH^{-} + OH^{\cdot} \quad (1)$$

These radicals harm proteins, DNA and lipids (Santo et al., 2008). Moreover, copper reacts with thiol moieties of cysteines and replace the actual the native metals of the proteins (Lippard & Berg, 1994). Copper should be supplied to the proteins for the survival and maintenance of the vital metabolic processes. Meanwhile, intracellular free copper concentration is kept as low as possible. For this reason, copper homeostasis in the bacterial cells need to be controlled strictly (Argüello et al., 2013; Burkhead et al., 2009).

1.2.1. Copper Homeostatic Mechanisms in *Escherichia coli*

As far as the copper homeostasis concerned, two regulatory systems are recognized which sense and respond to the internal copper concentration status in *Escherichia coli*. The first one is composed of two component signal transduction mechanism and named as Cus system whose denomination is coming from Cu-sensing (Munson et al.,

2000; Oshima et al., 2002). The second copper-responsive mechanism is called Cue. This mechanism takes its name from Cu efflux and is regulated by CueR (copper-induced transcriptional activator). CueR regulates *copA* and *cueO* genes (Outten et al., 2000; Petersen & Møller, 2000; Stoyanovet al., 2001a). CopA is considered as the central component of this mechanism and highly important for the copper homeostasis in bacterial cells (Rensing & Grass, 2003).

1.2.1.1. CopA as central component of Cue system

CopA is the main protein of the cytoplasmic copper homeostasis in *E. coli* and is a component of Cue system. Both in aerobic and anaerobic conditions, this protein is required for inherent copper resistance. CopA is included in P-type ATPase family. This protein family is ubiquitous and does the ATP-driven pumping of charged substrates across the membranes. Due to the formation of a phosphorylated intermediate during the reaction, they are called P-type ATPases (Axelsen & Palmgren, 1998; Moller et al., 1996). CopA translocates Cu(I) from the cytoplasm to the periplasmic space (Figure 1.1) and considered as the primary copper-efflux ATPase in *E. coli*. Regulation of CopA is done by CueR. CueR is a transcription factor and stimulates the transcription of CopA together with CueO. CueO is a periplasmic multi-copper oxidase and oxidizes Cu(I) to Cu(II) (Outten et al., 2000; Petersen & Møller, 2000). CueR is activated by the intracellular Cu(I) concentration, which results in the up-regulation of *copA* and *cueO* expressions (Outten et al., 2000; Petersen & Møller, 2000; Stoyanov et al., 2001b). Therefore, in the presence of excess copper, CopA makes the efflux of Cu(I) from the cytoplasm to the periplasmic space (Yamamoto & Ishihama, 2005).

1.2.1.2. Cus system

Protection of cytoplasm from excess copper concentration is provided by CopA. However, gram negative bacteria, such as *E. coli*, also have to translocate copper out of periplasmic space. That is necessary to protect the proteins working in inter-membrane area from copper-induced damage. In *E. coli*, periplasmic copper is translocated by Cus system. The system (Figure 1.1) is composed of two operons whose transcriptions occur in opposite directions; *cusCFBA* and *cusRS* (Rensing & Grass, 2003). CusCBA constitutes an RND-type Cu^{+}-efflux transporter. The transporter takes its name from the central pump protein, CusA, which is included in resistance nodulation cell division family (RND). RND family proteins utilize energy coming from proton-substrate antiport (Goldberg et al., 1999; Dietrich H Nies et al., 1989; Rensing et al., 1997; Tseng et al., 1999). The other two components of the transporter are CusB which is a membrane fusion protein (MFP) and CusC which is an outer membrane factor (OMF) (Dinh et al., 1994; Rensing & Grass, 2003). Another gene in the operon codes for periplasmic protein CusF. CusF chaperones Cu^{+} and transport to CusCBA for the efflux by forming direct interaction with CusB (Randall et al., 2014). Therefore, CusF performs two roles for the cell. The first one is capturing copper ions to decrease their accumulation in the periplasm and the second one is to shuttle copper to the corresponding transporter. CusF can discriminate copper from other metals, therefore, collateral removal of other metals does not take place (Bagai et al., 2008; Loftin et al., 2009; Loftin et al., 2005).

Moreover, CusRS constitutes a two-component regulatory mechanism. CusS is a cytoplasmic membrane-bound histidine-kinase. It senses periplasmic copper ions and subsequently phosphorylates the response regulator CusR. In turn, CusR activates the transcription of *cusCFBA* (Figure 1.1) (Kim et al., 2011; Rensing & Grass, 2003). It was shown that activation of CusS is necessary for the upregulation of *cusCFBA* expression in the presence of excess copper and the limitation of periplasmic copper accumulation (Gudipaty et al., 2013).

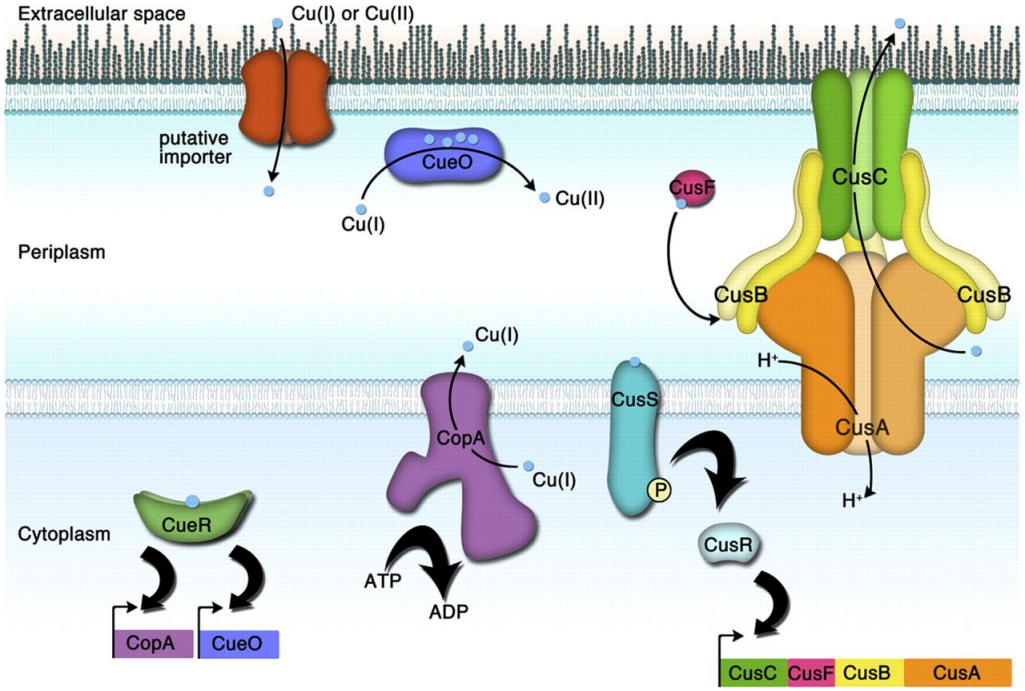

Figure 1.1. Copper homeostasis mechanisms in Escherichia coli (Kim et al., 2011).

1.3. Heavy Metals

Heavy metal term refers to the metals with a density higher than 5 g/cm³ (D. H. Nies, 1999). They are found on the Earth's crust naturally. Copper (Cu), mercury (Hg), cadmium (Cd), arsenic (As), chromium (Cr), thallium (Tl), and lead (Pb) are some examples of heavy metals (LennTech, 2017). Although, some of them do not have any known biological function like silver, aluminum, cadmium, gold, lead, and mercury, some have biological functions in microorganisms. These heavy metals, which are considered as essential, are calcium, cobalt, chromium, copper, iron, potassium, magnesium, manganese, sodium, nickel, and zinc (Bruins et al., 2000). In biochemical reactions they contribute to catalyses, stabilize the structures of proteins and the cell walls. Also they assist in osmotic balance maintenance (Hughes & Poole, 1991; Ji &

Silver, 1995; Poole & Gadd, 1989). For instance, magnesium and zinc participate in enzyme and DNA stabilization thorough electrostatic forces, potassium and sodium regulates the intracellular osmotic pressure (Dietrich H. Nies, 1992). Furthermore, among essential metals, iron, copper and nickel take part in redox reactions (Bruins et al., 2000).

Although some heavy metals are regarded as essential for biological processes, their high concentrations cause toxicity in microorganisms. That is due to heavy metals' ability to combine with a large range of biological molecules. They interact with the ligands of the proteins and acts as an inhibitor for a wide range of enzymes (H. Passow, 1961). Also, their high concentration cause conformational changes in proteins and nucleic acids; oxidative phosphorylation disturbance; and osmotic imbalance in the cell (Poole & Gadd, 1989). On the other hand, some heavy metals are transition metals, which mean that they have incomplete orbitals, resulting in formation of metal cations. This makes some of them readily enter into complex redox reactions. At high concentrations, these metal cations lead to non-specific binding to cellular components resulting in bizarre formations and toxicity in bacterial cells (D. H. Nies, 1999).

1.3.1. Use of Heavy Metals as Antimicrobial Agents

The use of heavy metals as antimicrobial agents dates far back in the human history (Lemire et al., 2013). From the Persian Period, for the disinfection of water and conservation of food, containers made up of Ag and Cu were used. Later, Phoenicians, Greeks, Romans and Egyptians also used these metals to preserve their food and water. By 1800, to keep wine, water, vinegar and milk pure, people had used silver vessels (Alexander, 2009). Also, the coins made of Ag were added to the bottom of the vessels in order to keep beverages clean by the settlers of North America. This method was also used by the Japanese soldiers in World War 2 to prevent dysentery (Alexander, 2009; Borkow & Gabbay, 2009).

In history, heavy metals were used in medicine as well. Silver nitrate was used to prevent the gonorrheal eye infections in newborns, while foils made up of silver were used to wrap surgical wounds in order to prevent infection (Crede, 1881; Silver et al., 2006). Te, Mg and As oxides and Cu and Hg salts were also used for the treatment of some diseases such as leprosy, tuberculosis, gonorrhea and syphilis in the last two centuries (Ehrlich & Bertheim, 1912; Frazer, 1930; Hodges, 1889; Keyes, 1920; Pereira, 1836). In agriculture, copper sulphate ($CuSO_4$) was used to make 'The Bordeaux mixture' in 1880s in order to preserve grape wines from downy mildew parasite (Ayres, 2004). This mixture is also used to cure plant-based diseases; potato blight, peach leaf and apple scab (Ayres, 2004; Dixon, 2004).

In the last decades, heavy metals have still been used for their antimicrobial property. In material science, Ag^+, Cu^{2+} and Zn^{2+} are used to produce antimicrobial ceramics to eliminate contamination on biomaterials (T. N. Kim et al., 1998). Moreover, nowadays, silver-drenched bandages are accessible for medical purposes. The fabrics prepared by the same method are available in sleeping bags and sports socks production to impede the growth of microorganisms and decrease the odor. Ceramic discs composed of metallic silver-copper are also retailed for being an alternative to laundry detergents for customers who are allergic (Silver, 2003). Meanwhile, in agriculture, copper salts are used as organic biocontrol agents for tomato and other crops of fruits in developing countries, Europe and North America (Dixon, 2004).

Therefore, understanding of antimicrobial activity of heavy metals is essential due to their increasing use both in medicine and industry. These substances became integral to retail products and in time started to disperse in the environment (Lemire et al., 2013). Increase of the heavy metal dispersal results in accumulation of heavy metals. Accumulating metals provide means for bacteria to get exposed and develop resistance mechanisms (Seiler & Berendonk, 2012).

1.4. *Escherichia coli* ATCC 8739

Escherichia coli ATCC 8739 is designated as Crooks strain. This strain is isolated from fecal samples and has an extended usage in laboratories for over 70 years (Esselen et al., 1939). Main areas which this strain is used include bioresistance testing, quality control studies, food testing, media testing, tests for pharmaceutical care and personal care and antimicrobial preservative studies (ATCC, 2017). Although *Escherichia coli* is a prokaryotic model organism and a rare pathogen, there is no reported case for pathogenicity of ATCC 8739 strain; therefore, biosafety level of this strain is 1 and can be handled with relative ease (Archer et al., 2011).

Also, ATCC 8739 strain only expresses OmpF porin compared to the E*scherichia coli* K12 strain which possesses both OmpF and OmpC porins (Pinto et al., 2011). Copper and other metal ions pass from the outer membrane into the cell via OmpF and OmpC porins (Lutkenhaus, 1977). However, *E. coli* mutants deficient in both OmpF and OmpC porins and the ones lack OmpC or OmpF individually exhibited no important difference in copper or silver resistance (Li et al., 1997).

1.5. Spontaneous and UV-induced Mutations in *Escherichia coli*

Modifications which take place by unknown mechanisms in chromosomal DNA during recombination, DNA replication or DNA repair are called spontaneous mutations (Drake, 1970; von Borstel, 1969). Spontaneous mutations mostly attributed to the errors during DNA replication, however, in *E.coli,* the mutants arise from error-prone DNA- repair mechanism (Sargentini & Smith, 1985; Smith, 1992).

On the other hand, induced mutations occur due to external physical or chemical mutagenic factors and in turn increasing the frequency of mutations in the cells (Elizabeth F. Rangel & Carvalho, 2017). UV light is a physical mutagenic agent which usually results in pyrimidine dimer formation (Coulondre& Miller, 1977). In the *Enterobacteriaceae* family, in general, *E.coli* is used in the studies involving

spontaneous and induced mutations or mutant formations (Elizabeth F. Rangel & Carvalho, 2017). In the UV-light spectrum, UVC light has the most dangerous effects on DNA due to its ability to cause GC-AT transitions, thymine dimers and back mutations at the specific sites of the genes (Wójcik & Janion, 1997).

1.6. Aim of the Study

In this study, spontaneous and UV-induced *E.coli* ATCC 8739 copper resistant mutants were generated by one time exposure to high concentration of copper. Any copper resistant *E.coli* mutants obtained through acute exposure was not reported in the literature so far. Investigating the copper resistance in gene expression level in conjunction to copper homeostasis was aimed in the study. To relate the expression of important genes for copper homeostasis and copper resistance, 3 genes were chosen; *copA*, *cusS* and *cusF*. Real Time Quantitative Polymerase Chain Reaction (RT-qPCR) technique was used to measure the expression level differences between parent control strain (*E.coli* ATCC 8739) and mutant strains (spontaneous and UV-induced, obtained from *E.coli* ATCC 8739). 16S Ribosomal RNA gene is customarily used as a housekeeping gene in RT-qPCR. In this study, as a housekeeping gene, 16S ribosomal RNA did not give stable results under heavy metal stress for *E.coli*. Therefore, an alternative housekeeping gene, *tus*, is found. This study is the first time that *tus* is used as reference gene in RT-qPCR experiments with *E.coli* under copper-driven stress conditions and related resistance mechanisms.

CHAPTER 2

MATERIALS AND METHODS

2.1. Culture Media

E.coli ATCC 8739 was used as the control strain in this study. The bacterial cultures were propagated in Nutrient Broth (Appendix C) at 37°C in incubator-shaker (Appendix A), at 180 rpm for 16 hours. For short-term storage, spread plates on nutrient agar of fresh bacterial culture was used (Appendix C) and the plates were stored at 4°C. For long-term storage, control strain was grown in nutrient broth as described above until the cells reach their mid-log growth phase. The culture was transferred to microfuge tubes and centrifugated at 3000 rpm for 5 minutes, the supernatants were discarded. The pellets were re-suspended in sterile glycerol (50%) solution and vortexed. Then, the aliquots in glycerol were stored at -80°C.

2.2. Heavy Metal-Containing Media for Mutants

The 310 μg/ml copper(II)chloride dihydrate (1,82 mM) containing Nutrient Agar plates (Appendix C) were prepared for the propagation of copper-resistant spontaneous and UV-induced mutants of *E.coli*. The colonies of mutant cells from solid media were streaked onto the copper-containing nutrient agar plates and incubated at 37°C, overnight (for about 16 hours)(Appendix A) then stored at 4°C. The culture passages were done on monthly basis by streaking for the short-term storage of the mutant cells. For long-term storage, the mutant cell cultures were kept in glycerol as described above.

2.3. Determination of Minimal Inhibitory Concentration

Minimal inhibitory concentration (MIC) of *E.coli* ATCC 8739 strain for copper(II) chloride dihydrate was determined in solid media (nutrient agar). European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology was followed for the MIC determination. 'Agar Dilution' technique was performed (EUCAST, 2000). The copper(II) chloride dihydrate solution was filter sterilized and agar plates containing different copper(II) chloride dihydrate concentrations were prepared (Appendix C). The concentrations were ranging from 10 μg/ml to 360 μg/ml of the media. The culturing of *E.coli* ATCC 8739 strain on copper-containing plates was done via spreading and the plates were incubated for 18 hours at 37°C. After incubation, the lowest concentration which inhibits the visible growth of *E.coli* ATCC 8739 strain colonies was accepted as the MIC of this strain for copper chloride dihydrate.

2.4. Spontaneous Mutant Detection and Selection

To obtain spontaneous mutants, *E.coli* ATCC 8739 was grown in nutrient broth media for 18 hours at 37°C in incubator-shaker (Appendix A and C). Then, inoculation of culture was done by spreading on nutrient agar plates having copper(II) chloride dihydrate concentrations ranging from 290 μg/ml to 360 μg/ml with 10 μg/ml increments. The plates, then, incubated at 37°C for 16 hours. The cells were exposed to high copper concentration acutely and without acclimation. The selectable copper resistant mutants were found upon screening 70 plates. After a bacterial colony was detected on an agar plate, the colony was streaked on an agar plate containing the same copper chloride dihydrate concentration. The concentration used for the maintenance of the spontaneous mutants was 310 μg/ml. The spontaneous mutants were named as TG-C01 and TG-C02.

