UV AND COLD TEMPERATURE EFFECTS ON MESSENGER RNA INTEGRITY FROM HUMAN SALIVA

A DISSERTATION SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTORATE OF EDUCATION IN SCIENCE

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BALL STATE UNIVERSITY
MUNCIE, INDIANA
DECEMBER 2011
ABSTRACT

DISSERTATION: UV and cold temperature effects on messenger RNA integrity from human saliva

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Messenger ribonucleic acid (mRNA) turns out to be an increasingly important molecule in forensic analysis of biological samples. Because of the specific role of mRNA in all living cells to transfer genetic information from DNA to proteins, mRNA is able to provide cell-specific information and regulate control of gene expression. mRNA analysis performed on an extracted mRNA sample isolated from a biological stain of a crime scene can be used to identify the nature of the tissue(s) comprising the stain. In this research, the effects of a couple of mRNA storage conditions such as cold temperature and ultraviolet light exposure on mRNA integrity from human saliva have been
evaluated. Human saliva samples have been sampled and exposed to UV light and freezing temperature (-20°C) for varying lengths of time. Extracted mRNA from each sample has been quantified spectrophotometrically and subjected to real time RT-PCR to evaluate stability and integrity of one of the saliva marker transcripts, KRT13 mRNA, of treated samples compared to untreated samples. The results of this study indicated that UV light and freezing temperature don’t have a significant effect on the integrity of KRT13 mRNA. There is also no apparent correlation between Ct values of treated samples and treating intervals. This research holds important implications for the use of mRNA for applications in forensic science, an area which has not been researched extensively.
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INTRODUCTION

**mRNA analysis in forensic science**

Biological samples such as blood, semen, saliva, and hair are often left in a crime scene which can be used later as forensic evidence in a court of law (Alvarez *et al.*, 2004). The importance of DNA analysis has dominated molecular forensic science during the past decade (Bauer, 2007). DNA analysis of forensic biological evidence is routinely done in forensic laboratories. Technicians usually extract DNA from biological samples left in a crime scene and subject those samples to DNA analysis. Establishing the genetic profile of donors is then performed in order to compare it against known profiles (Juusola and Ballantyne, 2003).

Although DNA analysis is useful in the identification of individuals from even minute amounts of biological evidence containing only perhaps a few nucleated cells, the data obtained do not reveal the type and origin of the evidence since the DNA profile of most cells is exactly the same (Bauer, 2007; Bauer and Patzelt, 2003; Alvarez *et al.*, 2004). There are many forensic cases in which the nature of the biological evidence becomes crucial. An example could be blood stains claimed to be left behind from a rape or a homicide case which are claimed to be of menstrual origin by the suspect but no distinction between the blood origin is possible using DNA samples.
Many biological samples found at crime scenes are heterogeneous in nature and are contaminated with a diverse array of other biological and/or chemical adulterants. These samples are mixtures of different body fluids, for example semen and saliva, perspiration, or vaginal secretions, to name a few. There are some conventional methods to identify the nature of biological evidence such as biochemical, serological, and immunological assays (Juusola and Ballantyne, 2003). For example, blood stains collected from crime scenes are tested using the tetrabase [4,4-bis(dimethylamino diphenylmethane)] assay (Zubakov et al., 2007; Lomholt and Keiding, 1977), the Kastle–Meyer phenolphthalein test the tetramethylbenzidine test (Zubakov et al., 2007; Garner et al., 1976), the orthotolidine test (Zubakov et al., 2007), or with luminol (3-aminophthalhydrazide) chemoluminescence which is specifically used for detecting blood stains after cleaning attempts (Zubakov et al., 2007; Creamer et al., 2005). All of the above assays tests for the ability of hemoglobin in blood to break down hydrogen peroxide. The disadvantage of using these methods is the possibility of having false positive results from strong oxidants such as chlorine-containing detergents or the presence of plant peroxidases (Zubakov et al., 2007; Kent et al., 2003).

Saliva stains collected from crime scenes are tested using either an enzymatic amylase test with Phadebas or by an ELISA (enzyme-linked immunosorbent assay)-based method (Zubakov et al., 2007; Quarino et al., 2005). The disadvantage of these tests is that the time window for performing them is limited due to amylase degradation (Zubakov et al., 2007; Keating and Higgs, 1994). Another disadvantage of using these methods is that most are not able to differentiate between salivary amylase and amylases from other sources, such as pancreatic amylase and urinary amylase. Therefore, these
assays are considered presumptive, meaning that results could be indicative but not absolutely confirmatory.

Another disadvantage of using presumptive methods is that these methods are labor-intensive, time-consuming, technologically diverse and expensive. There are also no existing confirmatory tests for some frequently encountered body fluids found in many crime scenes. For example, there is no reliable conventional test for the presence of saliva or vaginal secretions (Bauer and Patzelt, 2003; Juusola and Ballantyne, 2003). Therefore, developing a standard procedure in order to identify the nature of body fluid is highly desired.

Messenger RNA (mRNA) turns out to be an increasingly important molecule in forensic analysis of biological samples. Because of the specific role of mRNA in all living cells to transfer genetic information from DNA to make cell-specific proteins, mRNA is able to provide detailed information reflecting the state of gene expression very sensitively. Therefore, mRNA expression analysis performed on an unknown biological sample may be used to determine the nature of the biological sample.

