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Validation of Recently Discovered mRNA Stable Regions as Biomarkers for Body Fluids after Exposure to Environmental Hazards

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Abstract

The recognition of messenger RNA become common practice for body fluid identification in forensic field. Though, the degraded and rare nature of RNA from forensic samples point out that mRNA transcripts are not reliably spotted or remain undetected in practice. Thus, leading to hardening the process of forensic investigation and body fluids identification. Lately, using massively parallel sequencing technique, data obtained from degraded body fluids were used to design new primers to amplify transcript regions of messenger RNA of high read coverage, hence, exhibiting higher stability, as a substitute of the conventional primer that target span exon–exon boundaries which showed low stability and degradation susceptibility. In the current project, the relative gene expression was calculated for each biomarker to measure the stability of those messenger RNA regions. The criteria of testing involved exposing different messenger RNA biomarkers transcripts of blood, buccal cells and menstrual blood to various environmental hazards including UV irradiation, 40°C induced heat and 50% humidity. Degradation was in continuous exposure for a specific time span of 2 weeks. The results showed that each messenger RNA biomarker behaved differently among the different environmental insults.

Keywords: body fluids; degradation; environmental hazards; mRNA transcripts.

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1. Introduction

Evidences found at crime scenes are the key that lead the investigators to solve crime's mysteries. The type of evidence varies at crime scenes, depending on the type of the crime committed, various evidences are encountered and they are either physical or biological evidences. In forgery cases, the publication property or accounting theft, physical evidences are found. In murder cases, sexual assaults or burglary, biological evidences are detected; mainly body fluids [1]. Body fluid evidence are valuable since they contain nucleic acids that aid in relating an assailant and/or a victim to a crime. In fact, body fluids left at crime scenes will interact with the surrounding environment [2]. Since these body fluids are out of the human body, they will be affected with physical nature of earth. The ambient environment will influence the body fluids either by preserving the sample such as cold areas or salt environments or by degrading the fluids as in warm or hot environments [3]. Degradation is the main obstacle that face the investigators. This is because degradation will negatively affect the nucleic acid that carries all information that will assist in solving the case [4]. DNA has been used in forensic science to solve crime cases, and recently, RNA is being in the use. The unique thing about working with RNA that it can help to identify the gene expression of any sample. In recent past, a region within mRNA has been discovered, this region can resist degradation and remain stable, thus will overcome degradation obstacle [5]. This research paper examined the stability of these newly discovered regions of mRNA in different body fluids after exposing them to various environmental hazards for a period of two weeks. These conditions, in fact, simulate the environmental factors that the body fluids may be subjected to in nature. They include: UV irradiation by direct exposure to sunlight, 50% humidity and 40^{o}C induced heat.

2. Materials and Methods

2.1 Sampling and Degradation

Body fluids samples were obtained from volunteers of both genders with some specifications including: capillary blood, menstrual blood and buccal swabs in a total of 48 samples. An informed consent was given to each participant to obtain permission for sampling. [Appendix A]. 12 samples were fresh while the remaining 36 were subjected to degradation by three different environmental hazards namely: Solar UV irradiation, 40^{o}C induced heat and 50% relative humidity. The period of degradation spun for 2 weeks from the 5 the till the 19th of June 2017. 4 fresh capillary blood samples were obtained from volunteers using FreeStyle® lancet device and lancets and deposited on BROMED® sterile surgical sponges and stored at 0^{o}C. 4 Buccal swabs fresh and 4 fresh menstrual blood samples were collected using DUKAL cotton tipped applicators and stored at 0^{o}C. The same procedure of sampling was followed for the samples mandated for degradation. 12 samples from each body fluids were subjected to solar UV irradiation, 40^{o}C induced heat using POL-EKO APARATURA incubator and 50% relative humidity using Wisebath water bath.

2.2 Total RNA extraction

RNA was extracted from all biological samples using Promega® ReliaPrepTM RNA Cell Miniprep System following the provided protocol instructions. The extracted were stored at -20^{o}C. Successful extraction of total

RNA for each sample was checked using HIMEDIA 1.5 % Agarose Gel electrophoresis.