2.5. UV-Induced Mutant Detection and Selection

For UV-induced mutant detection, *E.coli* ATCC 8739 was grown in nutrient broth media for 18 hours at 37°C in incubator-shaker (Appendix A and C). Then, in a sterile environment (laminar flow hood), cell culture was directly exposed to UV for 10 seconds. UV-exposed cell culture was then spread onto nutrient agar plates having 310 μg/ml copper(II) chloride dihydrate. The copper concentration for the UV-induced mutants' detection was the same concentration at which the spontaneous mutants were detected. By keeping the copper-resistance levels at the same concentration, we tried not have an additional parameter which may complicate the analysis of gene expression studies. After spreading, the cells were incubated at 37°C for 16 hours. In the selection of UV-induced mutants, similar to the procedures that we followed in finding spontaneous mutants, no acclimation was done for the cells. Upon UV-exposure, the cells were spread on to 310 μg/ml copper containing solid media. To find a UV-induced mutant, over 100 plates were screened. After a bacterial colony was detected on nutrient agar plate, this colony was streaked to an agar plate containing the same concentration of copper(II) chloride dihydrate for the propagation of the strain. The UV-induced copper resistant mutant was named as TG-C03.

2.6. Primer Design

Due that qPCR does not provide consistent results if dimerization occurs between primers, the product sizes of primers were kept between about 90 and 250 bp. The repeats of cytosine and guanine bases longer than 3 was avoided. The primers were so designed that the melting temperatures (T_m) of primers were between 50-65°C and GC-contents were between 50-60 %.

The primers were designed for the genes; *copA, cusS, cusF,* and *tus.* The *tus* gene was used as reference gene in this study. *E.coli* ATCC 8739 genome (accession number of CP000946.1) was used as template for the design. The details for the primers were given in Table 2.1.

Table 2.1. *Genes and the primer sequences.*

Name of the Gene	Name of the Primer	Sequence of the Primer	Product Size
tus	Forward primer	GGGCAAGCGTGTTGTACTTG	103 bp
	Reverse primer	TGCCACAGAACGCGAAGTTA	
copA	Forward primer	TTGACGGGAAAGCAGTAGCC	134 bp
	Reverse primer	ATTGGCGGTGGTTGGGTTAT	
cusS	Forward primer	ATGTCTTTACCCGCCAGTCC	249 bp
	Reverse primer	CCGCCAGGTTGAGCATTTTC	
cusF	Forward primer	AAAGAAAGGTTGCCCTGCTG	89 bp
	Reverse primer	TTTACCATCACCCCGCAGAC	

2.7. Primer Optimization

In order to determine the optimal working conditions of primers, primer optimization was done. The optimizations were done by using conventional Polymerase Chain Reactions (PCR) technique. The genomic DNA of a mutant strain was used as template.

2.7.1. Genomic DNA Isolation

For the primer optimization, genomic DNA of one of the spontaneous mutants; TG-C01 was used as template for PCR. First, TG-C01 was streaked onto an agar plate containing 310 µg/ml copper(II) chloride dihydrate and incubated at 37°C for 18 hours. Then the cells were collected from the surface of the solid media. Isolation of genomic DNA was performed by the boiling method described in detail in Appendix

I. In this method, the cells are burst open at high temperature in GTE (glucose/Tris/EDTA) Mix. The mixture provides an optimum environment for the released cell content after the cells burst. The buffer contains 3 main components which are glucose, tris and EDTA. Glucose is used for the balance of the osmolarity between inside and outside of the cell. Tris is found in the buffer to maintain pH of 8.0 which is considered as optimum to prevent acid degradation of DNA and other undesired damages to the cell components. EDTA is used to clump metal ions found in the solution mixture thereby pathways using these metals to degrade DNA is inhibited (Boyle, 2005; Casali, 2010; Dashti et al., 2009; Steehler, 2009) Additionally, we included RNase (Appendix D) into the GTE mix to prevent the contamination of DNA by RNA sequences to reach a desired genomic DNA purity. Preparation of GTE mix was given in Appendix B.

2.7.2. Determination of Annealing Temperature

To determine optimum annealing temperature for each primer set, temperature gradient polymerase chain reaction (PCR) was done. The temperature gradient range was set between 54°C to 64°C and determined in accordance with the Tm values of the primers. The gradient PCR was performed with The Ampliqon PCR kit (Appendix D) and genomic DNA of TG-C01 was used as template. Optimum $MgCl_2$ concentration was determined for all primer sets and additional DMSO was added to reaction mix for the stabilization of the designed primers. The composition of PCR mixture was given in Table 2.3. The PCR cycles were repeated for 35 times. The PCR conditions used for all primer sets were given in Table 2.2.

Table 2.2. *PCR conditions for all primer sets.*

PCR Step	**Temperature**	**Duration**
Initial Denaturation	94°C	5 minutes
Denaturation	94°C	45 seconds
Annealing	54-64°C	25 seconds
Extension	72 °C	15 seconds
Final Extension	72 °C	7 minutes

Table 2.3. *Composition of temperature gradient PCR mixture.*

Component	**Final Concentration or Volume**
Taq 2X Master Mix	12,5 µl
Forward Primer	0,1 µM
Reverse Primer	0,1 µM
DMSO	4% (v/v)
25 mM $MgCl_2$	2,5 mM
Template DNA	10-50 ng
Total Volume (with molecular grade H_2O to reach final volume)	25 µl

2.7.3. Agarose Gel Electrophoresis and Visualization

After amplification of genomic DNA was done in different annealing temperatures with PCR, the PCR products were visualized. The visualizations were done by using 1,5% (w/v) agarose gels including 1X TAE Buffer (Appendix B). The amplified DNA fragment samples were loaded to agarose gels along with a size marker (Appendix E) and the gels were run for 50 minutes. The gel was soaked into 1X TAE buffer containing 0,5 μg/ml ethidium bromide. The DNA bands in gels were visualized under UV light with a shortwave UV transilluminator (Appendix A). The temperature giving the brightest band on gel was selected as the optimum annealing temperature for the specified primer set to be applied in RT-qPCR.

2.8. Total RNA Isolation

To isolate total RNA from control strain *E.coli* ATCC 8739 and copper mutants, Thermo Scientific RNA isolation kit (Appendix D) was used according to the manufacturer's instructions (Appendix F). In order to prevent any contamination, all RNA isolation steps were carried out in PCR cabinet (Appendix A). The kit was designed to be used for the cells growing in liquid, since we had to obtain the cells from solid media, the duration of the incubation at which the cells were collected was optimized and the incubation time providing the maximum RNA amount was selected. For the control strain, the optimum incubation time was found to be 4 hours while both for the spontaneous and UV-induced mutants it was found to be 5,5 hours at 37°C. All equipments used in the RNA isolation were given in Appendix A.

After total RNAs were isolated both for mutants and control strain, RNA quality was checked with Biodrop (Appendix A). Two parameters were controlled; absorbance ratio at 260/280 and at 260/230. The absorbance ratio 260/280 was expected to be around 2.0 for RNA samples. The ratio which is lower than 2.0 indicates that there is a contamination in the sample with protein, phenol or other contaminants. On the other hand, absorbance ratio of 260/230 is used to detect the nucleic acid purity. It was

expected to be around 2.0-2.2 for RNA samples in order to accept that the sample is pure and free from contaminants (Geuther, 2007). In the study, RNA samples which satisfy these parameters were accepted and the RNA isolation was repeated 3 times each with 3 technical replicates.

2.8.1. Qualification Determinations of Isolated RNA with Gel Electrophoresis

In order to qualify isolated RNA samples, gel electrophoresis was used. The agarose gel (2% w/v) was prepared with 1X TAE Buffer (Appendix B). The RNA samples together with a size marker (Appendix E) were loaded to the agarose gel and run for 50 minutes. Next, in order to visualize the RNA isolates under UV light, the gel was soaked into 1X TAE buffer containing 0,5 μg/ml ethidium bromide. After adequate time in soaking buffer, which was nearly 20 minutes, the bands were visualized under UV light. The equipment used was given in Appendix A.

2.9. DNase Treatment

Before cDNA synthesis, to make sure that there was no DNA contamination in the samples, the samples were treated with DNase. The DNase I enzyme used in the treatments is an endonuclease cleaving single and double stranded DNA and hybrids of RNA and DNA nonspecifically. DNase I kit of Thermo Fisher was preferred (Appendix D) and manufacturer's instructions were followed throughout the treatment and given in Appendix G. All steps were done in PCR cabinet to prevent contamination. The equipment used throughout the treatment was given in Appendix A.

2.10. cDNA Synthesis

After the treatment of samples with DNase I, in order to obtain cDNAs, cDNA Synthesis kit of Thermo Fisher was used (Appendix D). Manufacturer's instructions were followed throughout the treatment described in Appendix H. Instead of gene specific primer, random hexamers were used, and a pool of all possible cDNA was acquired for all samples. All preparation steps before synthesis were carried out in PCR cabinet. The equipment used throughout the cDNA synthesis step was given in Appendix A.

2.10.1. Control of cDNA with PCR

In order to be sure that the synthesis of cDNA was successful, by using the primer of the reference gene *tus*, a PCR operation was done for all samples (PCR kit from Ampliqon, Appendix D). The concentrations of $MgCl_2$ and DMSO were set according to the primer optimization results. Annealing temperature was set to the primer's optimum working temperature. The PCR conditions used were given in Table 2.4, while composition of PCR mixture was shown in Table 2.5. All preparation process was done in PCR cabinet. The information about PCR cabinet and PCR machine was given in Appendix A.

Table 2.4. *PCR conditions for the control of cDNA synthesis.*

PCR Step	**Temperature**	**Duration**
Initial Denaturation	94°C	5 minutes
Denaturation	94°C	45 seconds
Annealing	54°C	25 seconds
Extension	72 °C	15 seconds
Final Extension	72 °C	7 minutes

Table 2.5. *Composition of PCR mixture for the control of cDNA synthesis.*

Component	**Final Concentration or Volume**
Taq 2X Master Mix	12,5 µl
Forward Primer	0,1 µM
Reverse Primer	0,1 µM
DMSO	4% (v/v)
25 mM $MgCl_2$	2,5 mM
Template DNA	10-50 ng
Total Volume (with molecular grade H_2O to reach final volume)	25 µl

2.10.2. Qualification of cDNA with Gel Electrophoresis

The qualification of cDNA samples was determined with gel electrophoresis. The agarose gel (1,5% w/v) was prepared with 1X TAE buffer (Appendix B). All samples were loaded to gel along with a size marker (Appendix E) and run for 50 minutes. Then, the gel was soaked in 1X TAE buffer containing 0,5 μg/ml ethidium bromide for 20 minutes. The bands were visualized under UV light (Appendix A).

2.11. RT-qPCR Standard Curve Analysis

Standard curve analysis was carried out to determine the efficiency of RT-qPCR runs for the cDNA samples for each primer set. As standard curve samples, 5 different cDNA dilutions of the spontaneous mutant TG-C01 were used. Usage of TG-C01 was arbitrary. The dilutions (1/10, 1/20, 1/40, 1/80 and 1/160) were prepared by using molecular grade nuclease-free H_2O (Appendix D). These dilutions were used as template and their C_q values were expected to fall between the range of 15-25 to be considered as an efficient reaction. The standard curve analysis was carried out for each primer set. Bio-Rad RT-qPCR machine and Bio-Rad SYBR Green Mix (Appendix D) were used. During the analysis, manufacturer's instructions were followed.

The concentration of primers was set to 0.5 μM, and 3 μl of template cDNA was used with a total volume of 10 μl. The RT-qPCR conditions were 3 minutes at 95°C as initial denaturation followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 30 seconds. The melting curve step was added at the end of each run in order to ensure that only one template was amplified during the run corresponding to a single peak. The temperature range for melt curve analysis was 50-99°C with 1°C incremental increase at every 5 seconds.

2.12. Quantitative Real-Time PCR (RT-qPCR)

Quantitative real-time PCR technique was applied to 4 samples which were 2 spontaneous mutants (TG-C01 and TG-C02), 1 UV-induced mutant (TG-C03) and 1 control strain (*E. coli* ATCC 8739) for 4 different genes; *tus* (as reference gene), *copA*, *cusS* and *cusF*. BioRad SYBR Green Mix was used as qPCR kit (Appendix D) and during the preparations manufacturer's instructions were followed. BioRad CFX Real-Time PCR Detection System was used for the real-time detection of the samples (Appendix A). For each run, standard curve concentrations were used together with the samples of the mutants in order to ensure that the reaction progression is efficient. Moreover, at the end of each RT-qPCR run, melting curve analysis was added to ensure that there was no secondary product formation or contamination.

In a total volume of 10 µl reaction mixture, 3µl of template cDNA was used and the concentration of primers was set to 0.5 µM. The RT-qPCR conditions were 3 minutes at 95°C as initial denaturation followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 30 seconds. At the end of each run, melt curve analysis was added. During the analysis, the temperature was increased from 50°C to 99°C with 1°C increase for 5 seconds. This was done to ensure that only one template was amplified during the run without any contamination or primer dimer formation, therefore; one single peak was expected to be detected.

2.13. Livak ($2^{(-\Delta\Delta Cq)}$) Method for Gene Expression Analysis

For the RT-qPCR data analysis, relative quantification was used and expression change in the target gene was calculated relative to a control sample (Livak & Schmittgen, 2001) which had not been exposed to any copper ions. Control sample in this study was *E.coli* ATCC 8739.

In order to carry out this method, the efficiencies taken for the target gene and reference gene should be approximately equal or close to 100%. The acceptable percentage for the difference in efficiencies is 5% (Livak& Schmittgen, 2001). In this study, in each run, efficiency measurement was done and efficiencies which were approximately or exactly 100% were used during calculations, only.

Target genes were *copA*, *cusS* and *cusF* and their expressions were calculated relative to the reference gene; *tus*. While test samples were mutant strains (TG-C01 and TG-C02 are spontaneous, TG-C03 is UV-induced), control sample was used as the calibrator. For the calculations, steps given in Table 2.6 (Livak & Schmittgen, 2001) were followed.

Table 2.6. *Livak (2(-ΔΔCq)) Method for Gene Expression Calculation.*

1st Step: Target gene normalization relative to reference gene for test and control samples $\Delta Cq_{(test)} = Cq_{(target, test)} - Cq_{(reference, test)}$ $\Delta Cq_{(calibrator)} = Cq_{(target, calibrator)} - Cq_{(reference, calibrator)}$
2nd step: Test samples' ΔCq normalization relative to the control samples' ΔCq $\Delta\Delta Cq = \Delta Cq_{(test)} - \Delta Cq_{(control)}$
3rd step: Relative expression ratio calculation $2^{-\Delta\Delta Cq}$ = Giving expression ratio which is normalized relative to control

At the end of calculations according to Livak's method, ratio of target genes' expression was obtained relative to the control sample and with normalization to reference gene. When the expression ratios are determined for each mutant strain, statistical analysis was carried out.

2.14. Statistical Analysis

In the experiment, 3 biological replicates were used with 3 technical replicates each. The data were expressed as mean ± standard error of mean (SEM). Analysis of the results was done by one-way ANOVA (Analysis of variance) test by using GraphPad Prism 7.04 Software (California, USA) and for the comparison of mutants within each other Tukey's Multiple Comparison Analysis was performed. The confidence interval of 95% was used. The statistical level of significance was indicated as stars which corresponded to *($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Minimal Inhibitory Concentration Determination for Copper

According to Agar Dilution Method of EUCAST, colony counting was done on copper chloride dihydrate-containing nutrient agar plates. The method was performed as 3 replicates to obtain reliable results. During colony counting, the plates having colonies between 30-300 were taken into account. This is due to the fact that plates with more than 300 colonies are regarded as overcrowded and unsuitable for all the bacterial cells to generate individual colonies. On the other hand, plates having less than 30 colonies are ignored because they are not considered as significant statistically (Madigan et al., 2014). In Tables 3.1, 3.2, 3.3, and 3.4, the intervals chosen and the corresponding CFU's (viable count) results were shown.

Table 3.1. *CFU counts in media containing copper in 10-360 µg/ml range at 7 concentrations for MIC determination.*

	Number of Colonies		
Copper(II) Chloride Dihydrate Concentration	1st Replicate	2nd Replicate	3rd Replicate
10 µg/ml	Uncountable	Uncountable	Uncountable
60 µg/ml	Uncountable	Uncountable	Uncountable
120 µg/ml	Uncountable	Uncountable	Uncountable
180µg/ml	Uncountable	Uncountable	Uncountable
240 µg/ml	218	239	227
300 µg/ml	0	0	0
360 µg/ml	0	0	0

Firstly, large intervals for copper(II) chloride dihydrate concentration were tested in order to determine the interval which is going to be narrowed down in the later steps. Regarding the results shown in Table 3.1, at the concentrations of 10, 60, 120 and 180 µg/ml, the plates were rated as uncountable, whereas, no colonies were formed at 300 µg/ml copper. Therefore, the interval between 240-300 µg/ml of copper(II) chloride dihydrate concentration was narrowed down by 20 µg/ml increments and the CFU counts were shown in Table 3.2.