All differentiated cells in the human body have originally come from a fertilized egg. There are approximately 30,000 human genes in each human cell (Lander et al., 2001; Venter et al., 2001) except for red blood cells which lack DNA and immune cells in which some DNA is deleted. However, each cell type has a unique pattern of gene expression known as the transcriptome. In other words, different sets of genes are turned off or become transcriptionally silent while others sets are turned on - actively transcribed and translated into proteins in different cells. This is why there are many different types of cells with different functions in the body while having the same gene pool (Juusola
and Ballantyne, 2003). The genes that are actively on in blood monocytes or lymphocytes are different from those of spermatozoa, epithelial cells lining the oral cavity or epidermal cells from the skin, for example.

Due to the fact that there is a pattern of gene expression specific to each cell or tissue type, specific mRNA could be present in some cell types and absent in others. Moreover, the relative abundance of specific mRNA in some cell types versus the others could be different. Therefore, in order to develop a definitive test to identify unknown tissues or body fluids in crime scenes, the type and abundance of mRNAs could be tested in samples recovered at crime scenes using a number of amplification methods.

There are advantages to using mRNA detection methods compared to conventional biochemical assays, including greater specificity, the ability to have simultaneous and semi-automatic analysis, improved timeliness, more reliability, lower sample consumption, and lower cost. Even a small trace of mRNA in biological evidence can be detected and amplified using mRNA amplification methods under appropriate conditions, assuming the sample has been properly stored.

**mRNA stability**

RNA is generally believed to be unstable compared to DNA, primarily because RNA can be easily and rapidly degraded by endogenous and exogenous ribonucleases (RNases). RNases are either present in cells and/or they originate from bacterial cells and environmental contamination, such as those present on the skin of laboratory personnel or anyone who contacts the sample directly (Bauer, 2007). RNA is also believed to be very sensitive to physical and chemical factors. Therefore, forensic scientists have resisted
looking into the potential of RNA for use in forensic science. However, research indicates that mRNA can be detected and recovered after a long period of time after cell death. Multiple studies reveal that mRNA can be detected in post-mortem brain, liver, heart and collected within 12 hours of death (Johnson et al., 1986; Matsubara et al., 1992). One study reported that cytokeratin 19 (CK19) and progesterone receptor (PR) mRNAs were expressed in menstrual blood and not in peripheral blood (Juusola and Ballantyne, 2003, Zubakov et al., 2009). This study indicated that CK19 and PR mRNAs could also be detected in up to 6 month old dried menstrual blood. In another study, Juusola and Ballantyne (2003) showed that mRNA was stable in human blood, saliva, and semen, where tissue specific mRNAs were recovered in sufficient quantity and quality for further analysis. In this study samples were collected, aliquoted, and set to be dried at room temperature on either swabs or cotton gauze. For example, 50 μl aliquots of blood stains were stores at room temperature in dark up to nine months. The results indicated total RNA is able to keep its integrity in significant number of samples.

Rapid RNA degradation takes place in organs such as pancreas and liver, which contain a large amount of RNases (Bauer, 2007; Wong and Medrano, 2005) compared to other organs, specifically brain, in which mRNA seems to exhibit greater stability, up to 96h post-mortem (Zubakov et al., 2008). mRNA profiling for body fluid showed that mRNAs were stable up to 2-years in body fluid stains (Haas et al., 2009). Other studies indicate that mRNA extracted from dried stains which had been stored for up to 15 years could be amplified using reverse transcriptase polymerase chain reaction (RT-PCR) (Bauer, 2007).
Fetal and neonatal lung tissues have shown prolonged post-mortem RNA stability (De Paepe et al., 2002). Other human tissues such as human bone (Kuliwaba et al., 2005) have also shown long post-mortem RNA stability. Interestingly, brain has been shown to have the most stable mRNA content and liver the least stable (Inoue et al., 2002).

**Effects of physical factors on mRNA stability**

Few studies are published that address the effects of physical and chemical treatments on the stability of mRNA. Limited research has indicated that chemical and physical factors could greatly affect mRNA stability. For example, hypoxic induction of vascular endothelial growth factor (VEGF), a major regulator of physiological and pathological angiogenesis, is not only due to transcriptional activation but also to an increase in stability of mRNA (Ikeda et al., 1995). Results of that study indicated a slow increase of VEGF mRNA stability during hypoxia.

In another study, mice brain samples which had been stored at 4 °C and 37 °C with 80% humidity post-mortem were taken at six time points (immediately, 0.5h, 2h, 6h, 24h, 48h) (Zhu et al., 2011). Beta-actin mRNA and 18S rRNA were then amplified using real time RT-PCR. Results indicated that the RNA degradation rate was faster at 37 °C compared to 4 °C, as would be expected. Therefore there is a correlation between temperature and RNA degradation. The authors also found 18S rRNA to be more stable than beta-actin mRNA, suggesting differences in rates of degradation depending upon the specific mRNA.
**Tissue-specific genes**

Tissue-specific mRNA markers have been determined for most body fluids commonly found at crime scenes. Metalloproteinases, Zinc dependent enzymes able to cleave extracellular matrix proteins, have been identified as enzymes which were highly expressed in menstruating endometrium (Bauer, 2007), allowing reliable differentiation of menstrual from non-menstrual blood. There are different mRNA markers that are frequently used in forensic science in order to determine the nature of forensic samples. A series of time-wise degraded blood and saliva stain samples were tested using real time RT-PCR (Zubakov et al., 2008). In this study the blood and saliva samples were swabbed. Swabs were then left on a bench top at room temperature to be dried. After the drying step, the swabs were stored in dust-free nonhumid conditions (but subjected to normal daylight) for varying time intervals. The results of this study showed nine stable mRNA markers for blood including CASP1, AMICA1, C1QR1, ALOX5AP, AQP9, C5R1, NCF2, MNDA, and ARHGAP26, and five stable mRNA markers for saliva, including SPRR3, SPRR1A, KRT4, KRT6A, and KRT13 (Table 1). These markers showed tissue-specific expression in samples aged up to 180 days of age.