2.3 cDNA reverse transcription

cDNA was synthesized from all extracted RNA samples using ProtoScript® First Strand cDNA Synthesis Kit following the provided protocol instructions in a total volume of 50µL. Reverse transcriptase minus controls were prepared to ensure no genomic DNA contamination.

2.4 mRNA biomarkers and primers

Three mRNA biomarkers were used in the research for each body fluid; Solute Carrier Family 4 Member (*SLC4A1*), Histatin 3 (*HTN3*) and Matrix Metallopeptidase 11 (*MMP11*) of capillary blood, buccal swabs and menstrual blood samples respectively.

The primers used in this research paper were designed by Meng-Han Lin and his colleagues [6]. These primers target the high read coverage regions within the mRNA biomarkers. [Table 1].

Table 1: The forward and reverse primers sequences of SLC4A1, HTN3 and MMP11 mRNA biomarkers and *UBE2G2* as the housekeeping gene along with their corresponding annealing temperatures.

Primer	Annealing Temperature (Ta)	Forward primer (5'-3') sequence	Reverse primer (5'-3') sequence
SLC4A1	58°C	TGATGGAGGAGAATCTGGAGC	GTGATGTGGTGTGGTAGTCTGT
HTN3	65°C	TTCACATCGAGGCTATAGATC	GAGAATACACGAGTCCAAAGC
MMP11	58°C	CAAGACTCACCGAGAAGGGG	GCCTTGGCTGCTGTTGTGT
UBE2G2	58°C	GCCAAATGACAGTCCCTATCAGG	GCACTAAAGGATCATCTGGATTGGG

2.5 Real-Time PCR

Synthesized cDNA from fresh samples were amplified by Real-Time PCR using BIORAD CFX 1000 Touch Thermal cycler. The reaction total volume was 20µL, using BIORAD iTaq universal SYBR green as the master mix following the protocol conditions for a number of 40 cycles [Table 2].

An attached cycle threshold (C_T) values of each Real-Time PCR run is attached in appendix 1. Four reaction runs were performed. The first run was mandated for the fresh samples followed by the second run for the UV degraded samples, the third for the 50% humidity degraded samples and the forth for the 40°C induced heat degraded sample.

Table 2: Real-Time PCR conditions on BIORAD CFX 1000 Touch thermal cycler

Stage	Condition	Temperature	Time	Cycles
1	Polymerase Activation and DNA Denaturation	95°C	30 seconds	1
2	Denaturation	95°C	5 seconds	40
	Annealing/Extension	Depends on primer annealing temperature described in Table 1.	30 seconds	
3	Melt curve	65–95°C 0.5°C increments at 2–5 second/step		1

2.6 Relative gene expression

Gene expression from all biological samples were calculated using Livak Method ($2^{-\Delta\Delta CT}$) [7]. Gee expression data is attached in appendix A. This method relay on normalizing the target gene to the house keeping gene following the equations:

1. Normalizing the degraded target gene to the housekeeping gene:

   ΔCT of degraded sample = ΔCT (degraded sample) – ΔCT (UBE2G2)

2. Normalizing the fresh target gene to the housekeeping gene:

   ΔCT of fresh sample = ΔCT (fresh sample) – ΔCT (UBE2G2)

3. Normalizing the degraded target gene to the fresh target gene:

   ΔΔCT = ΔCT (degraded sample) – ΔCT (fresh sample)

4. Calculating the expression ratio:

   $2^{-\Delta\Delta CT}$

3. Results

3.1 Real-Time PCR

Real-Time amplification plots were generated along with the cycles threshold values. All the fresh and degraded cDNA templates were amplified. Reverse transcribed control didn't show any amplification which indicate free genomic DNA contamination. [Figures 1-4].