Table 3.2. *CFU counts in media containing copper in 240-300 μg/ml range at 4 concentrations for MIC determination.*

	Number of Colonies		
Copper(II) Chloride Dihydrate Concentration	1st Replicate	2nd Replicate	3rd Replicate
240 μg/ml	234	228	219
260 μg/ml	119	113	121
280 μg/ml	34	27	38
300 μg/ml	0	0	0

In the second set of concentration trials, the copper(II) chloride dihydrate concentration was taken between 240 and 300 μg/ml. Results showed that at 300 μg/ml, no colonies were formed. Therefore, in the next step, concentrations between 280 and 295 μg/ml were examined and the concentration intervals were taken as 5 μg/ml.

Table 3.3. *CFU counts in media containing copper in 280-295 μg/ml range at 4 concentrations for MIC determination.*

	Number of Colonies		
Copper(II) Chloride Dihydrate Concentration	1st Replicate	2nd Replicate	3rd Replicate
280 μg/ml	32	40	37
285 μg/ml	0	0	0
290 μg/ml	0	0	0
295 μg/ml	0	0	0

The results shown in Table 3.3 indicated that the MIC of copper(II) chloride dihydrate for *E. coli* ATCC 8739 is between 280-285 μg/ml due to no significant growth above 280 μg/ml. Therefore, as a last step, incremental increase of concentrations was tried at 1 μg/ml increments and the results were shown in Table 3.4.

Table 3.4. *CFU counts in media containing copper in 280-284 μg/ml range at 5 concentrations for MIC determination.*

	Number of Colonies		
Copper(II) Chloride Dihydrate Concentration	1st Replicate	2nd Replicate	3rd Replicate
280 μg/ml	36	33	42
281 μg/ml	0	0	0
282 μg/ml	0	0	0
283 μg/ml	0	0	0
284 μg/ml	0	0	0

The last CFU counts showed that the MIC of copper for the *E. coli* ATCC 8739 was 281 μg/ml (1,65 mM) because it was the lowest concentration which prevents the visible growth of bacterial colonies on agar plate.

In the literature, MIC for copper(II) chloride dihydrate was reported to be 3402 μmol/L (0,58 μg/ml) determined with broth dilution method for *E. coli* (Patel et al., 2012). In addition, it has been showed that MIC investigations give different results for broth dilution method and agar dilution method for a particular antimicrobial agent (Di Bonaventura et al., 2002; Jiang, 2011). This may be the result of difference in the principles between the methods, the microorganisms or strains used and variations in the solubility of the test compound (Valgas et al., 2007). For *E.coli* ATCC 8739, the MIC value we measured represents the quantity of the compound in solid media. Therefore, in any comparison, errors caused by methodology, microorganism and strain differences or forms of copper used were taken into account.

3.2. Spontaneous and UV-induced Mutants

In this study, 2 spontaneous mutants were obtained as described in 2.4, although it was indicated that spontaneous mutations rarely occurs due to its deleterious effects on cells (Drake, 1991). In the literature, the mutants mentioned as spontaneous are the mutants which passed through a long process of adaptation in different environments (Sniegowski et al., 1997). However, in our study, the mutants mentioned as spontaneous were obtained by acute exposure to high concentration of copper(II) chloride dihydrate. In that respect, our study is significantly important. These spontaneous mutants were entitled as TG-C01 and TG-C02. Furthermore, one more mutant was obtained by UV induction. This mutant was named as TG-C03. TG-C03 was also acquired by sudden exposure to high copper concentration after exposure to UV light as explained in 2.5.

3.3. Gene Expression Measurement Studies

3.3.1. Annealing Temperature Optimization of Primers for PCR

In order to identify the optimum annealing temperatures for the genes *tus*, *cusF*, *cusS* and *copA*, temperature gradient PCR was run and the samples were loaded to agarose gels and after electrophoresis the visualization was done. The gel electrophoresis results and gel images were given in Figures 3.1, 3.2, 3.3, and 3.4.

The gene *tus* was used as housekeeping gene in this study. This gene expresses the Tus protein which is responsible for the DNA replication termination by binding to the terminator sequences (Coskun-ari & Hill, 1997). Although 16S rRNA gene is commonly used in RT-qPCR as reference gene for *E.coli* (Zhou et al., 2011), this gene did not give stable results for our samples. This indicates that the 16S rRNA gene was not suitable to use as a reference under copper-driven stress environments of *E.coli* for the RT-qPCR analysis. Therefore, the gene *tus* was preferred in our study due to its expression stability under our experimental conditions. The *tus* gene was previously used as reference gene in other RT-qPCR studies in which the strain UPEC (*E.coli*) CFT073 was used (Cai et al., 2013; Ma et al., 2018). However, our study was the first time that *tus* was used for *E.coli* ATCC 8739 and its mutants to examine the copper-related stress effects on copper homeostasis genes' expressions. Also, in the literature, there is no study covering RT-qPCR analysis of spontaneous mutants in which *tus* used as a reference gene.

The product size for the primers selected for *tus* was 103 bp and gel image recorded for optimum annealing temperature determination was given in Figure 3.1.

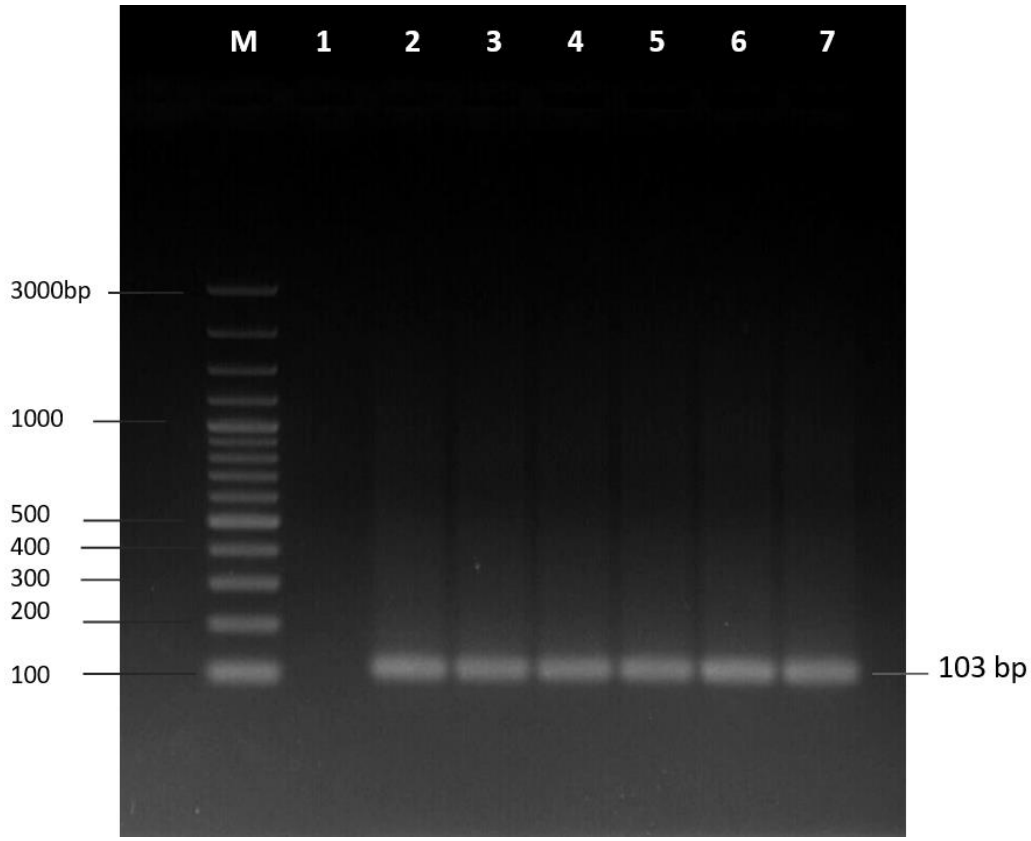

Figure 3.1. Annealing temperature optimization with temperature gradient PCR for *tus*: M, GeneRuler 100 bp Plus DNA Ladder; No template control (1); 54°C (2); 55,5°C (3); 58°C (4); 59,6°C (5); 62,3°C (6); 64°C (7).

The protein CopA is responsible for ATP-driven pumping of charged substrates from cytoplasm to the periplasmic space of the bacterial cell (Axelsen & Palmgren, 1998; Moller et al., 1996). In this study, the primers designed for the amplification of *copA* gene was 134 bp long. The temperature gradient PCR results were given in Figure 3.2.

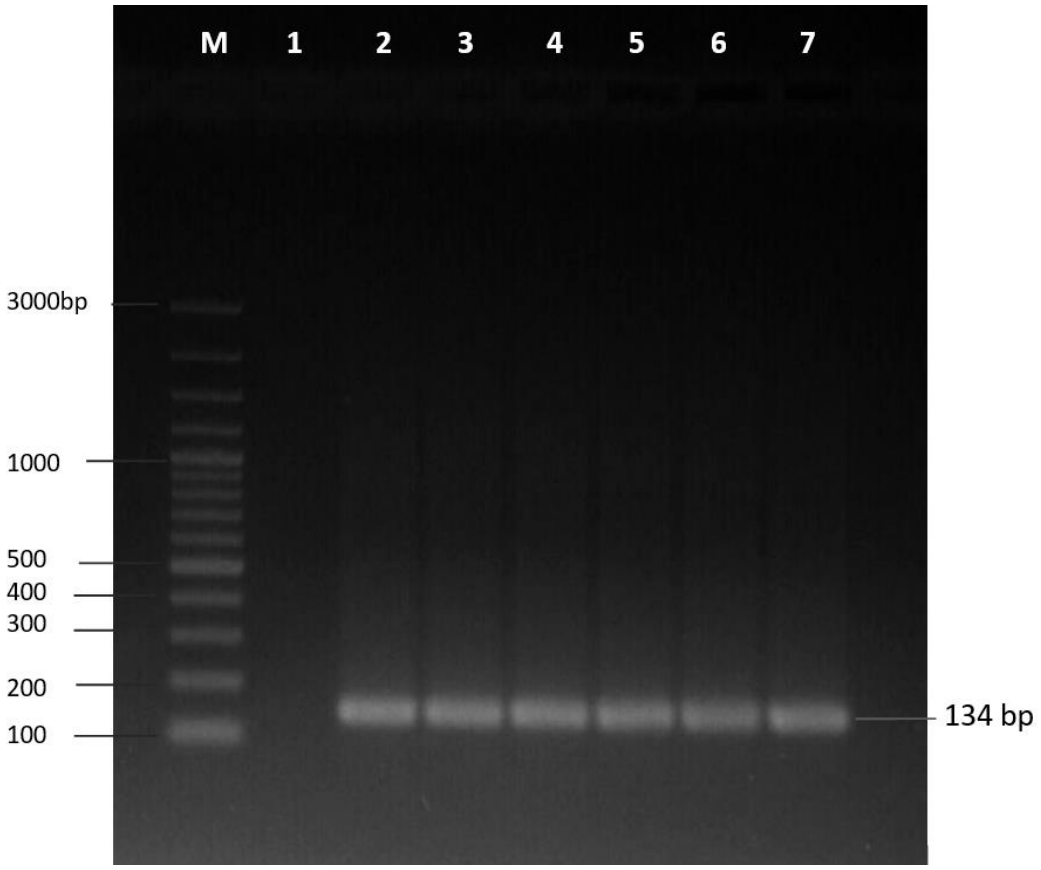

Figure 3.2. Annealing temperature optimization with temperature gradient PCR for *copA*: M, GeneRuler 100 bp Plus DNA Ladder; No template control (1); 54°C (2); 55,5°C (3); 58°C (4); 59,6°C (5); 62,3°C (6); 64°C (7).

Another gene chosen in this study was *cusS* which expresses the CusS protein. This protein is a sensory protein found in the cytoplasmic membrane (E. H. Kim et al., 2011). The product size of the designed primers for the *cusS* gene was 249 bp and temperature gradient PCR results were given in Figure 3.3.

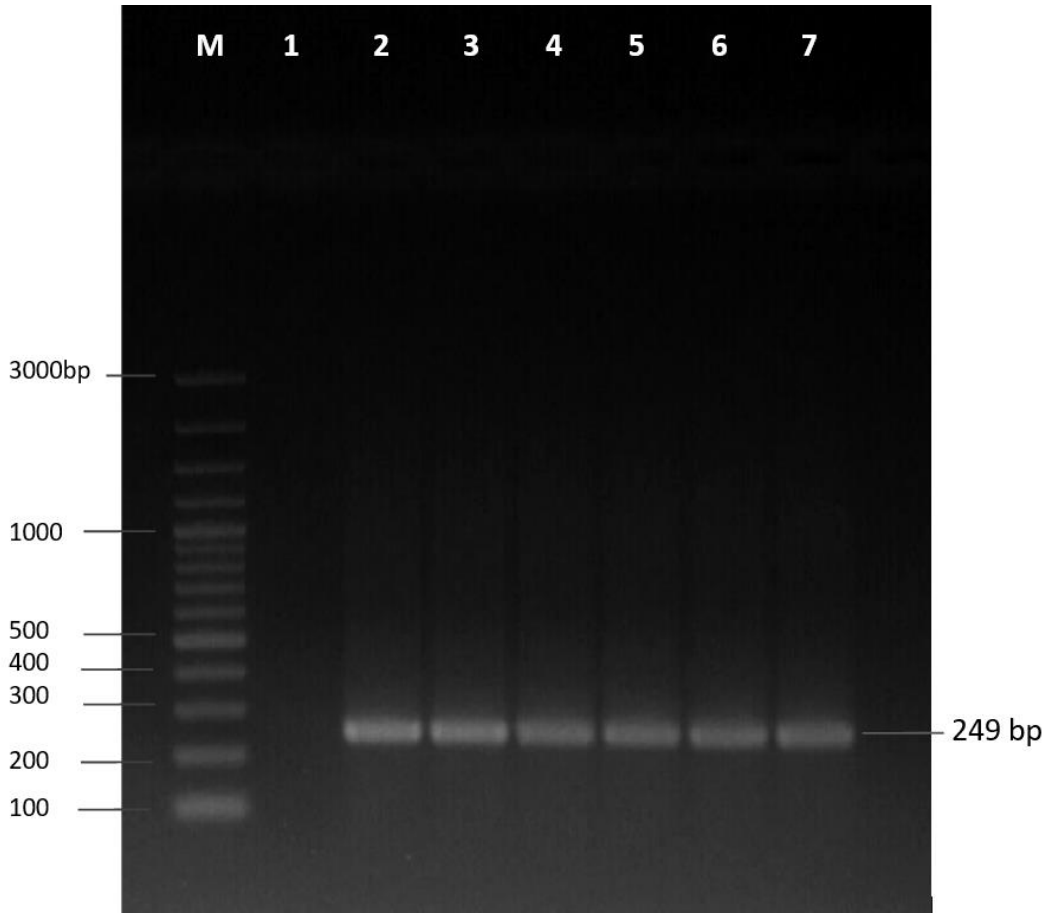

Figure 3.3. Annealing temperature optimization with temperature gradient PCR for *cusS*: M, GeneRuler 100 bp Plus DNA Ladder; No template control (1); 54°C (2); 55,5°C (3); 58°C (4); 59,6°C (5); 62,3°C (6); 64°C (7).

The last gene chosen in this study was *cusF*. This gene codes for the periplasmic protein CusF which chaperons Cu^{+} in the periplasmic space carrying them to CusCBA complex (Randall et al., 2014). The product size of the primers designed for *cusF* was 89 bp. Figure 3.4 shows the temperature gradient PCR results for this gene.

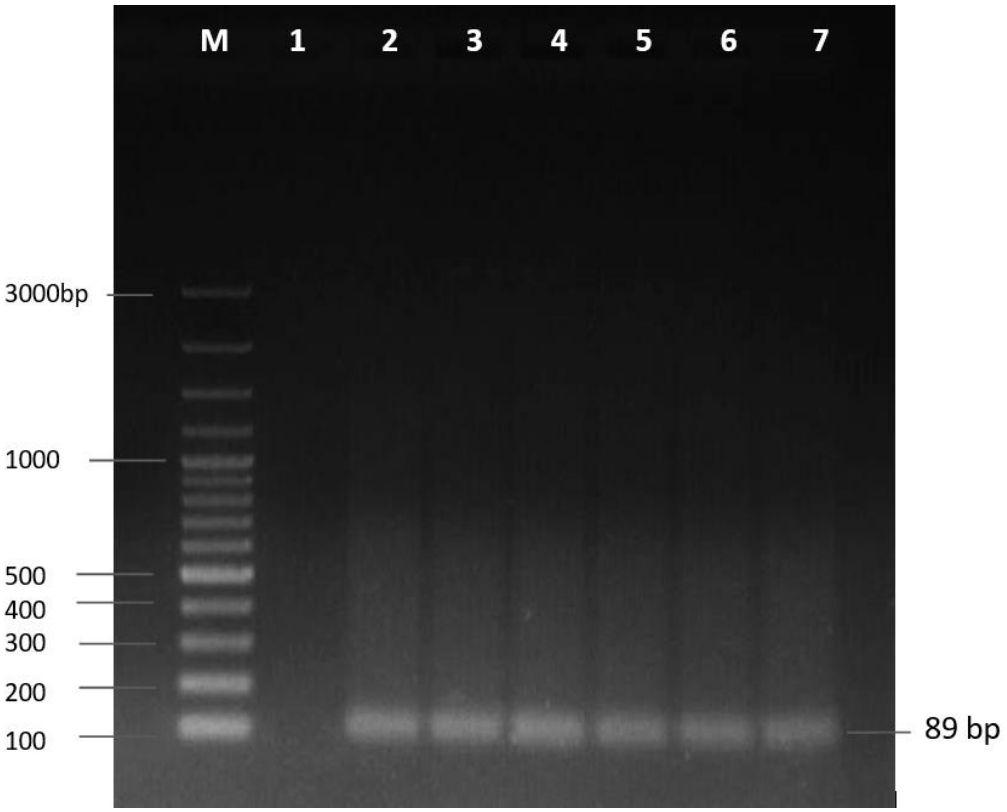

Figure 3.4. Annealing temperature optimization with temperature gradient PCR for *cusF*: M, GeneRuler 100 bp Plus DNA Ladder; No template control (1); 54°C (2); 55,5°C (3); 58°C (4); 59,6°C (5); 62,3°C (6); 64°C (7).