Another study revealed differentiation between several body fluids as well (Nussbaumer et al., 2006). In this study, three tissue-specific genes including hemoglobin-alpha locus1 (HBA), kallikrein 3 (KLK) and mucin4 (MUC) were identified. The human MMP-7 gene was recently identified as a useful indicator for differentiation of menstrual blood from other stains (Bauer and Patzelt, 2008). Detecting MMP-7 mRNA using real time RT-PCR indicated that this method was reliable and sensitive.
Cytokeratin 13 (KRT13) is a protein encoded by the *krt13* gene in humans (Romano et al., 1992, Schweizer et al., 2006). KRT13 is a member of the type I intermediate filament protein family existing in the cytoskeleton of epithelial cells. There are 19 unique cytokeratins with molecular masses between 68 and 40 kDa. It has been reported that expression of cytokeratin molecules are tissue-specific. For example, nonstratified epithelia contain simple cytokeratin molecules, including cytokeratins eight and 18, unlike stratified squamous epithelia that contains more complex cytokeratin molecules. KRT13 is one of the markers frequently used in forensic science in order to determine the nature of body fluids in crime scenes.

**Real-time RT-PCR**

In RT-PCR, mRNA is transcribed into DNA (complementary DNA, cDNA) using the reverse transcriptase (RT enzyme) and a transcript-specific primer (Bauer and Patzelt, 2005). The cDNA is amplified using primers binding to the target sequence of interest (Fig.1). Real-time PCR and real-time RT-PCR have catalyzed dramatic changes in many disciplines of biology. Real-time PCR is the technique that allows collection of data throughout the process of PCR. Real-time PCR is capable of combining amplification and detection into a single step (Wong and Medrano, 2005). This ability is supported by using different fluorescent chemistries that allows one to draw a correlation between the concentration of PCR product and fluorescence intensity.

SYBR Green I is one quantification method used in real time PCR. SYBR Green I is a fluorescent dye that has the ability to intercalate with double-stranded DNA produced
during RT-PCR. SYBR Green I provides the simplest and most economical approach for detecting and quantitating real time RT-PCR products. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution alone, but emits a strong fluorescent signal upon binding to double-stranded nucleic acids. As more double-stranded amplicons are produced, SYBR Green I dye signal increases correspondingly. The disadvantages of using this method is that SYBR Green I will bind to any double-stranded DNA in the reaction, including primer-dimers and other nonspecific reaction products. Amplicon identity can easily be confirmed using melting curve analyses, in which the dissociation-characteristics of double-stranded DNA during heating are evaluated. There are many advantages related to using SYBR Green I such as relatively low cost, ease of use, and high sensitivity of detection (Bauer and Patzelt, 2008).

The real time feature of the amplification process allows for data acquisition starting at the point or the cycle at which the product is first detected rather than accumulation of amplification product after a fixed number of cycles. This value is usually referred to as cycle threshold (Ct). Ct is the PCR cycle number at which the fluorescence of the reaction passes over the background fluorescence (Wong and Medrano, 2005). There is a relatively quantitative relationship between amount of starting target transcript and the Ct value. The higher the Ct value, the lower the amount of starting target transcript and vice versa. Therefore, a low Ct value indicates a large amount of target transcript in the reaction in the beginning. In other words, the greater the quantity of target mRNA at the starting point of reaction, the faster a significant increase in fluorescent signal will appear, yielding a lower Ct (and vice versa).
There are many advantages in using real time RT-PCR. This amplification method does not require post-amplification manipulation, such as agarose gel electrophoresis (Wong and Medrano, 2005). Real-time PCR is 10,000- to 100,000-fold more sensitive than other traditional amplification assays for nucleic acid detection (Wang and Brown, 1999). For example, real-time PCR is 1000-fold more sensitive than dot blot hybridization (Malinen et al., 2003). Real-time PCR is theoretically able to detect a single copy of a specific transcript (Palmer et al., 2003). Real time RT-PCR is also able to differentiate between mRNAs with similar sequences. Real time RT-PCR needs much less RNA template than other methods of gene expression analysis. A disadvantage of using real time PCR in general is that real time PCR requires expensive equipment and reagents compared to traditional methods of analysis.

**Purpose of the study**

The role of mRNA in forensics has not been thoroughly explored. There is much potential in studying mRNA, since it is possible to accurately and sensitively determine body fluid type in biological evidence specimens, which may in some cases, circumvent expensive DNA typing procedures, and expedite casework handling. Cold temperature and ultraviolet light exposure represent abuse storage conditions to which extracted mRNA is frequently exposed. This research examines the possibility of using mRNA to determine the nature of forensic samples such as saliva and to evaluate several poor mRNA storage conditions on the integrity of extracted mRNA as a target molecule in forensic evidence analysis.
MATERIALS AND METHODS

Saliva Sample Collection

Twenty healthy subjects (ten male, ten female) volunteered for saliva donation. Volunteers were recruited as they responded to posted flyers in various locations in Cooper Science Building, Ball State University. All sample collections were performed according to protocols set forth by the Ball State University Institutional Review Board (IRB), including signing a consent form for donating saliva. Donors avoided consumption of food, gum, or flavored beverages for 1h prior to sample acquisition. Each donor’s mouth was thoroughly rinsed with tap water before sampling to avoid any outside contaminants. At least 3 ml of saliva was collected from each donor in a sterile container (Fisherbrand, Mexico, #06-443-18) in order to have enough samples for 3 replicates. Each sample was then immediately subjected to RNA extraction.