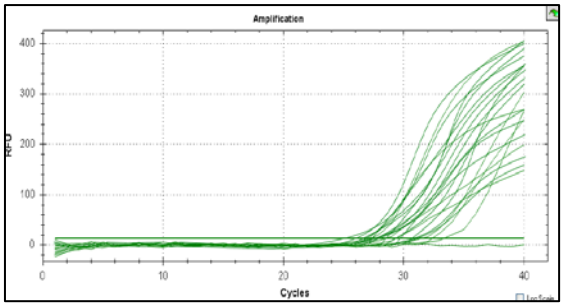

Figure 1: Real-Time PCR amplification plot of fresh samples

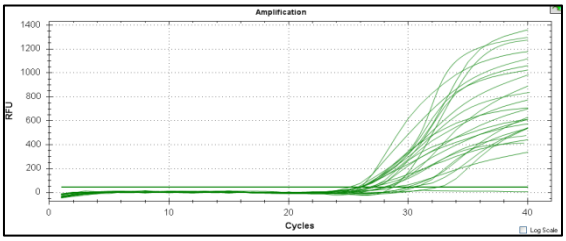

Figure 2: Real-Time PCR amplification plot of UV degraded samples

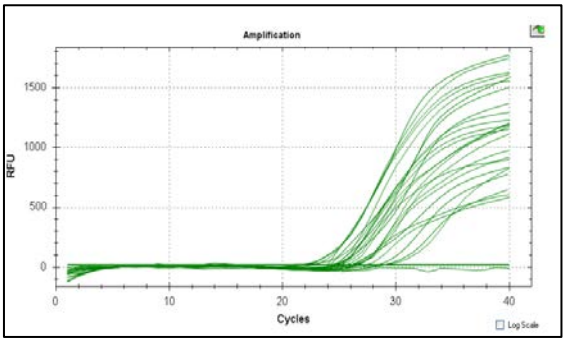

Figure 3: Real-Time PCR amplification plot of humidity degraded samples

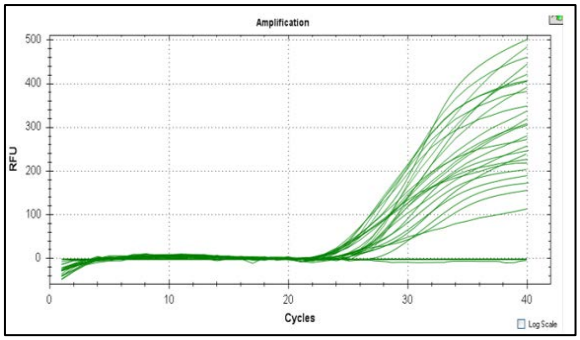

Figure 4: Real-Time PCR amplification plot of 40^{o}C degraded samples

3.2 Relative gene expression

Each mRNA biomarker showed a unique gene expression data under the different degrading environmental hazards. The gene expression of fresh samples was: 1.2 folds of *SLC4A1*, 1.7 folds for *HTN3* folds and 1.9 folds for *MMP11*. Under solar UV irradiation, *SLC4A1* biomarker showed a higher gene expression level than the fresh samples by 1.55 folds. Whereas *HTN3* and *MMP11* showed a lower gene expression levels by 0.8 folds and 1.76 folds respectively. [Figure 5]. Under humidity stress, both *SLC4A1* and *HTN3* biomarkers showed a higher gene expression by 2.3 folds and 2.6 folds respectively, while *MMP11* Showed an equal gene expression with the fresh sample; 1.9 folds [Figure 6]. In 40°C induced heat degradation, once again *SLC4A1* showed a higher gene expression by 1.4 folds whereas *HTN3* and *MMP11* biomarkers showed a lower gene expression levels; 1.5 folds and 1.7 folds respectively [Figure 7].