In this study, because the Tm values of all primers alternated between 57 and 59°C, the gradient temperature range was started from 54°C and increased by 2°C at a time up to 64°C. The first wells which were labeled as (1) in the figures contained no template controls. The annealing temperature used for no template controls were 54°C. No bands were detected in these lanes. This states that there was no primer dimer formation for the primer pair or contamination. Moreover, all primer sets gave the products at the expected alignment with respect to the DNA ladder.

Regarding the gel images in Figures 3.1 through 3.4, although *tus* demonstrated no apparent difference between changing temperatures (Figure 3.1), *copA* and *cusF* showed slightly lower brightness from 54°C to 64°C, which were given in Figure 3.2 and Figure 3.4, respectively. Moreover, in Figure 3.3, at the first two temperatures; 54°C and 55,5°C, *cusS* primers gave the brightest bands. This indicates that the optimum annealing temperatures for *cusS* primer set should be around 54-56°C. Therefore, the annealing temperatures for all 4 primer sets were chosen as 54°C, since they gave the brightest bands on gels for all the primer sets. In the following procedures, using the same annealing temperature for all primer sets eased the RT-qPCR runs.

3.3.2. Isolation of Total RNA from Spontaneous and UV-induced Mutants

The total RNAs were isolated from 2 spontaneous and 1 UV-induced mutants and 1 control strain which is *E.coli* ATCC 8739. The isolated RNA molecules then checked for purity at OD_{260}/OD_{280}, and at OD_{260}/OD_{230}. The expected range was 2.0-2.2 for RNA samples to accept that the sample is pure and free from contaminants (Geuther, 2007). The RNA isolates in this study satisfied these parameters meaning that they were pure RNA samples and free from any contaminants. Then, they are run on a 2% agarose gel and visualized in order to ensure that the isolation is successful. After a successful RNA isolation, 2 clear bands are seen on agarose gel belonging to 16S and 23S rRNAs in prokaryotes (Oelmiiller et al., 1990). Figure 3.5 shows our total RNA isolation from all samples on agarose gel.

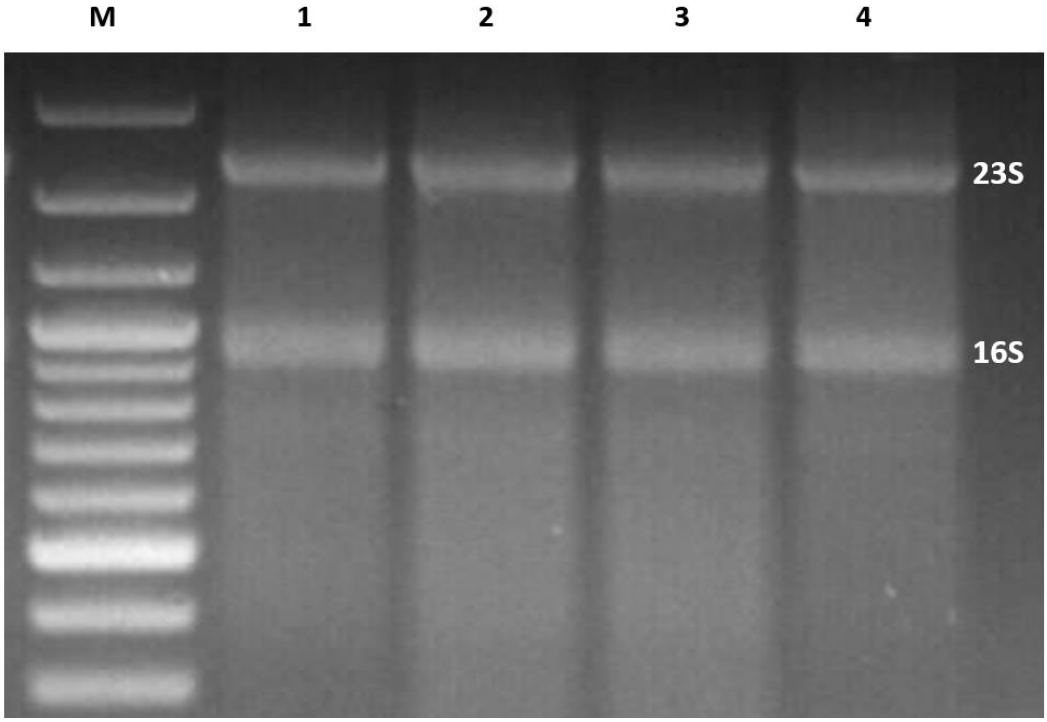

Figure 3.5. Total RNA banding patterns of Control (E.coli ATCC 8739), T-C01, T-C02 and T-C03 on agarose gel: M, GeneRuler 100 bp Plus DNA Ladder; E.coli ATCC 8739 (1); TG-C01 (2); TG-C02 (3); TG-C03 (4).

3.3.3. cDNA Synthesis

After the cDNA synthesis from 2 spontaneous (TG-C01 and TG-C02), 1 UV-induced mutant (TG-C03), and 1 control strain (*E.coli* ATCC 8739) were done, a PCR was performed with the primers belonging to housekeeping gene *tus.* This was done to control the success of cDNA synthesis. The annealing temperature used was 54°C and a no template control was included. After PCR run, the samples were loaded to 1,5% agarose gel and visualized as seen in Figure 3.6.

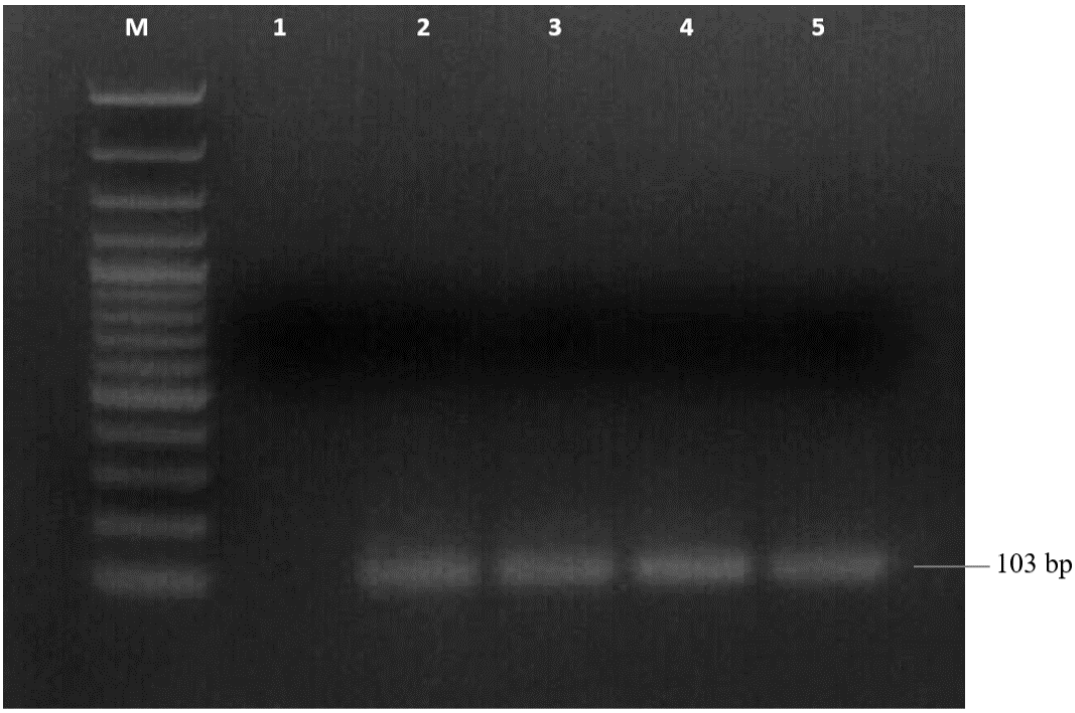

Figure 3.6. Banding pattern of cDNA control with *tus* primers: M, GeneRuler 100 bp Plus DNA Ladder; No template Control (1), E.coli ATCC 8739 (2); TG-C01 (3); TG-C02 (4); TG-C03 (5).

Absence of any band in the no template control lane means there were no contamination, non-specific bindings, or primer dimers. The bands for all samples were clear, bright and free from smears or non-specific bands, which indicated that the cDNA synthesis was successful.

3.3.4. RT-QPCR Standard Curve Analysis

In this study, standard curve analysis was performed for each and every run for each gene's primer set in order to demonstrate the RT-qPCR efficiency for the current runs. No template controls were conventionally used for each gene's standard curve analysis. Also, 5 different concentrations of standards were used and outliers were excluded from the analysis.

For the analysis of standard curve generated for *copA*, cDNA of TG-C01 was used in varying concentrations starting from the dilution of 1/10 up to 1/160 by diluting out 1/2 the concentration at a time. The standard curve was given in Figure 3.7. The reaction repeated 35 cycles, and melt curve analysis was done at the end of the RT-qPCR operation (Figure 3.8).

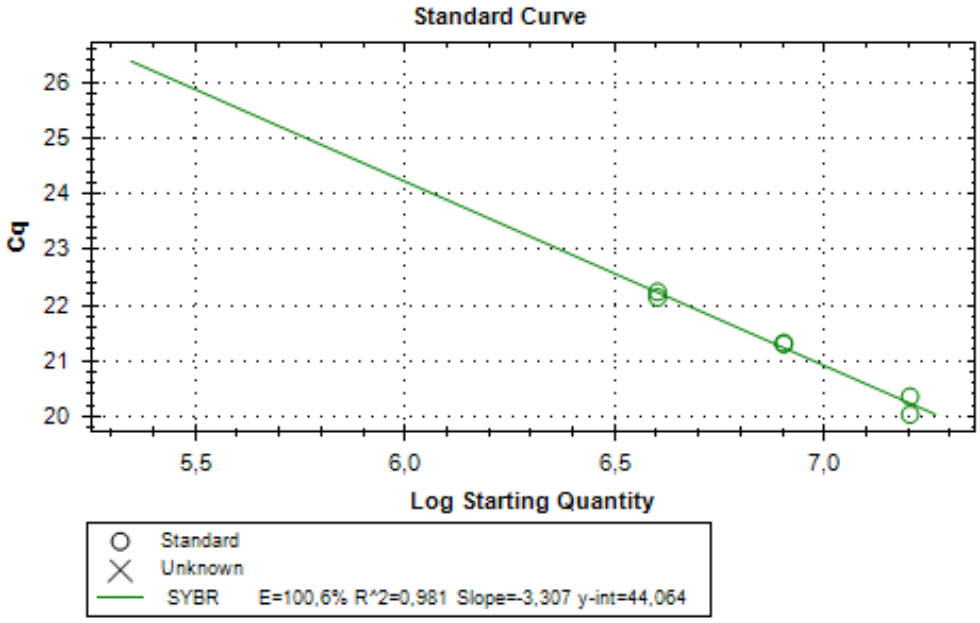

Figure 3.7. Standard curve analysis of *copA* gene.

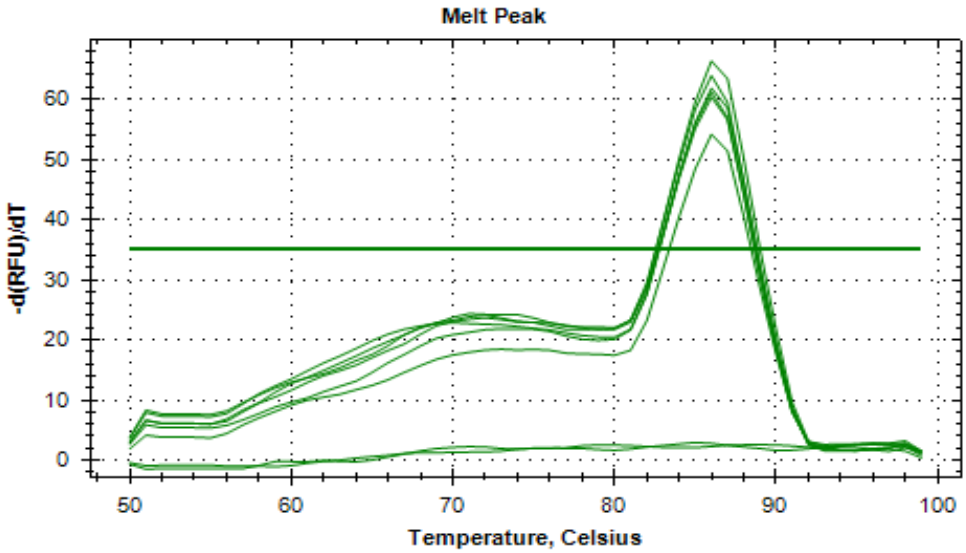

Figure 3.8. copA melt curve analysis for standard curve determination.

In the standard curve analysis of *copA* (Figure 3.7), the reaction efficiency was found to be 100,6% and slope was -3,307. Also, no-template controls gave no amplification throughout the run, which can be seen below the threshold line. Figure 3.8 shows melt curve analysis of standards. No non-specific melting peaks were seen, only one peak was observed and no-template control samples did not give any melting peaks.

In the standard curve analysis for *cusF*, cDNA of TG-C01 was used. The concentrations1/10, 1/20, 1/40, 1/80 and 1/160 were applied for standards and runs were done as 35 cycles. The standard curve analysis was given in Figure 3.9, while melt curve analysis which was done at the end of the RT-qPCR given in Figure 3.10.

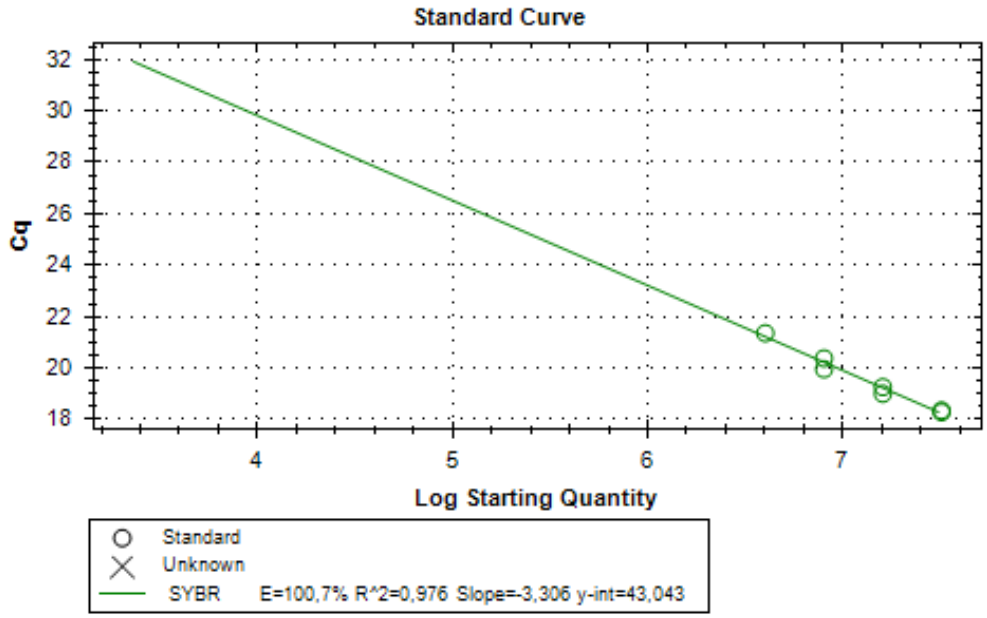

Figure 3.9. Standard curve analysis for *cusF* gene.

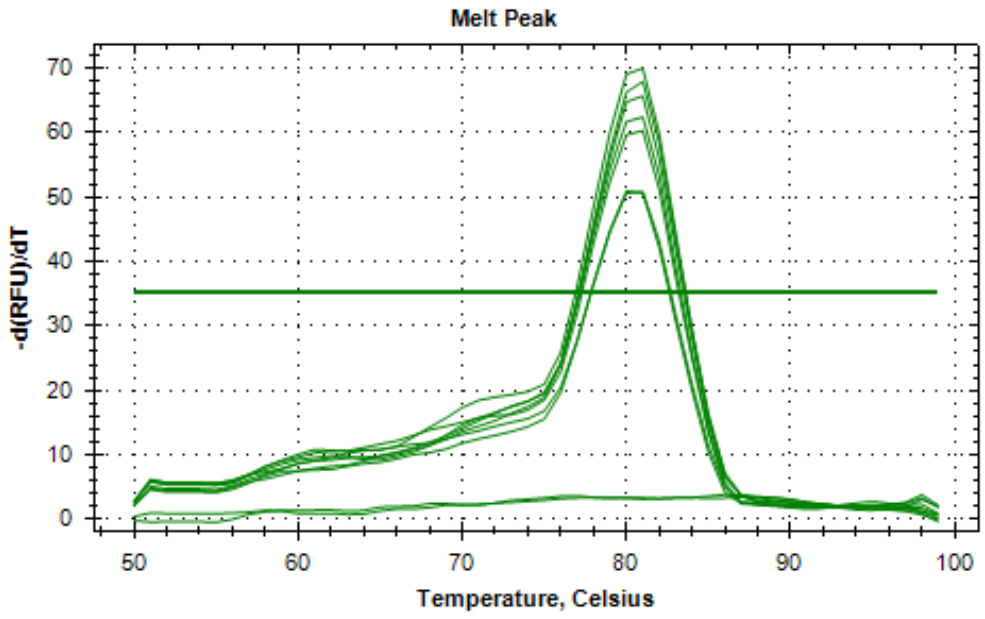

Figure 3.10. cusF melt curve analysis for standard curve determination.