RNA Extraction

750 µl of Ribozol reagent (Amresco, Solon, Ohio, # 0951C493) was used for each 250 µl of collected saliva. Saliva was treated in Ribozol by aspiration. Homogenized samples were incubated for 5 minutes at ambient temperature allowing the complete separation of nucleic acids from associated proteins. Then, 200 µl of chloroform (EM
Science, Gibbstown, NJ, # CX1055-6) (per 750 µl of Ribozol reagent) was added. Tubes were shaken vigorously by hand for 15 seconds and incubated at 23 °C for 5 minutes, and then centrifuged at 12,000 × g for 15 minutes at 4°C. The mixture separated into three phases after centrifugation: a lower pink, phenol-chloroform phase; a white interphase and; a colorless upper aqueous phase which contains the RNA portion of the cells.

Aqueous phases were transferred to fresh RNAase-free tubes. In order to precipitate the RNA from the aqueous phase 500 µl of isopropyl alcohol (Sigma, S. Louis, MO, # 036K3716) was added per 750 µl of Ribozol reagent used for the initial homogenization of saliva samples. Samples were incubated at 23 °C for 5-10 minutes and centrifuged at 12,000 × g for 8 minutes at 4°C. The RNA pellets were usually invisible after centrifugation.

After removing the supernatant, RNA pellets were washed once with 1 ml of cold 75% ethanol (Pharmco-AAPER Alcohol, Shelbyville, KY, #KJK12F) per 750 µl of Ribozol reagent used for the initial homogenization. Samples were mixed completely by vortexing and centrifuged at 7,500 × g for 5 minutes at 25°C. At the end of the procedure RNA pellets were briefly air dried not more than 5-10 minutes to avoid decreasing RNA solubility. RNA sample pellets were dissolved in 10 µl of RNase-free water by aspiration. Tubes containing RNA solution were incubated for 10 minutes in a 55 to 60 °C waterbath to help RNA pellets to dissolve more easily.

**UV light treatments**

Six extracted RNA samples from female subjects and six extracted RNA samples from male subjects were each divided into three aliquots of 1 ml in order to have
triplicates for each treatment. Then samples were randomly chosen to be subjected to UV light for 0 min, 2 min, 5 min, 10 min, 20 min, 30 min, 60 min, 240 min, 480 min, 720 min, 1080 min, and 1440 min (Table 2). The 254 nm UV light source (Fischer Scientific PCR hood, Pittsburgh, PA) was placed 50 cm above the purified RNA samples for the specified time period in the absence of visible light. Each treatment was performed in triplicate. Samples were then subjected to quantification and Real-time RT-PCR reactions as described below.

- 20 °C freezing treatments

Four extracted RNA samples from female subjects and four extracted RNA samples from male subjects were each divided into three aliquots of 1 ml in order to have triplicates for each treatment. Then samples were randomly chosen to be subjected to 0 days, 1 day, 7 days, 21 days, 42 days, 56 days, 70 days, and 84 days of -20 °C freezing treatments (Table 3). Each treatment was performed in triplicate. Samples were then subjected to quantification and real-time RT-PCR reactions as described below.

RNA quantification

RNA samples were quantified spectrophotometrically (Beckman Coulter™, DU 530, Fullerton, CA) at 260 nm (A$_{260}$). Samples with A$_{260}$ readings of 0.09-0.9 were collected and used for RNA amplification, as these were considered to be within the linear range of the instrument’s capabilities. A$_{260}$/A$_{280}$ ratio values also were recorded in order to estimate the purity of RNA. Ratio values of 1.8 to 2.0 were favorable, meaning that RNA sample was less contaminated with protein and DNA.
Quartz cuvettes were washed using 95% ethanol (Pharmco-AAPER Alcohol, Shelbyville, KY, #KJK12F) carefully. TE buffer (10 mM Tris-Cl, pH 7.5; Fisher Scientific, Fair Lawn, New Jersey, # BP152-500) was used to blank the spectrophotometer.

**Example RNA quantification:**

\[ A_{260} = 0.240 \]

Concentration of RNA sample: \( 44.19 \times A_{260} \times \text{dilution factor} \)

\[ 44.19 \times 0.08 \times 100 = 353.52 \mu\text{g/ml} = 0.354 \mu\text{g/\mu l} \]

The concentration of RNA in each sample was then standardized to 0.5 µg/µl.

**Example RNA standardization:**

\[ 0.5/0.354 = 1.41 \mu\text{l extracted RNA used in a Real-time RT-PCR reaction} \]

**Real-time RT-PCR reactions**

A commercial MasterAmp RT-PCR kit (Epibio, Madison, WI, # 10517) was used for performing Real-time RT-PCR reactions. The general protocols for real-time RT-PCR amplification of KRT13 transcript, internal control (GAPDH), and negative control are summarized in Table 4. Information about all primers used in real-time RT-PCR amplification of KRT13, GAPDH transcripts are included in Table 5.