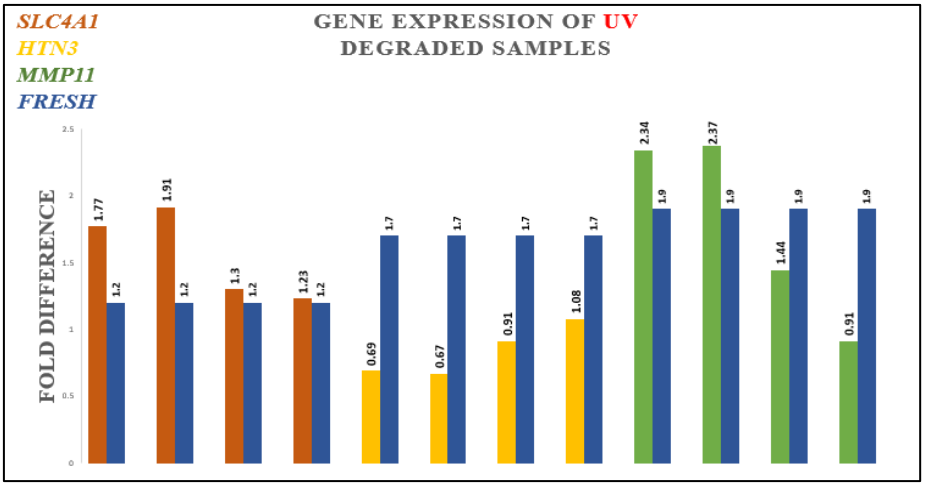

Figure 5: Relative gene expression of UV degraded samples

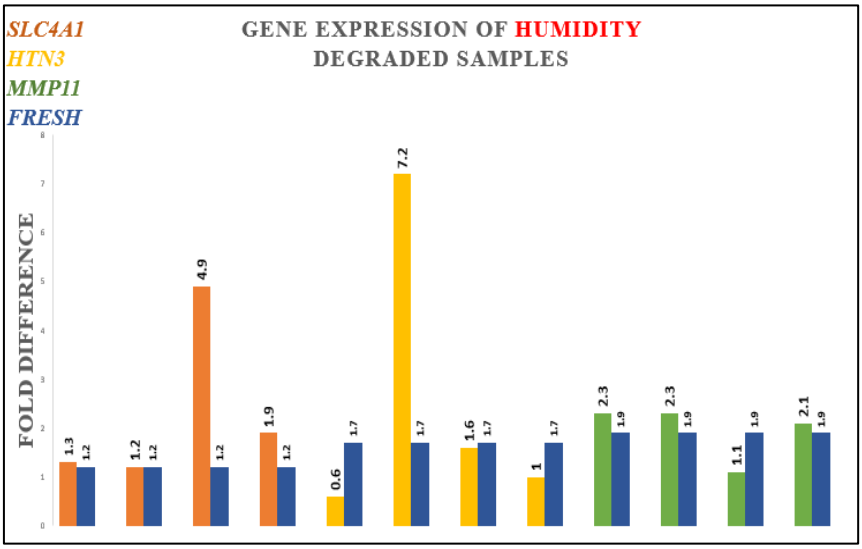

Figure 6: Relative gene expression of humidity degraded samples

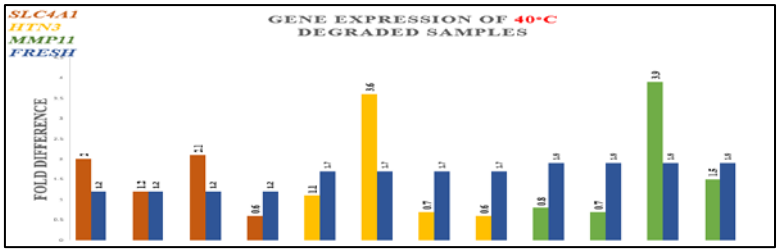

Figure 7: Relative gene expression of 40°C degraded samples

3.3 Statistical analysis

Analysis of variance (ANOVA) statistical test was performed to detect any significant differences between the three groups of degradation on each biomarker. The statistical test was conducted using SPSS. [Tables 3-5]

Table 3: ANOVA of the three groups of degradation; Solar UV, humidity and 40°C on *SLC4A1* biomarker.

SUMMARY						
	Groups	Count	Sum	Variance	Variance	
	40°C	4	5.9	0.5025	0.5025	
	Humidity	4	9.3	3.0425	3.0425	
	UV	4	6.21	0.114292	0.114292	
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1.767017	2	0.883508	0.724327	0.510881	4.256494729
Within Groups	10.97788	9	1.219764			
Total	12.74489	11				

Table 4: ANOVA of the three groups of degradation; Solar UV, humidity and 40°C on *HTN3* biomarker.