At the end of the analysis of standard curve for *cusF*, running efficiency was found to be 100,7% and slope was -3,306 (Figure 3.9). In the melt curve analysis given in Figure 3.10, no-template controls did not give any peaks. The standard concentrations showed one single peak and non-specific curves were not detected.

In the standard curve analysis for *cusS*, cDNA of TG-C01 was used. Standards were prepared in the concentrations covering 1/10, 1/20, 1/40, 1/80 and 1/160 range. The standard curve analysis was given in Figure 3.11. Melt curve analysis which was done at the end of the RT-qPCR reaction was given in Figure 3.12.

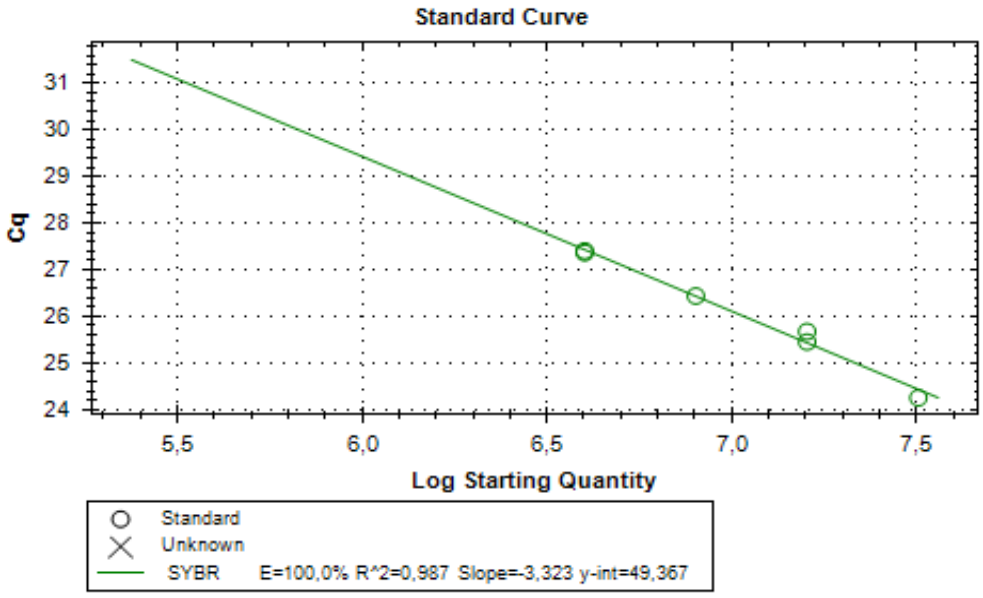

Figure 3.11. Standard curve analysis for *cusS* gene.

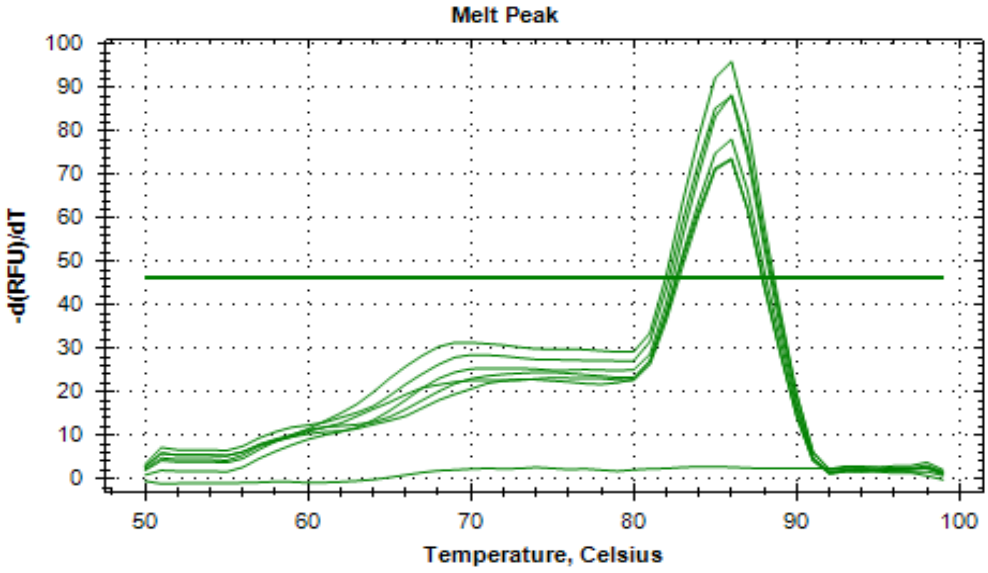

Figure 3.12. cusS melt curve analysis for standard curve determination.

In the standard curve analysis of *cusS*, run efficiency was found to be 100,0% and slope was -3,323, which was given in Figure 3.11. At the end of the melt curve analysis, no-template controls did not give any peaks and standard samples exhibited one single peak, and non-specific curves were not formed as can be seen in Figure 3.12.

For the *tus* gene standard curve analysis, cDNA of TG-C01 was used. Varying 5 concentrations were prepared starting from the dilution of 1/10 to 1/160 as described previously. The standard curve analysis result was given in Figure 3.13. The melt curve analysis was done at the end of the RT-qPCR run which was given in Figure 3.14.

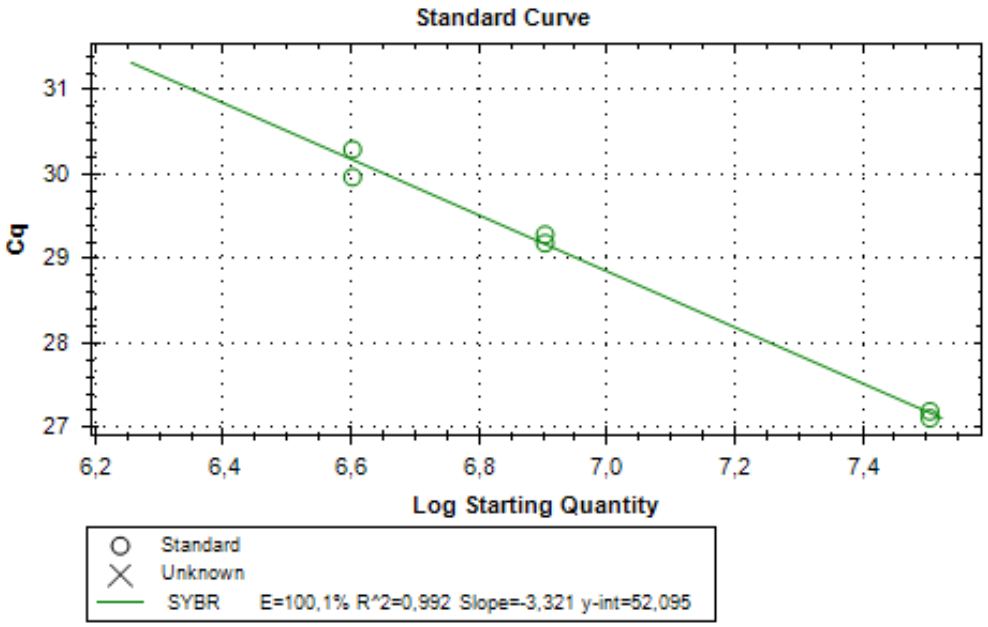

Figure 3.13. Standard curve analysis for *tus* gene.

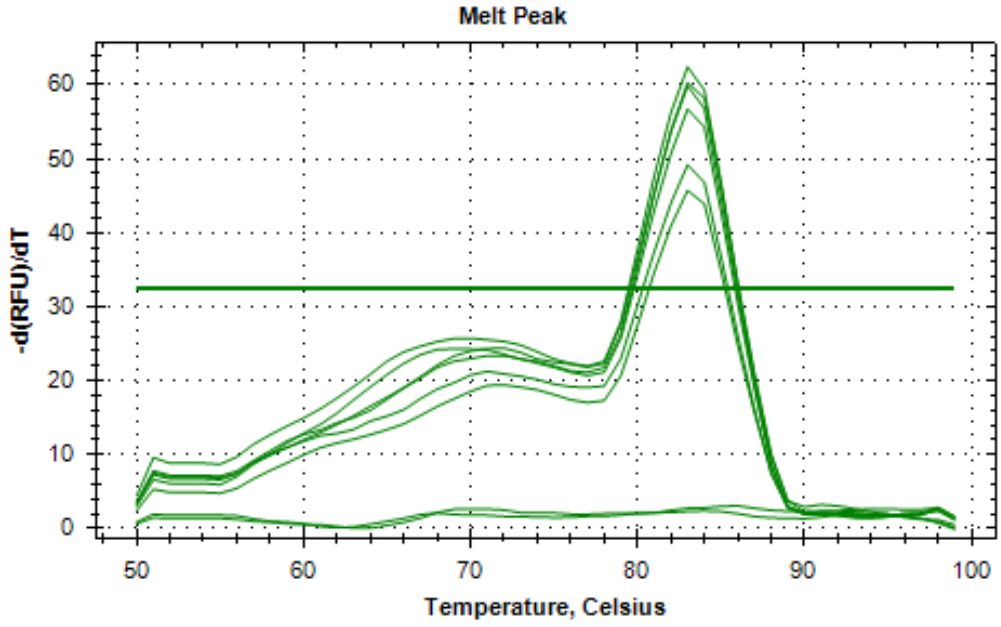

Figure 3.14. tus melt curve analysis for standard curve determination.

At the end of the standard curve analysis of the *tus* gene, efficiency of the run was found to be 100,1% and slope was -3,321, given in Figure 3.13. In the melt curve analysis, no peaks were detected for the no-template controls and standard samples gave a single peak. Also, non-specific curves were not formed, which can be seen in Figure 3.14.

Standard curves establish the efficiency of PCR for the current run in a simple, easy and repeatable manner. The maximum number of PCR efficiency is 1.00 or 100%, theoretically. This theoretical maximum value indicates that the amount of target sequence is doubled in each PCR cycle. Although, 100% PCR efficiency is considered as ideal for a reaction, the efficiencies falling between the ranges of 90-110% are accepted. The efficiencies falling below 90% are considered as poor efficiencies. The result coinciding with below 90% may be due to several reasons; firstly, inadequate concentrations of primers, magnesium, *Taq* DNA polymerase; secondly, the T_ms of primers differing from each other more than 5°C; thirdly, suboptimal PCR conditions for the reaction. Under those cases, poor reaction efficiency may be observed. On the other hand, efficiencies above 110% indicate that there is an inhibition effect in the reaction. The reasons which lead to inhibitions are poor quality of DNA/RNA, residuals from purification steps of nucleic acids and high concentrations of templates (Bonab et al., 2015). Moreover, the PCR efficiency is linked with the slope of the calibration curve's log-linear portion. The efficiency is calculated from the equation: $10^{-1/slope} - 1$. Therefore, they are directly interconnected (Bustin et al., 2009). The optimal result for slope in order to achieve an efficiency close to 100% is tabulated as -3,32 (Biosystems, 2013). In this study, standard samples were prepared in 5 different concentrations which were 1/10, 1/20, 1/40, 1/80 and 1/160. At the end, the outliers were extracted from the analysis. Because 2-fold dilution was done, the expected C_q change between the varying concentrations was 1 C_q, in agreement with the equation; 2^n = Dilution factor (Bustin & Huggett, 2017). In the standard curve analysis of each gene, the C_q change was 1 as expected. Moreover, for each gene's standard curve assay, the reaction efficiency was found as 100%. This indicated that the ideal RT-

qPCR reaction was obtained and in our experiments all optimal conditions for the reactions were satisfied. These results were also supported with the results of calibration curve slopes which were -3,32 up to second decimal. Furthermore, melt curve analysis were done at the end of each standard curve analysis run. Regarding the melt curve results, a linear line was achieved from the melt peaks of no-template controls for each gene. This indicates that there occurred no contamination or primer-dimer formations during the reaction. Also, in each melt curve, standard samples gave one single peak and no non-specific peaks were formed. This means that there were no primer-dimers, secondary products, non-specific bindings of primers or nucleic acid contamination.

3.3.5. Expression Analysis of RT-qPCR for Spontaneous and UV-induced Mutants

After standard curve analyses were completed, RT-qPCR runs were done for control strain (*E. coli* ATCC 8739), the spontaneous mutants (TG-C01, TG-C02) and the UV-induced mutant (TG-C03) for 3 different genes (*copA*, *cusS* and *cusF*) and 1 house-keeping gene (*tus*). In each run, 3 technical replicates were made and the results were obtained in 3 biological replications. A corresponding standard curve was used in each run in order to be sure about the reaction efficiency of the run. 5 different concentrations of standards (1/10, 1/20, 1/40, 1/80 and 1/160) were used and the outliers were excluded from the expression analysis as described previously in 3.3.4.

RT-qPCR run for measuring *copA* expression, the samples were control strain, spontaneous, and UV-induced mutants. The cDNA dilution was chosen as 1/40. No-template controls and standards were used. Figure 3.15 shows the real-time amplification results, and Figure 3.16 gives standard curve and efficiency of the run. Figure 3.17 and Figure 3.18 show melt curve and melt peak, respectively.

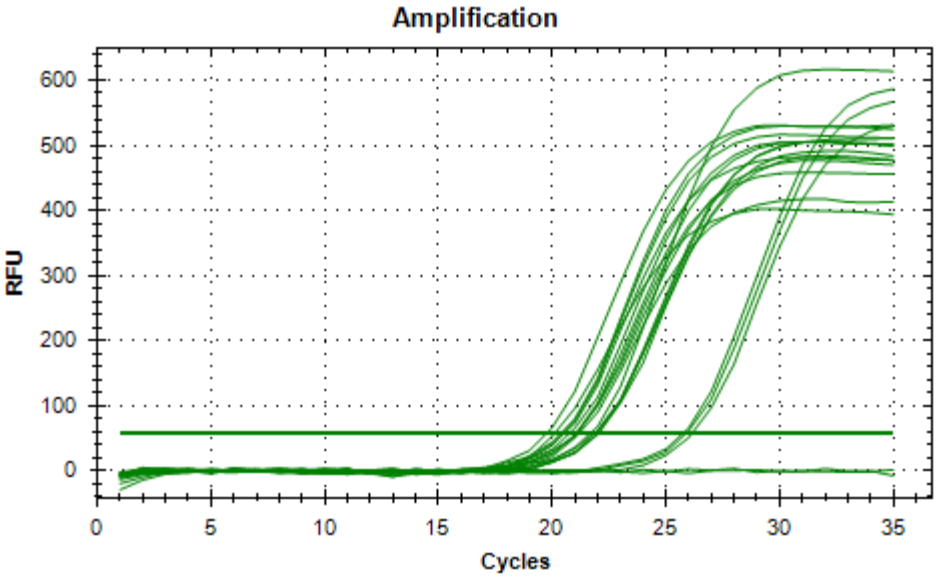

Figure 3.15. Amplification curves of all samples for *copA*.

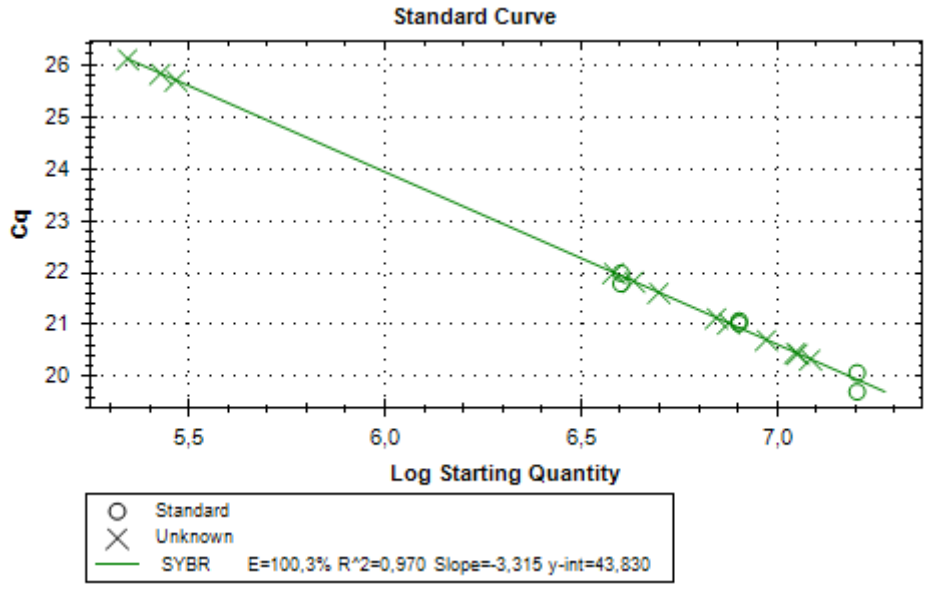

Figure 3.16. Standard curve analysis of all samples for *copA*.