Sixteen reactions were usually set up at each real-time RT-PCR run. Each treated or untreated sample was used in triplicate. The general protocol for setting up one Real-time RT-PCR reactions was as follows:

1. A master mix was prepared by adding the following:
   a. 1.25 µl 20X RT-PCR buffer
b. 3 ul 25mM MgCl₂ 

c. 2.5 ul MasterAmp 10X PCR enhancer 

d. 0.5 ul 25mM MnSO₄ 

e. 0.25 ul Retramp RT DNA polymerase 

f. 4 ul dNTPs 

g. 0.63 ul forward primer (krt13F; Integrated DNA Technologies, Coralville, IA) 

h. 0.63 ul reverse primer (krt13R; Integrated DNA Technologies, Coralville, IA) 

Since there was usually more than one reaction at each run, a larger amount of master mix was prepared and then allocated for each reaction. Owing to the light sensitivity of SYBR Green dye, aluminum foil was used to wrap up the Master mix tube from this step forward. 

i. 0.5 ul SYBR Green I (1,000X concentration, Invitrogen, Eugene, OR, #27521W): Reagents were mixed by gentle aspiration. 

2. RNA samples with volume adjusted to 0.5 µg/µl were added to each test tube (except for negative control and internal control). 

3. 18.26 µl of reagents was then added to each test tube except the internal control which had a different set of primers. 

4. RNase-free water was then added to the reaction tube to bring the reaction volume up to 25 µl.
5. The Smart Cycler (Cephied, Sunnyvale, CA) was programmed to run up to 42 cycles. The cycling regime included 3 stages. The first stage completed in 120 sec at 95°C. In this stage, real-time tubes were heated in the real-time Smart Cycler. The second stage was holding step completed in 1400 sec at 60°C. The last stage included 42 repeats of 3 cycles: 20 sec at 95°C, 75 sec at 53°C, and 3-60 sec at 72°C.
RESULTS

UV light treated saliva samples

Real-time RT-PCR was used in this study to analyze the integrity of mRNA under poor storage conditions such as storing in a defrosting freezer or UV light exposure. Samples were analyzed in triplicate. Real-time amplification of the KRT13 transcript level was graphically depicted as log scale of fluorescence (SYBR Green I) versus cycle number (Fig. 2).

An average of cycle threshold (Ct) values was used in this study to assess the results (Tables 6 and 7). Ct is the PCR cycle number at which the fluorescence of the reaction exceeds the background fluorescence. In this study, the threshold was set to 1 in order to detect all fluorescence presence (Fig. 3).

Figure 4 shows an example of real time RT-PCR amplification graph for UV light treated samples. This graph also shows the Ct values corresponding to each sample. Figure 5 shows the melting curves for the same UV light treated samples. The melting data exported from smartcycler indicated that KRT13 transcript was successfully amplified.
No significant effect was measureable by UV light treatment on KRT13 mRNA quality

Comparison of average Ct values among UV light-treated saliva samples showed that Ct values among treated samples are widely spread (Table 6). Comparison of average Ct values between untreated and UV light-treated saliva samples shows that Ct values increased in 83.3% of the UV light treated samples (Table 6) indicating that integrity of KRT13 mRNA was negatively affected in 83.3% of the UV light treated samples. Although a general decrease in integrity of KRT13 transcript in UV light treated saliva samples was observed, no significant effect of UV light treatment on the quality of the KRT13 mRNA could be detected as attested by R-squared value and error bars in Figure 8.

No measureable correlation exists between Ct values of UV light-treated samples and UV light treating intervals

Although most of the UV light treated samples showed a decrease in integrity of KRT13 mRNA, no significant positive or negative pattern or trend was seen among samples exposed to UV light at different intervals (Fig. 8).

-20 °C freezing treated saliva samples

Real-time RT-PCR was used in this study to analyze the integrity of mRNA in -20 °C freezing-treated saliva samples. Samples were run in triplicate. Real-time amplification of KRT13 transcript levels was graphically depicted as log scale of
fluorescence (SYBR Green I) *versus* cycle number. Figure 6 shows an example of real time RT-PCR amplification graph for -20 °C freezing treated samples. This graph also shows the Ct values corresponding to each sample. Figure 7 shows the melting curves for the same -20 °C freezing treated samples. The melting data exported from smartcycler indicated that KRT13 transcript was successfully amplified.

**No significant effect was measureable by the -20 °C freezing treatment on KRT13 mRNA quality**

Comparison of average Ct values between untreated and -20 °C freezing-treated saliva samples showed that Ct values increased in 75% of the -20 °C freezing treated samples (Table 7), indicating integrity of KRT13 mRNA was negatively affected in most of the -20 °C freezing treated samples. Although comparison of average Ct values between untreated and -20 °C freezing treated saliva samples indicated a general decrease in integrity of KRT13 transcript in -20 °C freezing treated saliva samples (Table 7), no significant effect by -20 °C freezing treatment on the quality of the KRT13 mRNA could be detected as attested by R-squared value in Figure 5.

**No apparent correlation between Ct values of -20 °C freezing treated samples and 20 °C freezing treating intervals was measureable**

Although 75% of the -20 °C freezing treated samples showed a decrease in integrity of KRT13 mRNA there was not a significant positive or negative pattern or trend seen among samples exposed to -20 °C freezing treatment at different intervals.
DISCUSSION

The role of mRNA in forensics has not been thoroughly explored. There is great potential, however, in studying mRNA during biological evidence analysis, since it is possible to accurately and sensitively determine body fluid type in tissue and body fluid specimens, which may in some cases, circumvent expensive DNA typing procedures, and expedite casework handling (Alvarez et al., 2004).

Cold temperature and ultraviolet light exposure represent conditions to which extracted mRNA might be exposed to. It is widely believed that RNA is not stable, being prone to rapid degradation by exogenous ribonucleases (Setzer et al., 2008). This research examined the possibility of using mRNA to semiquantitatively evaluate the effect of UV light and cold temperatures on the integrity of KRT13 mRNA.