SUMMARY						
	Groups	Count	Sum	Average	Variance	
	40°C	4	6	1.5	2.006667	
	Humidity	4	10.4	2.6	9.573333	
	UV	4	3.35	0.8375	0.037958	
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	6.340417	2	3.170208	0.818614	0.471371	4.256495
Within Groups	34.85388	9	3.872653			
Total	41.19429	11				

Table 5: ANOVA of the three groups of degradation; Solar UV, humidity and 40°C on *MPP11* biomarker.

SUMMARY				
Groups	Count	Sum	Average	Variance
40°C	4	6.9	1.725	2.229167
Humidity	4	7.8	1.95	0.33
UV	4	7.06	1.765	0.5111

ANOVA					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.115267	2	0.057633	0.056314	0.945572	4.256495
Within Groups	9.2108	9	1.023422			
Total	9.326067	11				

4. Discussion

The present study investigated the ability of these mRNA biomarkers to resist degradation. Gene expression was calculated after the degradation period. The primers used in this research were designed to targets the regions of mRNA with high reading coverage in the reconstructed sequence. qPCR amplification plots of stable region primers revealed an excellent amplification curves for the all samples. Moreover, the study revealed that all mRNA biomarkers showed a noticeable gene expression level. Besides, when looking for the effect of each method of degradation, it's clear that each mRNA biomarker behaved differently in each method but all the biomarkers showed an expression. In general, the highest gene expressions of all three biomarkers were seen humidity degraded samples. In addition, the next highest gene expression of *SLC4A1* and *MMP11* biomarkers were seen in UV degraded samples while *HTN3* biomarker exhibited its next highest gene expression in 40°C degraded samples. This gives an indication that humidity is the least effective degradation methodology compared with solar UV irradiation and 40°C induced heat.

Remarkably, *SLC4A1* biomarker showed a greater expression than the fresh samples in all degradation methodologies. *HTN3* showed a greater gene expression level than the fresh samples only under humidity stress, while *MMP11* higher expression was seen in under 40°C stress. Gene expression level reflects the level of stability, despite the method used in this research which relied on qPCR, the final overall hypothesis (mRNA stable regions can resist degradation) matches the ones presented by LinHin and his colleagues research despite the techniques used. Moreover, as this study covered samples from different genders, there were no variance in terms of stability in either male's nor female's samples. The sex doesn't affect due to the presence these mRNA biomarkers are expressed in both genders. The objective of collecting samples from both genders is for the purpose of gender balance in research. Menstrual blood samples, were obtained during the first cycle were *MMP11* biomarker is expressed the most, and its expression were noticeable in all degradation methodologies.

In this context, the ANOVA was conducted to reveal any significant variation in terms of effectiveness among the different types of degradation methods on each biomarker followed in this study. The test assured that the three methods of degradation utilized in this study did not have a preference over each other or had a greater or lesser effect on the three mRNA biomarkers. The P values obtained in the three biomarkers were greater than the α significance level (≥0.05) which supports the null hypothesis. In addition, the critical F values were greater than the F values, which also supports the null hypothesis. Furthermore, the upshots of this research gave a wider knowledge about these newly discovered stable regions of mRNA. And it enhanced our understanding regarding their level of stability and how they can reveal a robust expression regardless the degradation methodology nature for a period of 2 weeks. Moreover, in field, this research idea can be applied in cases were a further identity confirmation of a suspicious body fluid is needed.

5. Conclusion and Limitations

In conclusion, this study reflects the ever-growing worldwide attention to the newly discovered mRNA stable =regions. As they represent a new aspect of forensic science, validating such regions is crucial in research fields, since it gives us a wider perspective towards how these regions react.

In addition, and as crime scene evidence degradation is the main obstacle that confronts forensic scientists, these regions were subject to various environmental hazards that simulate what might naturally happen to the evidence in the aftermath of a crime. Throughout this research, mRNA stable regions consistently showed an observable expression across the different degradation methodologies. Each biomarker behaved uniquely in each environmental thread.