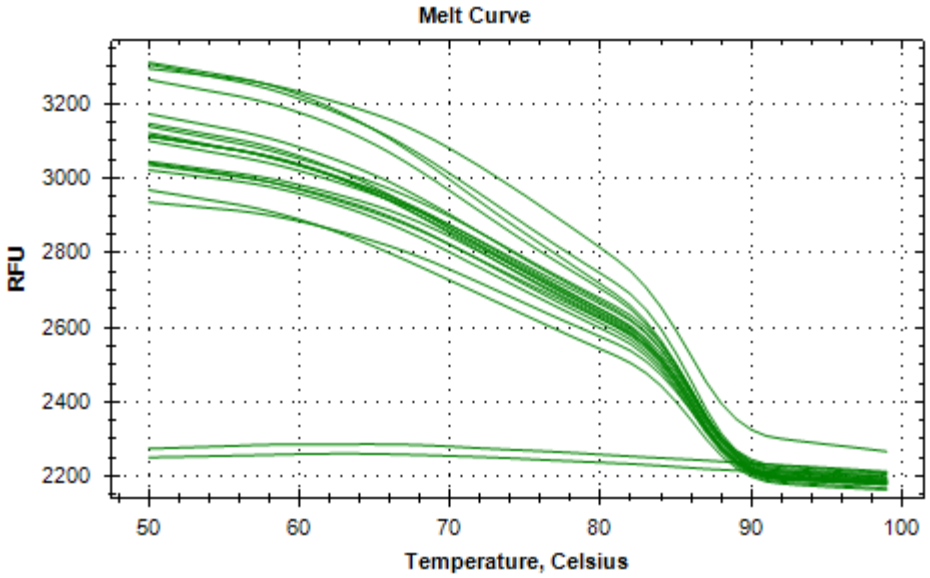

Figure 3.17. Melt curves of all samples for *copA*.

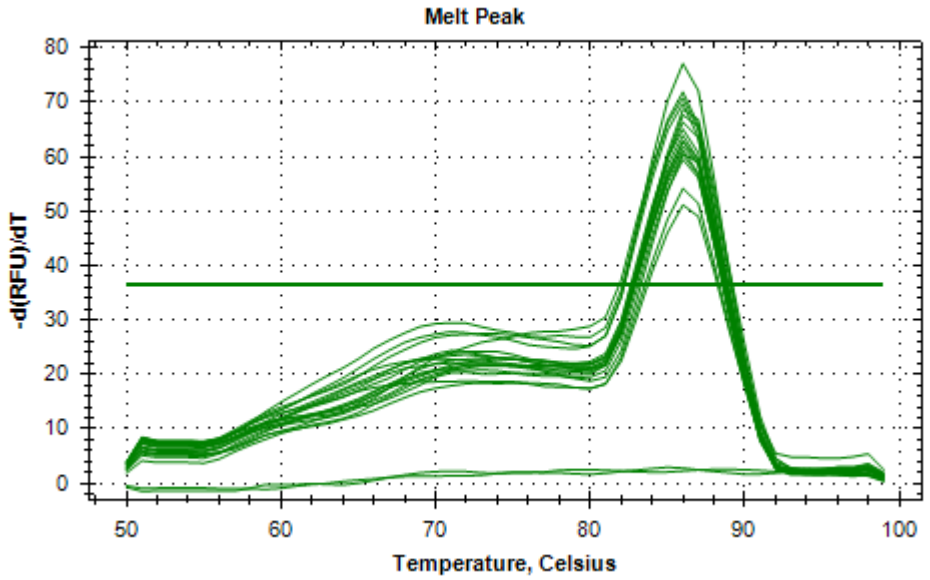

Figure 3.18. Melt peaks of all samples for *copA*.

In RT-qPCR run for *copA*, the efficiency of run was found to be 100,3% and slope of the curve was -3,315. Also, in the melt peak of the run, there was only one peak for each sample (Figure 3.18) and no aberrations were seen in the melt curve (Figure 3.17).

RT-qPCR run for *cusF* was done for all samples and cDNA dilution concentration for samples was chosen as 1/40. No-template controls and standards were used. In Figure 3.19, real-time amplification of the run and in Figure 3.20 standard curve and efficiency results were given. Figure 3.21 and Figure 3.22 show the melt curve and the melt peak results, respectively.

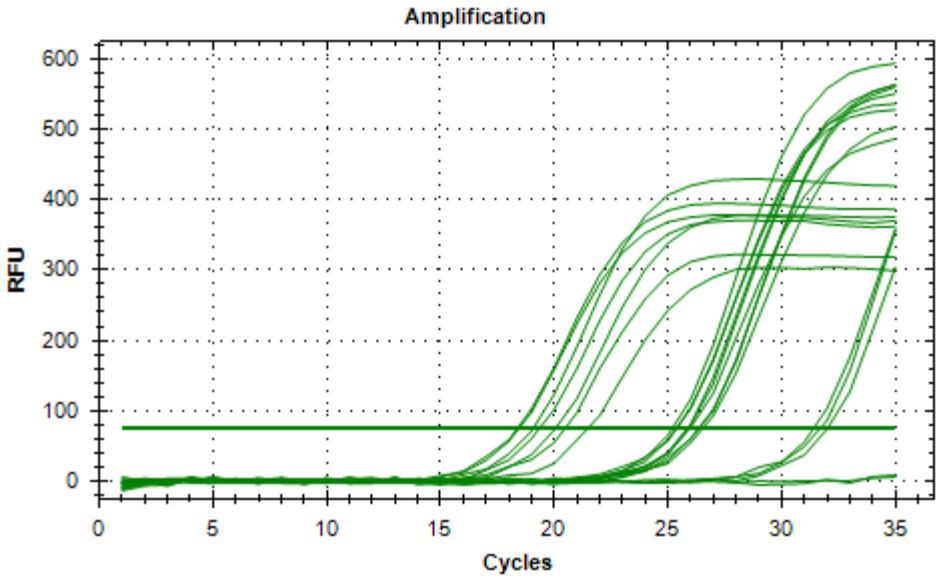

Figure 3.19. Amplification of all samples for *cusF*.

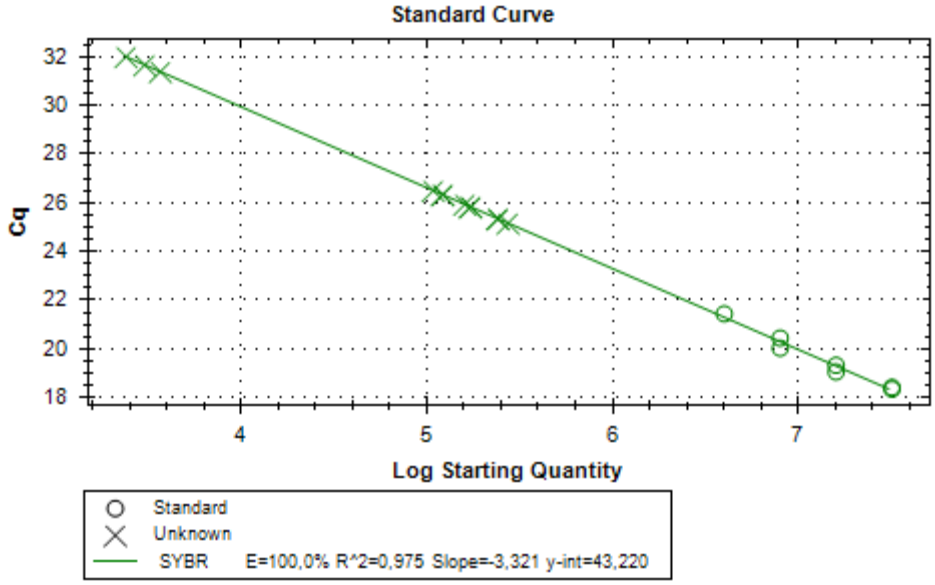

Figure 3.20. Standard curve analysis of all samples for *cusF*.

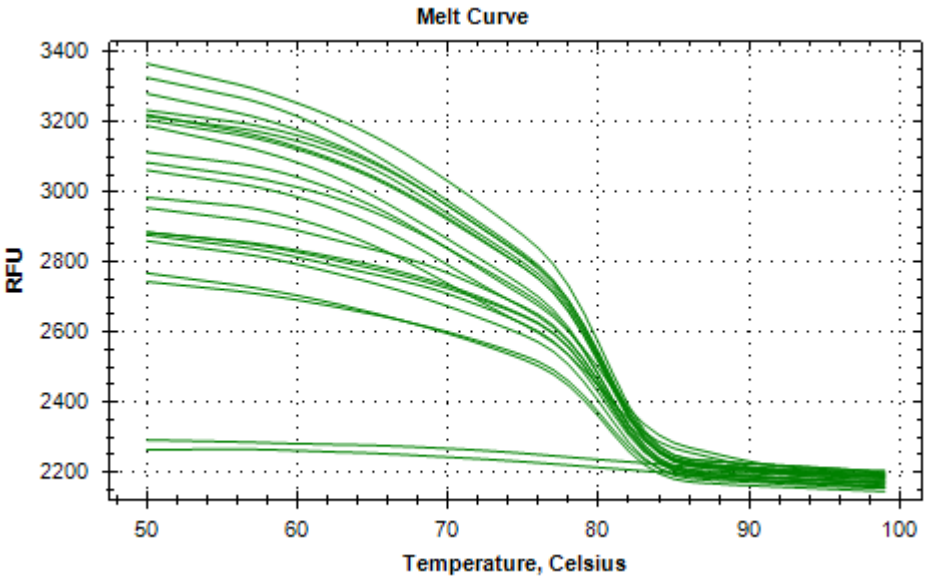

Figure 3.21. Melt curve of all samples for *cusF*.

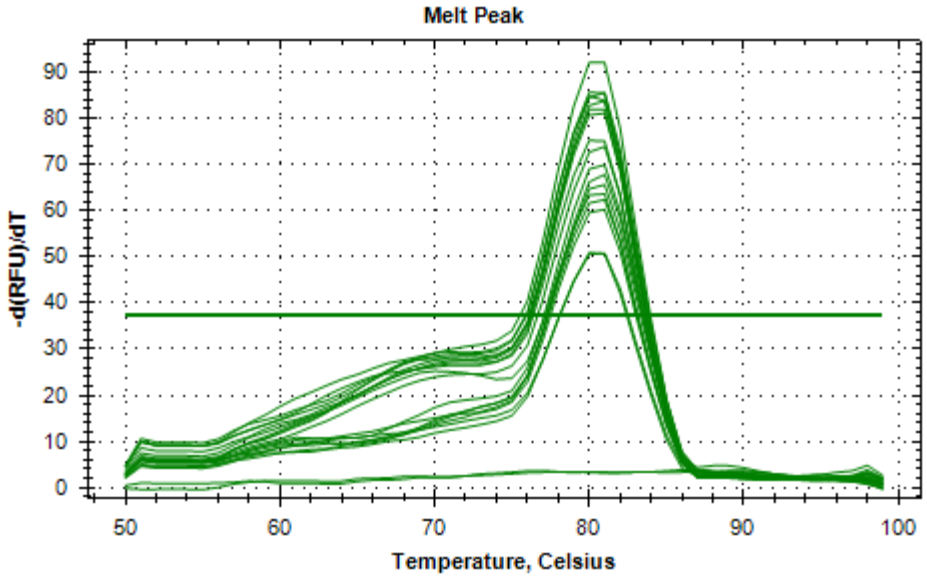

Figure 3.22. Melt peak of all samples for *cusF*.

Result of RT-qPCR run for *cusF* shows that the efficiency of run was found to be 100,0% and slope of the curve was -3,32. Furthermore, the melt peak of the run demonstrated only one peak for each sample (Figure 3.22) and there were no aberrations in the melt curve (Figure 3.21).

In the RT-qPCR run for *cusS*, cDNA dilution concentration for all samples was chosen as 1/40. No-template controls and standards were used. Figure 3.23 shows real-time amplification of run and Figure 3.24 gives standard curve and efficiency results of the run. Figure 3.25 and Figure 3.26 demonstrates melt curve and melt peak results, respectively.

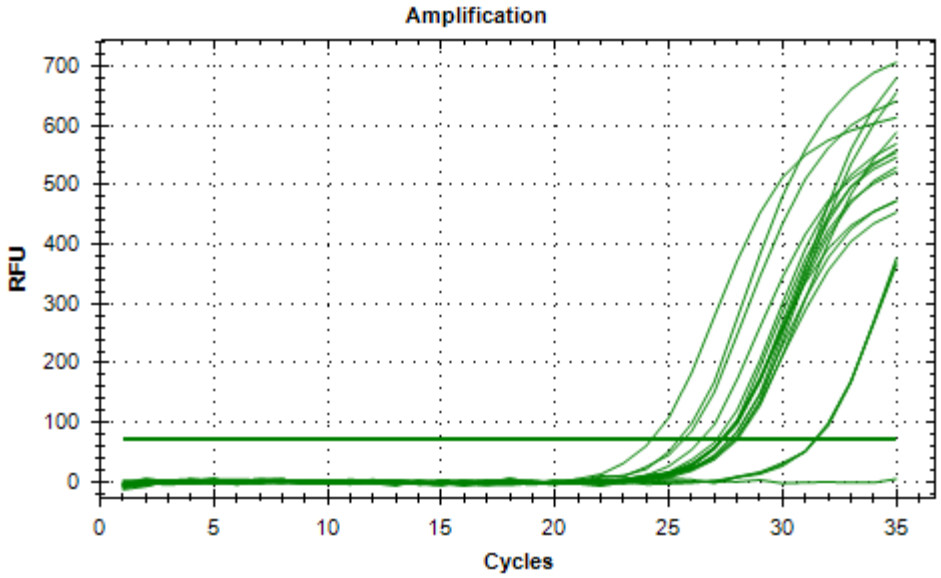

Figure 3.23. Amplification of all samples for *cusS.*

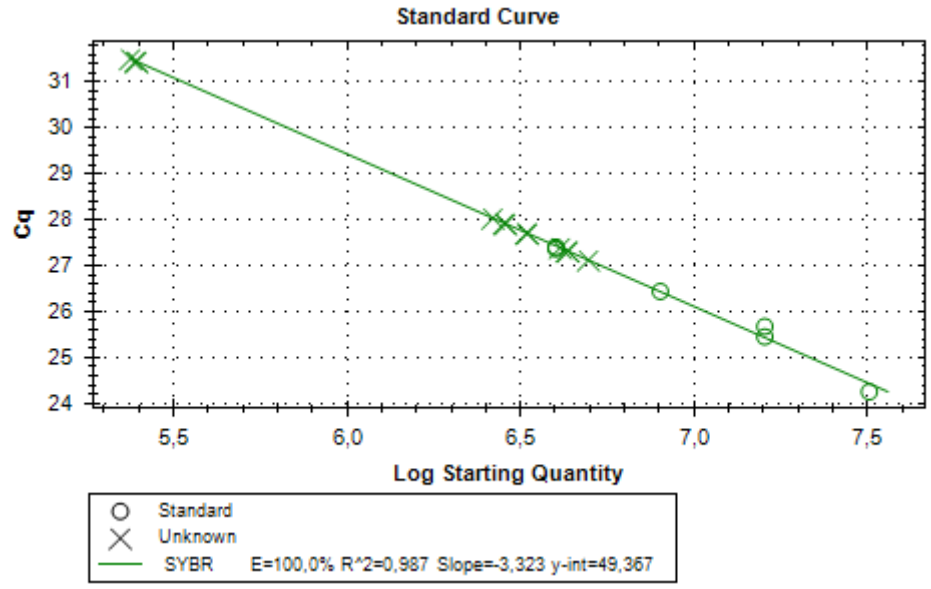

Figure 3.24. Standard curve analysis of all samples for *cusS.*

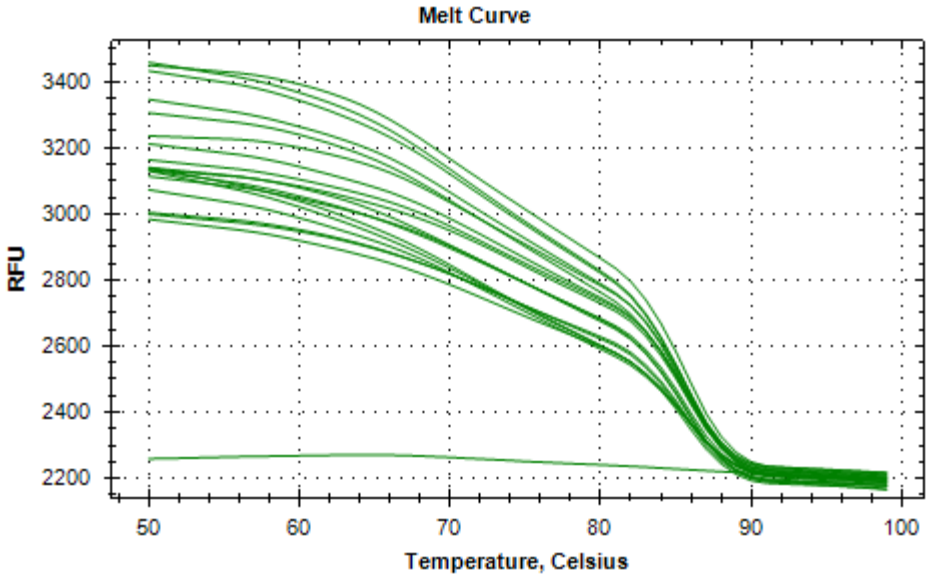

Figure 3.25. Melt curve of all samples for *cusS.*

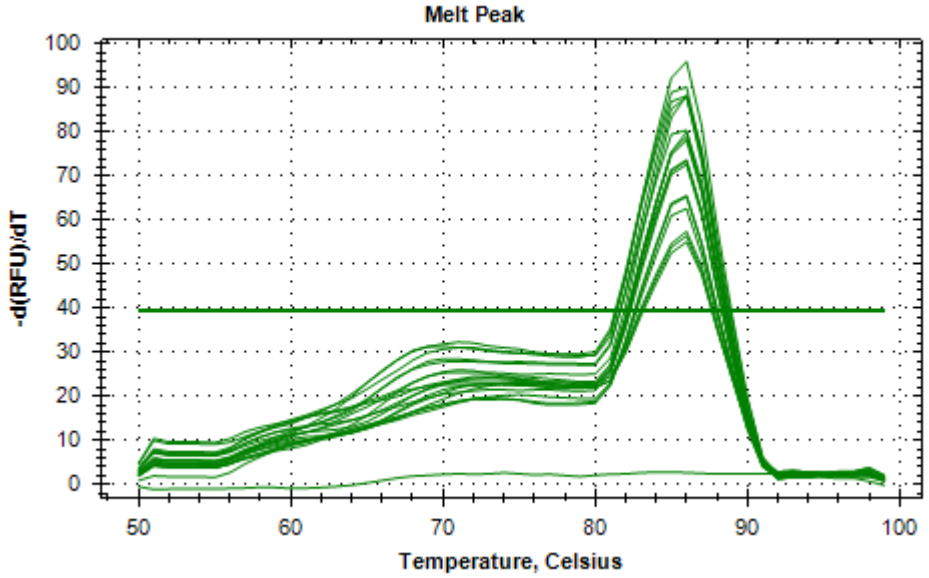

Figure 3.26. Melt peak of all samples for *cusS.*

With respect to the RT-qPCR run results of *cusS*, the efficiency of run was found to be 100,0% and slope of the curve was -3,32. Moreover, only one peak for each sample was observed in the melt peak of the run (Figure 3.26) and no aberrations were seen in the melt curve (Figure 3.25).