The presumptive tests that are currently used such as the Phadebas test for saliva, the ABAcard HemaTrace method for blood, or prostate specific antigen detection for semen (Virkler et al., 2009) can either give false positive results, or their sensitivity is under question (Visser et al., 2001). For example, the forensic luminol test which is commonly used in crime scene evaluation to detect blood has a major disadvantage of not having selectivity (Creamer et al., 2005). Substances other than blood such as sodium
hypochlorite solution (bleach) which is a common ingredient in many cleaning agents in homes and industries can give false positive results using the luminol test.

Some research showed that mRNA molecules are capable of providing the necessary specificity, sensitivity and analysis by automation that modern forensic biology laboratories look for in order to determine the cellular origin of biological evidence. Studies have shown that there are various genes which over-express their mRNAs in peripheral blood, menstrual blood, saliva, semen and vaginal secretions. Therefore, these mRNA markers could be used with high sensitivity in order to determine the origin of forensic evidence in a short amount of time (Bauer and Patzelt, 2002; Fleming et al., 2010; Sakurada et al., 2009).

There is some evidence showing RNA is in fact a very stable molecule. There are even a few studies indicating that RNA can be used in order to estimate the postmortem interval (PMI) which is very important in forensic medicine (Li et al., 2011). In another study to evaluate the PMI, the stability of mRNAs for 3 different genes of bone and bone marrow was shown to be about 21 days (Van Doorn et al., 2001). In this study, total RNA was extracted from bone and bone marrow at different time points between zero and 31 days after death. Using real time PCR, the stability of three different mRNAs was evaluated. The results of this study showed stability of examined mRNAs up to 21 days postmortem. In another study, CDSN, LOR and KRT9 were identified as mRNAs overexpressed in skin. Therefore, these mRNA markers could be used in differentiating even tiny skin epidermal tissue evidence left in crime scenes (Visser et al., 2011).

In another study, nine blood-specific mRNA markers and five saliva-specific mRNA markers showed stability and sensitivity in samples which had dried up to 16
years of age (Zubakov et al., 2009). There are some studies indicating that the stability of RNA in post-mortem tissue is dependent upon the type of tissue and the conditions in which the postmortem tissue has been stored (Bahar et al., 2007; Fitzpatrick et al., 2002).

There are studies supporting each of the two sides of the issue of RNA stability. Although some studies indicate that RNA is very stable in some human post-mortem tissues (Cummings et al., 2001; Yasojima et al., 2001), many others have indicated that RNA quality can be negatively affected by several physical and chemical factors (Barrachina et al., 2006).

Effects of poor storage conditions on integrity of mRNA have not fully been understood. In one recent study, stains from blood, saliva, semen, and vaginal secretions were exposed to visible light versus UV light, temperatures, and humidity from 1 to 547 days (Setzler et al., 2008). The stability of 8 unique housekeeping and tissue-specific mRNA transcripts was evaluated using RT-PCR. The results of this study indicated that mRNA can be recovered from samples in sufficient quantity and quality for downstream mRNA analysis. mRNA could be detectable in those samples which had been stored at room temperature for at least 547 days. Samples which had been protected from direct rain impact showed housekeeping and tissue specific mRNA stability up to 7 days for saliva and semen samples, 30 days for blood samples, and 180 days for vaginal samples.

In this study, extracted RNA from 20 healthy individuals was exposed to UV light and -20 °C freezing temperature at different intervals. Then target KRT13 mRNA was amplified in each sample using real time RT-PCR. Each sample was analyzed in triplicate. Average of cycle threshold (Ct) values were used in this study to assess results.
There is a quantitative relationship between the amount of starting target transcript and the Ct value. The higher the starting copy number of the transcript target, the sooner a significant increase in fluorescence is observed. Therefore, the lower the Ct value the higher the number of target transcript and vice versa. Therefore, a Ct value can be used as a relative measurement of integrity of mRNA transcripts in treated versus untreated samples. In this study, the threshold was set to 1 in order to detect all fluorescence presence.

Comparison of average Ct values between untreated and UV light and -20 °C treated saliva samples showed that average Ct values increased in most of the treated samples (Table 6 and 7). Higher Ct values indicated that there were not a high number of integrated KRT13 mRNA copies in the reaction tube to start with. Higher Ct values for most of the treated sample could be an indication of the negative effect of UV light and 20 °C freezing treatment on the integrity of mRNA. Most researchers accept the notion that RNA degradation is faster at higher temperatures (Zhu et al., 2011). However, because the Ct values are widely spread among the treated samples and the R-squared values are very small, there is no reliable evidence in this research to support that statement. Therefore, the main conclusion is that there is no significant negative effect observed by exposure to UV light or -20 °C temperature on the integrity of isolated KRT13 mRNA.

The results of this study indicated that although there could be some negative effects resulting from UV light or -20 °C freezing treatments on the quality of KRT13 mRNA, RT-PCR data support the idea that KRT13 could still be used as a saliva marker in evaluation of crime scenes. The results of this study indicated that mRNA was able to
maintain sufficient integrity to be amplified when it was exposed to harsh physical storage conditions.

Ct value comparisons between untreated and UV light and -20 °C treated samples showed no significant increase in the average Ct values of treated samples as the treatment period increased (Table 6 and 7). Therefore, the results of this study indicated that increased periods of mRNA physical treatment did not necessarily cause more mRNA degradation. There was no significant correlation between the period of treatment and values for average Ct (Figures 8 and 9).

Using real time RT-PCR, forensic scientists would be able to cut through the labor extensive presumptive tests to identify the nature of stains left in crime scenes (Juusola and Ballantyne, 2005). There are several studies which showed the successful use of either RT-PCR or real time RT-PCR in amplification of body fluid marker genes (Bauer, M. and D. Patzelt. 2002; Bauer, M. and D. Patzelt. 2003). Other studies showed mRNA molecules are indeed very stable molecules. The results of this study also reinforced those latter findings, indicating that KRT 13 mRNA has the capability to survive harsh physical storage conditions, and that this target can be successfully amplified and detected using real time RT-PCR. The future direction for this research would be to either expose whole saliva to the same treatments or other physical and chemical treatments and also to expose other body fluids to physical and chemical treatments representing treatments that forensic evidence might be exposed to, in order to evaluate the stability of their marker transcripts under different treatments.
Figure 1: RT-PCR procedure (www.Takara-bio.com). In the RT-PCR process, mRNA is transcribed back into DNA (complementary DNA, cDNA) using the enzyme reverse transcriptase (RT enzyme). Then cDNA is amplified using primers binding to the target DNA.
**Figure 2: Real-time amplification of the KRT13 transcript.** Real-time amplification of the KRT13 transcript level was graphically depicted as log scale of fluorescence (SYBR Green I) *versus* cycle number. SYBR Green I is a fluorescent dye used commonly in real time PCR.
**Figure 3: Ct values of real time RT-PCR reactions.** This picture shows an example of recorded Ct values in the Smartcycler. Ct is the PCR cycle number at which the fluorescence of the reaction exceeds the background fluorescence. In this study, the threshold was set to 1 in order to detect any fluorescence present.
Figure 4: A real time RT-PCR example graph showing Ct values of several UV light treated samples. Ct values for several UV light treated samples which have been exposed to different UV light exposure intervals have been recorded in this graph.
Figure 5: A melt curve analysis for several UV light treated samples. This graph is an example graph of melt curve analysis for several UV light treated samples. Smartcycler was able to detect the temperature at which the dsDNA dissociated. Melting temperature is based on the GC content of the dsDNA, and its length. The results of melting curve analysis indicated that the melting temperature of the target cDNAs among samples was in a same range.
Figure 6: A real time RT-PCR example graph showing Ct values of several - 20 °C freezing treated samples. Ct values for several - 20 °C freezing treated samples which have been exposed to different - 20 °C freezing treatment intervals have been recorded in this graph.
**Figure 7: A melt curve analysis for several - 20 °C freezing treated samples.** This graph is an example graph of melting curve analysis for several - 20 °C freezing treated samples. The results of melting curve analysis indicated that melting temperature of the target cDNAs among samples was in the same range.
Figure 8: Effect of UV light treatment on KRT13 mRNA quality. Real-time RT-PCR was used to analyze the integrity of KRT13 mRNA in collected UV light-treated human saliva samples. Samples were analyzed in triplicate. Average of cycle threshold (Ct) values for three replicates of each treatment was used in this study to assess the results. Although 83.3% of the UV light-treated samples showed a decrease in integrity of KRT13 mRNA, no significant effect by UV light treatment on the quality of the KRT13 mRNA could be concluded as attested by R-squared value and error bars. Also, no apparent correlation between Ct values of UV light-treated samples and UV light treating intervals was measureable as attested by the poor R-squared value.
Real-time RT-PCR was used to analyze the integrity of KRT13 mRNA in collected -20 °C freezing-treated human saliva samples. Samples were analyzed in triplicate. Average of cycle threshold (Ct) values for three replicates of each treatment was used in this study to assess the results. Although 75% of the -20 °C freezing treated samples showed a decrease in integrity of KRT13 mRNA, no significant effect by -20 °C freezing treatment on the quality of the KRT13 mRNA could be concluded as attested by R-squared value. Also, no apparent correlation between Ct values of -20 °C freezing treated samples and -20 °C freezing treating intervals was measureable as attested by the poor R-squared value.

Figure 9: Effect of the -20 °C freezing treatment on KRT13 mRNA quality.
**Table 1: A summary of several mRNA markers.** Several mRNA markers, their abbreviations, full names, and the tissues expressed in are listed below.