RT-qPCR run for *tus* was done for all samples. cDNA dilution concentration for all samples was chosen as 1/40 while no-template controls and standards were used in the run. Figure 3.27 gives real-time amplification of run and Figure 3.28 shows standard curve and efficiency results of the run. Figure 3.29 and Figure 3.30 demonstrates melt curve and melt peak results, respectively.

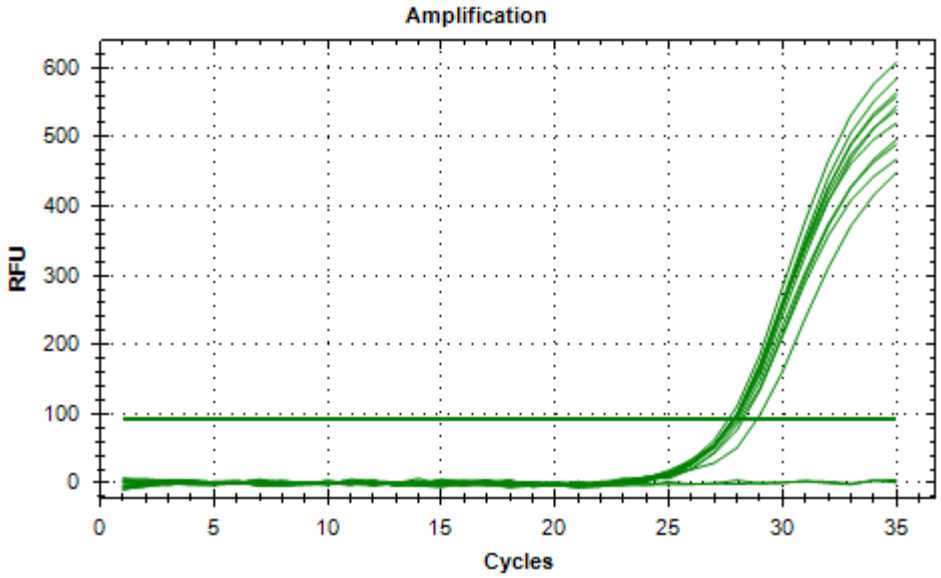

Figure 3.27. Amplification of all samples for *tus*.

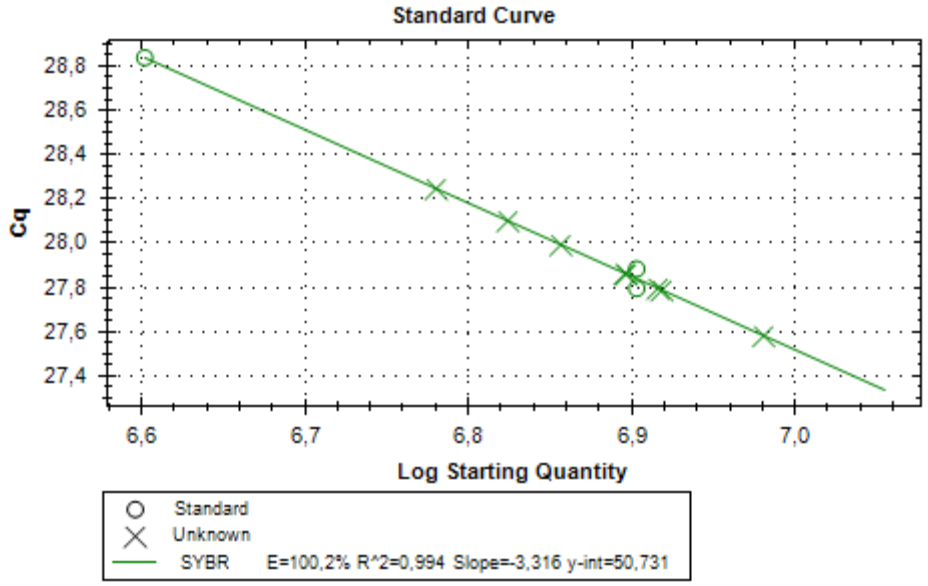

Figure 3.28. Standard curve analysis of all samples for *tus.*

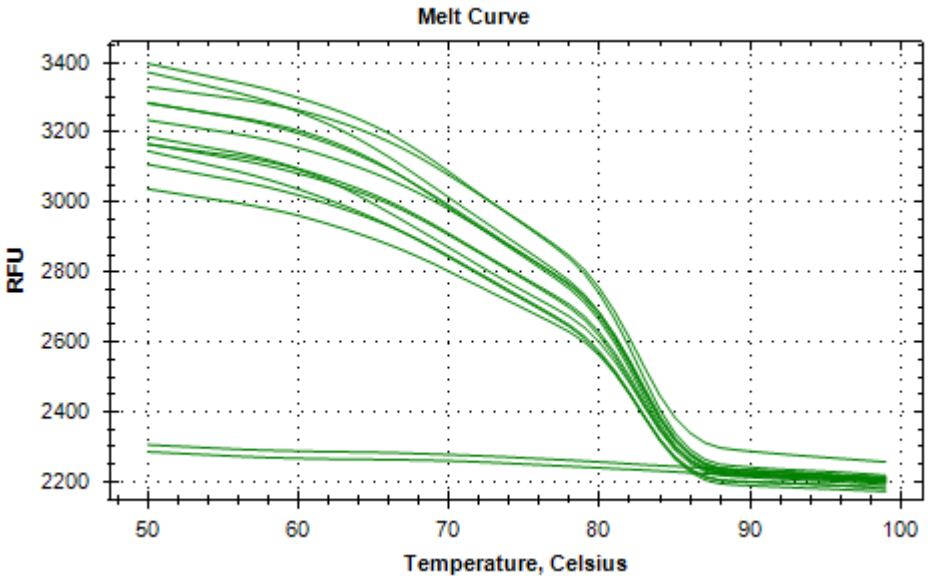

Figure 3.29. Melt curve of all samples for *tus.*

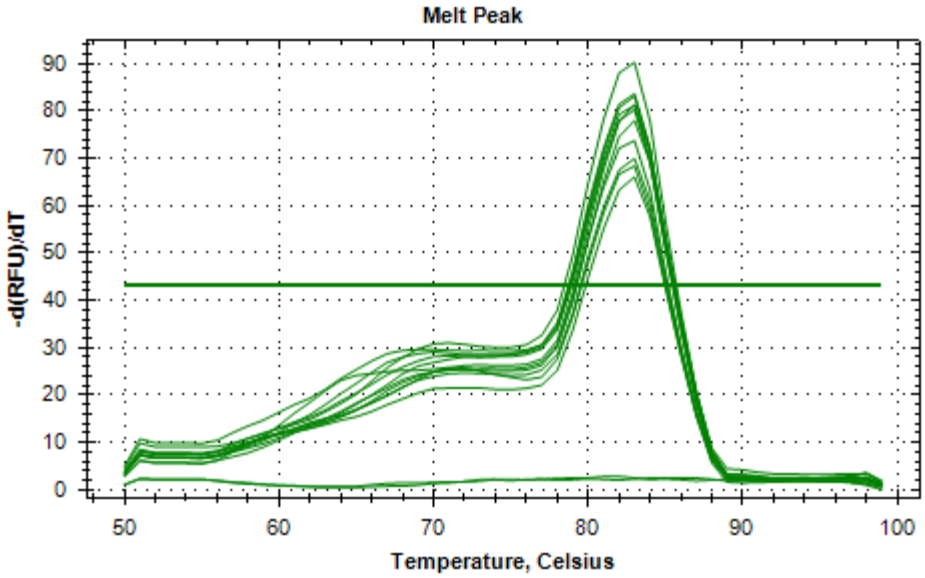

Figure 3.30. Melt peak of all samples for *tus.*

Regarding to the result of the RT-qPCR run of *tus*, the efficiency of the run was 100,2% and slope of the curve was found as -3,316. Also, there was only one peak for each sample in the melt peak of the run (Figure 3.30) and no aberrations were seen in the melt curve (Figure 3.29).

In the RT-qPCR analyses for every 4 samples (TG-C01, TG-C02, TG-C03 and control *E.coli* ATCC 8739), 4 genes' expression were investigated; *copA*, *cusS*, *cusF* and the reference gene *tus.* In the melt peaks and melt curves of the runs, there was only one peak for each sample and no secondary peaks. This shows that no primer-dimers formed. Also, secondary products, non-specific bindings of primers, or nucleic acid contamination were not present in our samples. Moreover, there were no amplification and no melt peak for no-template controls, which indicated that no contamination or primer-dimer formation was present in our runs. Furthermore, efficiencies of RT-

qPCR runs of all samples were 100% and the slopes of the curves were almost -3,32. Our results demonstrated that the run was ideal and the duplication of target cDNA was occurred in each cycle successfully (Biosystems, 2013).

3.3.5.1. Relative Expressions of *copA, cusF* and *cusS*

The expressions of all 3 genes (*copA, cusF* and *cusS*) were normalized to *tus* expression by using Delta-delta C_t Method. The calculations were done for each mutant strains relative to the control strain *E.coli* ATCC 8739. Due to the fact that the achieved efficiency results for the RT-qPCR runs were 100%, this method was preferred in all calculations. Otherwise, possible efficiency changes might result in aberrations in the fold calculations of the relative expression.

In Figure 3.31, the relative expression of *copA* for the spontaneous and UV-mutants in reference to control strain was given. One-way ANOVA with Tukey's multiple comparison test results was shown in Table 3.5.

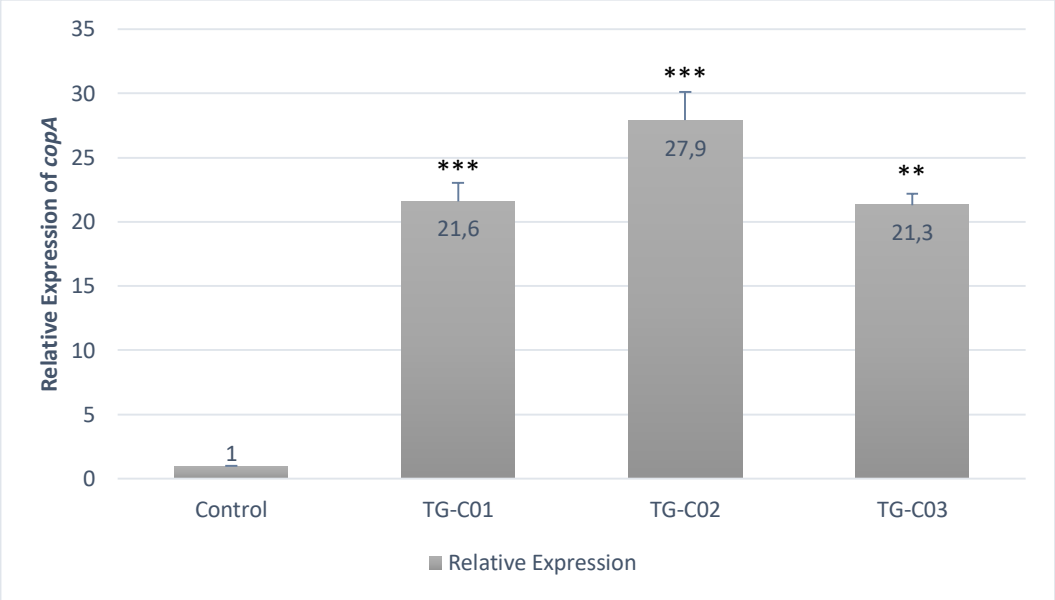

Figure 3.31. Relative expression of *copA* in spontaneous (TG-C01 and TG-C02) and UV-induced (TG-C03) mutants. The relative expression of *copA* was normalized in reference to *tus* gene. Fold changes are given within the bars for each mutant and calculated relative to the control strain; *E.coli* ATCC 8739. The error bars represent Standard Error of Mean (SEM). The confidence interval was taken as 95%. P values; * ($p \leq 0.05$), ** ($p \leq 0.01$), ***($p \leq 0.001$) by using one-way ANOVA test with Tukey's multiple comparison test.

Table 3.5. *One-way ANOVA with Tukey's Multiple Comparison Test Results of copA for spontaneous and UV-induced mutants with control strain E.coli ATCC 8739.The confidence interval was 95%. The statistical significance levels were indicated as stars; * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) while ns indicated no significance between strains.*

	Significance Level	**P Value**
Control Strain vs. TG-C01	***	0.0002
Control Strain vs. TG-C02	***	0.0002
Control Strain vs. TG-C03	**	0.0015
TG-C01 vs. TG-C02	ns	0.9907
TG-C01 vs. TG-C03	ns	0.2910
TG-C02 vs. TG-C03	ns	0.1985

With respect to the relative expression results of mutants TG-C01, TG-C02 and TG-C03 relative to control strain (*E.coli* ATCC 8739), all mutants showed at least 21.3 fold increase for the gene *copA*. These results correspond with the results of previous studies which reported increase of *copA* gene expression with the increasing copper concentration in the growth environment (Kershawet al., 2005) and 12-fold increase in *copA* expression with the incremental increase of copper concentration from 1μM to 1 mM (Outten et al., 2001).

The significance of the fold increases was checked with statistical analysis. Considering the statistical analysis results, fold increase in all 3 mutants were significant relative to the control strain (Table 3.5). The comparison of mutants among themselves showed that there was no significant difference (Table 3.5).

The relative expression of *cusF* in the spontaneous and UV-mutants relative to control strain was shown in Figure 3.32. In Table 3.6, one-way ANOVA with Tukey's multiple comparison test results was given for the *cusF* gene.

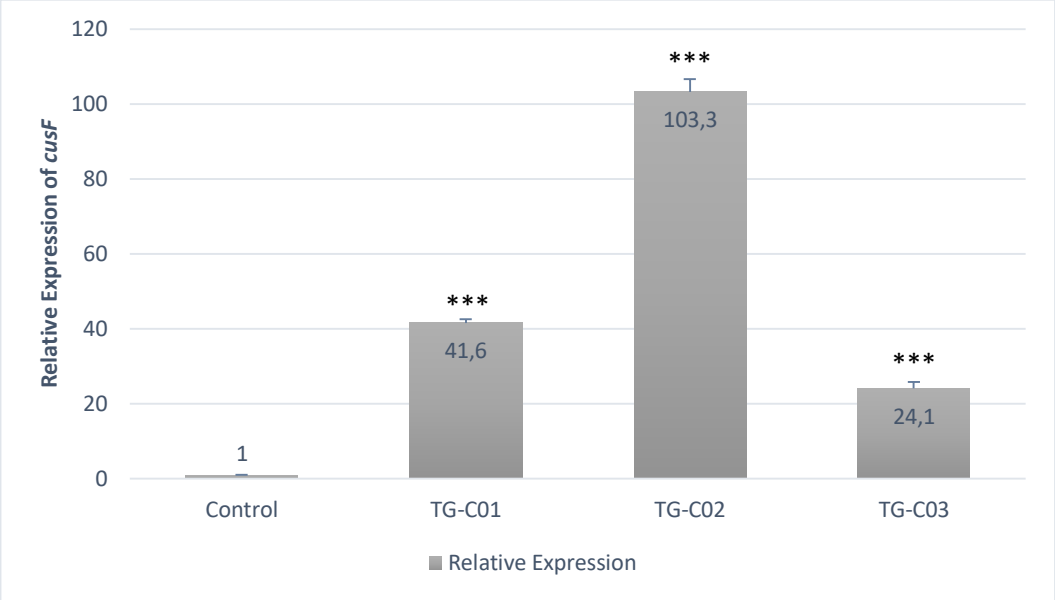

Figure 3.32. Relative expression of *cusF* in spontaneous (TG-C01 and TG-C02) and UV-induced (TG-C03) mutants. The relative expression of *cusF* was normalized in reference to *tus* gene. Fold changes are given within the bars for each mutant and calculated relative to the control strain; *E.coli* ATCC 8739. Standard Error of Mean (SEM) was used to represent the error bars. The confidence interval was 95%. P values; *($p \leq 0.05$),** ($p \leq 0.01$), *** ($p \leq 0.001$) by using one-way ANOVA test with Tukey's multiple comparison test.

Table 3.6. *One-way ANOVA with Tukey's Multiple Comparison Test Results of cusF for spontaneous and UV-induced mutants with control strain E.coli ATCC 8739. The confidence interval was 95%. The statistical significance levels were indicated as stars; * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) while ns indicated no significance between strains.*

	Significance Level	**P Value**
Control Strain vs. TG-C01	***	0.0001
Control Strain vs. TG-C02	***	<0.0001
Control Strain vs. TG-C03	***	0.0003
TG-C01 vs. TG-C02	ns	0.8931
TG-C01 vs. TG-C03	ns	0.8352
TG-C02 vs. TG-C03	ns	0.4591

The relative expression results of *cusF* for spontaneous mutants (TG-C01 and TG-C02) and UV-induced mutant (TG-C03) relative to the control strain (*E.coli* ATCC 8739) showed that all mutants gave increase in the expression with at least nearly 24.1-fold increase. This result was compatible with the previous study which showed expression increase in *cusF* gene both in low (750 μM) and high concentration (2 mM) of copper in the growth medium ranged from 12.05 fold to 59.56 fold, relative to no-copper added control sample of *E.coli* (Kershaw et al., 2005).

With respect to the statistical analysis results, expression increase in all mutants was significant relative to the control strain. However, analysis showed no significance between mutants as given in the Table 3.6.

Figure 3.33 shows the relative expression of *cusS* for the spontaneous and UV-induced mutants relative to control strain. One-way ANOVA with Tukey's multiple comparison test results for *cusS* was given in Table 3.7.

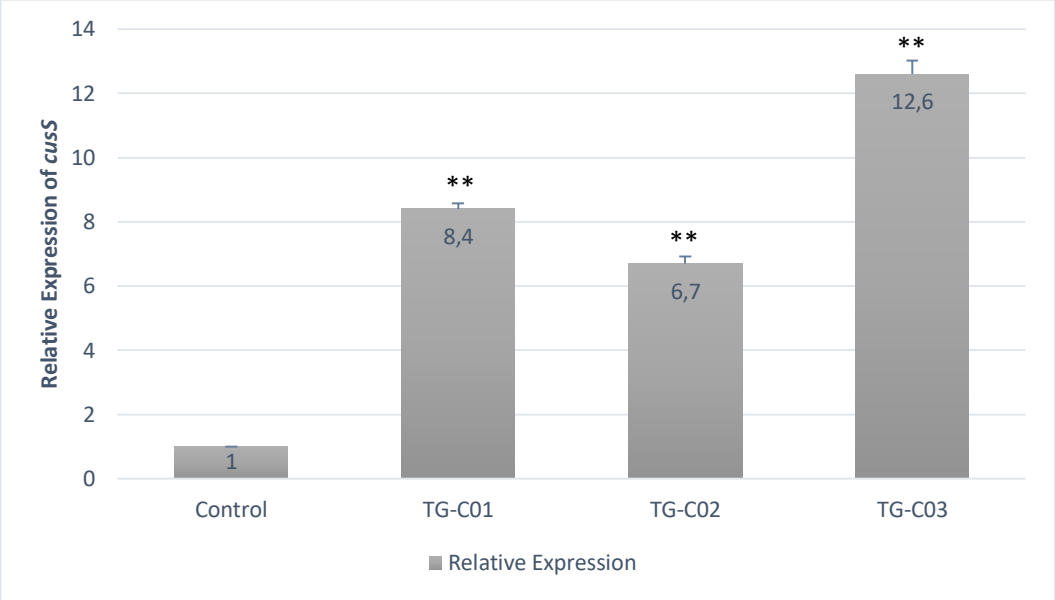

Figure 3.33. Relative expression of *cusS* in spontaneous (TG-C01 and TG-C02) and UV-induced (TG-C03) mutants. The relative expression of *cusS* was normalized in reference to *tus* gene. Fold changes are given within the bars for each mutant and calculated relative to the control strain; *E.coli* ATCC 8739. Standard Error of Mean (SEM) was used to represent the error bars. The confidence interval was 95%. P values; *($p \leq 0.05$),** ($p \leq 0.01$), *** ($p \leq 0.001$) by using one-way ANOVA test with Tukey's multiple comparison test.

Table 3.7. *One-way ANOVA withTukey's Multiple Comparison Test Results of cusS for spontaneous and UV-induced mutants with control strain E.coli ATCC 8739. The confidence interval was 95%. The statistical significance levels were indicated as stars; * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) while ns indicated no significance between strains.*

	Significance Level	**P Value**
Control Strain vs. TG-C01	**	0.0072
Control Strain vs. TG-C02	**	0.0087
Control Strain vs. TG-C03	**	0.0014
TG-C01 vs. TG-C02	ns	0.9987
TG-C01 vs. TG-C03	ns	0.5496
TG-C02 vs. TG-C03	ns	0.4710

With respect to the results in Figure 3.33, expression of *cusS* relative to the control strain (*E.coli* ATCC 8739) for 2 spontaneous mutants (TG-C01 and TG-C02) and 1 UV-induced mutant (TG-C03) increased in all strains significantly. The minimal fold increase was 6.7. This increase was in accord with the study which showed *cusS* transcription with the trigger of copper ions in *E.coli* (Yamamoto & Ishihama, 2005).

Although the expressions levels were significantly higher than the level in the control, mutants compared to each other showed no significant increase (Table 3.7).

These results indicate that while expressions of all 3 genes in all copper mutants increased significantly relative to control strain, they did not show significant increase among themselves. Considering that TG-C01 and TG-C02 were spontaneous mutants and TG-C03 is UV-induced mutant, the results imply that the method of mutagenesis did not have a significant role in the extent of expression in copper-related stress conditions.

CHAPTER 4

CONCLUSION

In this study we aimed to measure the relative expression of copper homeostasis genes in spontaneous and UV-induced mutants of *E.coli*. Minimal inhibitory concentration (MIC) for the strain *E.coli* ATCC 8739 was found to be 281 μg/ml for copper(II) chloride dihydrate via Agar Dilution Method. Above this concentration, we found two spontaneous *E.coli* mutants and 1 mutant UV-induced mutant. The relative expressions of the genes were measured by using RT-qPCR technique. The gene expressions of fundamental copper homeostasis proteins *copA*, *cusF* and *cusS* increased in all copper resistant mutants independently of being spontaneous or UV-induced. The results indicated that the way in which the mutants formed did not affect the expression of genes encoding fundamental copper homeostasis proteins significantly in copper resistant *E.coli* mutants. Our results verified that *copA*, *cusF* and *cusS* genes are crucial for the copper-resistant mutant strain formation. Moreover, *tus* gene provided stable expression under copper concentration driven stress conditions. Therefore, *tus* can be used as a reference gene in the RT-qPCR studies dealing with *E.coli* or at least for ATCC 8739 strain. Our results are significant from the stand point that we report, for the first time, the use of *tus* gene as a housekeeping gene in RT-qPCR studies under copper-driven stress conditions on *E.coli* previously and also copper-resistance homeostasis genes have not been studied in spontaneous mutants so far.

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APPENDICES

A. EQUIPMENTS

Table A.1. *Equipments and their suppliers*.

EQUIPMENT	SUPPLIER
Incubator	Binder, Germany
Shaker Incubator	Zhicheng, China
Magnetic Stirrer	VelpScientifica, Italy
Autoclave	Nuve, Turkey
pH Meter	Jenco, USA
Vortex	VelpScientifica, Italy
Microcentrifuge	Sigma, Germany
Class II Biological Safety Cabinet	ESCO, USA
UV-Spectrophotometer	SOIF, China
Biodrop	Biodrop, UK
Heat-block	Bioer, China
Gel Electrophoresis System	Biometra, Germany
Shortwave UV Transilluminator	UVP, Canada
PCR Machine	Thermo Scientific, USA
RT-qPCR Machine	Bio-RAD, USA
PCR Cabinet	N-Biotek, Korea

B. SOLUTIONS AND BUFFERS

TAE Buffer (50X):

- Tris-base……..242 g.
- Glacial acetic acid……..57.1 ml.
- EDTA (0.5 M, pH 8.0)……..100 ml.
- Distilled water (completed to) ……....1000 ml.

DNA Loading Dye (6X):

- Tris-HCl (pH 7.6)……..10 mM
- Bromophenol Blue (w/v)……0.03 %
- Xylene cyanol FF (w/v)…….0.03 %
- Glycerol (w/v)…….. 60 %
- EDTA…….60 mM

GTE Mix:

- Glucose……..50mM
- EDTA……10 mM
- TRIS-HCl (pH 8.0)……...25 mM
- RNase ……0.1 mg/ml

TE Buffer:

- TRIS-HCl ……10 mM
- EDTA……1 mM

The final pH was adjusted to 8.0.

50 µg/ml Copper chloride dihydrate Stock Solution:

- Copper chloride dihydrate……....3,17 g
- Distilled Water (complete to) ……50 ml

Copper chloride dihydrate was dissolved completely in distilled water. The solution was filter sterilized with 0,22 µl filter (Pall, USA).

C. CULTURE MEDIA COMPOSITIONS AND PREPARATIONS

Nutrient Agar:

- Meat Extract……..……...3.0 g
- Peptone from meat…….……..5.0 g
- Agar……...15.0 g
- Distilled Water (completed to)……. 1000 ml

After final pH was adjusted to 7.0 (+/- 0.2), sterilization of medium was done at 121°C for 20 minutes.

Nutrient Broth:

- Meat Extract……3.0 g
- Peptone from meat……..5.0 g
- Distilled Water (completed to)…… 1000 ml

After final pH was adjusted to 7.0 (+/- 0.2), sterilization of medium was done at 121°C for 20 minutes.

310 µg/ml Copper chloride dihydrate-containing Nutrient Agar:

- Meat Extract……..3.0 g
- Peptone from meat……....5.0 g
- Agar…….15.0 g
- Distilled Water (completed to)…….. 1000 ml

After final pH was adjusted to 7.0 (+/- 0.2), sterilization of medium was done at 121°C for 20 minutes.

Just before the agar media was solidified, 310 µg/ml copper chloride dihydrate was added to the media.

D. SUPPLIERS OF KIT, CHEMICALS AND ENZYMES

Agar...……Sigma

Agarose..…….……...Sigma

Copper chloride dihydrate…..……...Merck

DMSO……..…Thermo Fisher Scientific

EDTA ……..……..Sigma

Ethanol (Molcular Biology Grade) ……..….Sigma

Ethidium Bromide ……..……..AppliChem

Glacial Acetic Acid ……..…….…Merck

Glycerol …….…….……Fluka

HCl ……..……..Merck

NaCl……..…….Sigma

NaOH ……..……..Merck

Nutrient Broth ……..…….Merck

Trizma Base……..…….Sigma

Tris-HCl……..…….……...Fluka

Water (Molecular Biology Grade) ……..……Fisher BioReagents

2-mercaptoethanol ……...……Aldrich

Kits:

DNase I Kit, RNase-free ……..…Thermo Fisher

GeneJet RNA Purification Kit…….……Thermo Scientific

RevertAid First Strand cDNA Synthesis Kit ……..…….Thermo Fisher

SsoAdvanced Universal SYBR Green Super..……...……Bio-Rad

Taq DNA Polymerase 2X Master Mix …….…..Ampliqon

Enzymes:

RNase……...…….5Prime

E. DNA LADDER

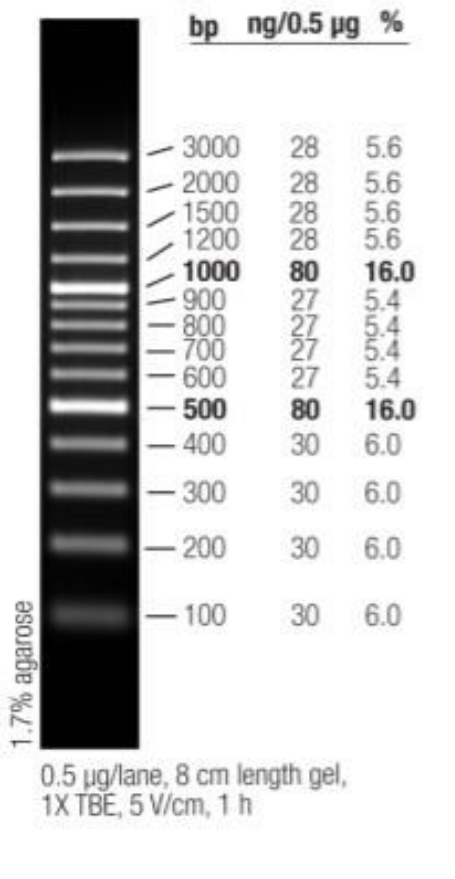

Figure E.1. DNA Ladder. GeneRuler 100 bp Plus (Thermo Scientific #SM0321).

F. RNA ISOLATION KIT PROCEDURE

Prior to Isolation

- Wash Buffer 1 and Wash Buffer 2 were prepared by adding specified amount of ethanol (96-100%).

Table F.1. *Preparation of wash buffers.*

	Wash Buffer 1	**Wash Buffer 2**
Wash Buffer (Concentrated)	40 ml	23 ml
Ethanol (added)	10 ml	39 ml
Total Volume	50 ml	62 ml

- Prior to each RNA purification, 20 µL of 14.3 M β-mercaptoethanol was supplemented to each 1 mL volume of Lysis Buffer.
- Lysozyme was added to TE buffer containing 10 mM TrisHCl and 1 mM EDTA with a pH of 8.9 by adjusting 0.4mg/mL final concentration.

Isolation Procedure

- Bacteria cells were collected during the growth of the cells were in their exponential phase.
- After 1.5 mL of bacterial culture (up to 1×10^9cells) was transferred to a 1.5 mL microcentrifuge tube, the collection of the cells was done by 2 min centrifugation at ≥12000 x g. The removal of the supernatant was done in a careful way to obtain a dry pellet as much as possible.
- TE buffer was prepared freshly by using lysozyme having a final concentration of 0.4mg/mL. Then, in 100 µL TE buffer, the pellet was dissolved by inverting the tubes multiple times.
- The resuspended cells were incubated for 5 minutes at 15-25°C.
- 300 µL Lysis Buffer which was prepared with the proper amount of β-mercaptoethanol were added to each tube and vortex was done for 15 seconds to get a homogeneous mixture.
- 180 µL of ethanol (96-100%) was added to each tube and pipetting was done.
- Maximum amount of 700 µL of lysate was taken and put into the 'GeneJET RNA Purification Column' which is found onto a collection tube. The centrifugation of the columns was done for 1 minute at ≥12000 × g. The flowthrough was discarded and the column was put back into the collection tube.
- Into each column, 700 µL Wash Buffer 1 was added. Then, centrifugation was done for 1 minute at ≥12000 × g. After the flowthrough was discarded, the column was put into the collection tube.
- To each column, 600 µL Wash Buffer 2 was added and centrifugation was done for 1 minute at ≥12000 × g. After the flowthrough was discarded, the column was put into the collection tube.

- Into each column, 250 μL Wash Buffer 2 was added. Then, centrifugation was done for 2 minutes at ≥12000 × g. The tube containing the flowthrough was discarded and the columns were taken into a new sterile microcentrifuge tube (1.5 mL) (RNase-free).
- 100 μL of nuclease-free water was put into the center of the purification column membranes. After centrifugation for 1 minute at ≥12000 × g, RNA was obtained.
- The used purification column was then discarded.

G. DNase APPLICATION PROTOCOL

- To an RNase –free tube, RNA, enzyme and reaction buffer is added in the following order;

Table G.1. *DNase mixture preparation.*

RNA	1 µg
10X Reaction Buffer (with $MgCl_2$)	1 µl
DNase I (RNase-free)	1 µl (1U)
RNase-DNase-free Water (completed to)	10 µl

- The samples were incubated at 37 °C for 30 minutes.
- 1 µL of 50 mM EDTA was added and incubation at 65 °C for 10 minutes was done.

H. cDNA SYNTHESIS PROTOCOL

- In the following order given in Table H.1, the indicated reagents were added to a nuclease–free tube placed on ice.

Table H.1. *cDNA synthesis mixture prepapation.*

Template RNA	0.1 ng - 5 µg
Primer	1 µl
Nuclease-free Water (completed to)	12 µl
5X Reaction Buffer	4 µl
RiboLock RNase Inhibitor (20U/µL)	1 µl
10 mMdNTP Mix	2 µl
RevertAid M-MuLV RT (200 U/µL)	1 µl

- The tubes was mixed in a gently way and centrifugation was done.
- Then, the tubes were incubated at 25°C for 5 minutes and then at 42°C for 60 minutes.
- The reaction was finished by incubation at 70°C for 5 minutes.

I. BOILING METHOD FOR DNA EXTRACTION

- The cells were collected from the overnight culture of TG-C01.
- The collected cells were dissolved in 1 ml of GTE Mix solution with RNase by vortexing.
- The solution containing the cells was placed in heat-block at 100°C for 10 minutes.
- Centrifugation at 1000 rpm for 5 minutes was done and the supernatant was taken which contains the extracted DNA.