<table>
<thead>
<tr>
<th>mRNA Marker Abbreviation</th>
<th>mRNA Marker Full Name</th>
<th>Expressed Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASP1</td>
<td>apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>AMICA1</td>
<td>adhesion molecule, interacts with CXADR antigen 1</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>C1QR1</td>
<td>Homo sapiens complement component 1, q subcomponent, receptor 1 (C1QR1)</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>ALOX5AP</td>
<td>arachidonate 5-lipoxygenase-activating protein</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>AQP9</td>
<td>Aquaporin9</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>CSR1</td>
<td>complement component 5a receptor 1</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>NCF2</td>
<td>Neutrophil cytosolic factor 2</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>MNDA</td>
<td>Myeloid cell nuclear differentiation antigen</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>ARHGAP26</td>
<td>Rho GTPase activating protein 26</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>SPRR3</td>
<td>small proline-rich protein 3</td>
<td>Saliva</td>
</tr>
<tr>
<td>SPRR1A</td>
<td>small proline-rich protein 1A</td>
<td>Saliva</td>
</tr>
<tr>
<td>KRT4</td>
<td>keratin 4</td>
<td>Saliva</td>
</tr>
<tr>
<td>KRT6A</td>
<td>keratin 6A</td>
<td>Saliva</td>
</tr>
<tr>
<td>KRT13</td>
<td>Keratin 13</td>
<td>Saliva</td>
</tr>
</tbody>
</table>
**Table 2: UV light treatments.** UV light period of treatments (min) to which the collected RNA samples from the subjects were exposed. Comparison of average Ct values between untreated and UV light-treated saliva samples showed that Ct values increased in 83.3% of the UV light-treated samples, indicating integrity of KRT13 mRNA was negatively affected in 83.3% of the UV light-treated samples.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV light treatment (min)</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>60</td>
<td>240</td>
<td>480</td>
<td>72</td>
<td>1080</td>
<td>1440</td>
</tr>
</tbody>
</table>
**Table 3: -20 °C freezing treatments.** The -20 °C freezing period of treatments (days) to which the collected RNA samples from subjects were exposed. Comparison of average Ct values between untreated and -20 °C freezing-treated saliva samples showed that Ct values increased in 75% of the -20 °C freezing treated samples, indicating integrity of KRT13 mRNA was negatively affected in 75% of the -20 °C freezing treated samples.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20 °C freezing treatment (day)</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>21</td>
<td>42</td>
<td>56</td>
<td>70</td>
<td>84</td>
</tr>
</tbody>
</table>
**Table 4: Typical reaction set up for KRT 13 Real-time RT-PCR.** The Real-time RT-PCR reaction was set up according to protocol of the MasterAmp RT-PCR kit (Epicentre, Madison, WI) for amplifying KRT13 transcript.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.63µl</td>
<td>KRT13 F</td>
<td>100 pmol/µl</td>
</tr>
<tr>
<td>0.63 µl</td>
<td>KRT13 R</td>
<td>100 pmol/µl</td>
</tr>
<tr>
<td>1.25 µl</td>
<td>RT-PCR buffer 20X</td>
<td></td>
</tr>
<tr>
<td>0.5 µl</td>
<td>SYBR Green I 1,000X</td>
<td></td>
</tr>
<tr>
<td>3 µl</td>
<td>MgCl₂ 25 mM</td>
<td></td>
</tr>
<tr>
<td>0.5 µl</td>
<td>MnSO₄ 25 mM</td>
<td></td>
</tr>
<tr>
<td>0.25 µl</td>
<td>RT polymerase -</td>
<td></td>
</tr>
<tr>
<td>4 µl</td>
<td>dNTPs -</td>
<td></td>
</tr>
<tr>
<td>2.5 µl</td>
<td>PCR enhancer 10X</td>
<td></td>
</tr>
<tr>
<td>… µl</td>
<td>RNA adjusted to 0.5µg/µl</td>
<td></td>
</tr>
<tr>
<td>… µl</td>
<td>Rnase-free H₂O -</td>
<td></td>
</tr>
</tbody>
</table>

Total volume: 25µl
Table 5: *GAPDH* and *KRT13* primer sequences. Primer sequences for both *GAPDH* (internal control) and *KRT13* (target gene) used in Real-time RT-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ to 3’</th>
<th>Melting Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH Forward</td>
<td>GGAGTCAACGGATTTGGT CG Add T7 promotor site to this primer (TAATACGACTCACTATAGGGAGA)</td>
<td>56.0</td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>GATGGTGATGGGATTTCATG</td>
<td>52.4</td>
</tr>
<tr>
<td>KRT13 Forward</td>
<td>AATTCTAATACGACTCACTATAGG GAGAAGGCCCCGTAGCACCTCTGT TA</td>
<td>67.3</td>
</tr>
<tr>
<td>KRT13 Reverse</td>
<td>ACGACCTGGGGTCCGGAGTCA</td>
<td>60.6</td>
</tr>
</tbody>
</table>
Table 6: Average Ct values of UV light untreated and treated samples. No significant effect was measureable by UV light treatment on KRT13 mRNA quality. Comparison of average Ct values among UV light-treated saliva samples shows that Ct values among treated samples are widely spread. Comparison of average Ct values between untreated and UV light-treated saliva samples shows that Ct values increased in 83.3% of the UV light treated samples, indicating that integrity of KRT13 mRNA was negatively affected in 83.3% of the UV light treated samples. Although a general decrease in integrity of KRT13 transcript in UV light treated saliva samples, no significant effect by UV light treatment on the quality of the KRT13 mRNA could be concluded.

<table>
<thead>
<tr>
<th>UV light treatment (min)</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>60</th>
<th>240</th>
<th>480</th>
<th>720</th>
<th>1080</th>
<th>1440</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Ct values</td>
<td>15</td>
<td>20</td>
<td>23</td>
<td>22</td>
<td>18</td>
<td>25</td>
<td>22</td>
<td>31</td>
<td>19</td>
<td>23</td>
<td>26</td>
<td>14</td>
</tr>
</tbody>
</table>
Table 7: Average Ct values of -20 °C freezing untreated and treated samples. No significant effect was measureable by the -20 °C freezing treatment on KRT13 mRNA quality. Comparison of average Ct values between untreated and -20 °C freezing-treated saliva samples showed that Ct values increased in 75% of the -20 °C freezing treated samples, indicating integrity of KRT13 mRNA was negatively affected in most of the -20 °C freezing treated samples. Although comparison of average Ct values between untreated and -20 °C freezing treated saliva samples indicates a general decrease in integrity of KRT13 transcript in -20 °C freezing treated saliva samples, no significant effect by -20 °C freezing treatment on the quality of the KRT13 mRNA could be concluded.

<table>
<thead>
<tr>
<th>-20 °C freezing treatment (day)</th>
<th>0</th>
<th>1</th>
<th>7</th>
<th>21</th>
<th>42</th>
<th>56</th>
<th>70</th>
<th>84</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Ct values</td>
<td>15</td>
<td>16</td>
<td>18</td>
<td>20</td>
<td>23</td>
<td>17</td>
<td>19</td>
<td>15</td>
</tr>
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</table>
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