This gives a clean idea about the resistance level of these stable biomarkers. Some few barriers were rising during this research, one of them is the difficulty in obtaining large number of menstrual blood samples of the second cycle from different volunteers at the same time. The second barrier was in using a specific kit that didn’t extracted the RNA from the samples with a good quality, thus we have changed it. The third limitation was the high risk of RNA degradation during the transfer of samples from the sampling area to the laboratory due to the far distance and the high climate temperature.

6. Recommendations

As far as this research goes, it is recommended that body fluids collected from crime scenes would need further exposure to various degrading factors and for longer period.

This is imperative to improve our learning knowledge and envisage how these regions would react in longer degradation periods and whether such conditions would limit or otherwise affect their stability. What would be the time factor beyond which no body fluid evidence will be significant?

Is increasing the number of samples will have an effect in supporting or rejecting the null hypothesis? All these quarries and a few more, are legitimate questions that are crying for answers, which only further research would answer.

Acknowledgments

I would like to express my warm thank to The University of Modern Sciences for funding this research and providing the necessary kits and equipment. Also, my appreciation is extended to Dr. Rania Gomaa and Dr.Lamis Nader the supervisors of this research project for their support and guidance to complete the task till the end. Moreover, I show my thankfulness to all the volunteers who were pleased to be a part in this research by donating specimens. Besides, I likewise would like to express my honest appreciations and gratitude to my parents, family and friends who supported me massively through the period of my work.

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7. Appendix A

7.1 Informed consent

College of Biotechnology

Forensic Biotechnology

Informed Consent for the Collection of Biological Samples for Research Study

Validation of recently discovered mRNA stable regions as biomarkers for body fluids after exposure to environmental hazards

Invite to Participate:

You are being invited to participate in this research proje**ct.**

Why is This Study Being Done?

The purpose of this research is to test the stability of a specific regions found in your genome called: mRNA transcript stable regions (StaRs) by exposing it to different environmental factors.

What are the Procedures Involved in This Study?

Samples of Blood, saliva and menstrual blood (if you are female) will be collected from you and exposed to three different environmental factors: UV irradiation, humidity and 40°C induced heat.

What Are the Dangers of the Study?

Donating blood, saliva and menstrual blood for this research purpose will not add any risks to your health or any other procedures you have had or will have.

Are There Welfares of Taking Part in the Study?

Your contribution to this study will be for research purposes only. You will not be paid for participating in this study.

Use of Specimens:

Any body fluid sample obtained for the purposes of this study becomes the property of the University of Modern Sciences. The University may retain, preserve, or dispose of these specimens.

Is There Return for the Study?

There will be no financial compensation for participation in this study.

Confidentiality?

All information collected in this study will be kept strictly confidential.

What Are My Rights?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time.

Disclaimer

Your participation in this research study is completely voluntary. If you choose not to participate, that will not affect your present or future care at the University of Modern Sciences. If you decide to participate, you are free to withdraw your consent and discontinue participation at any time.

In Case of Queries or Problems?

For questions about the study or a research-related injury and for questions about your rights as a research participant, contact the research student of this study at the telephone numbers on page two of this form.

Signature

Your signature below indicates that you have been given the opportunity to read this consent form and to ask questions. Your questions have been answered to your satisfaction. You voluntarily agree to participate in this research study. Upon signing below, you will receive a copy of the consent form.

_____	_____	_____
Name of Participant	Signature of Participant	Date
_____	_____	_____
Name of Person Obtaining	Signature of Person Obtaining	Date

Research student: Einas Mohamad Ahmad

ID: S0000000196

Mobile: 00971509279019

7.2 Cycle threshold values

Samples codes:

BLFF1 / BLFD1 = Blood Female Fresh 1 / Blood Female Degraded 1

BLMF1 / BLMD1 = Blood Male Fresh 1 / Blood Male Degraded 1

BUFF1 / BUFD1 = Buccal Female Fresh 1 / Buccal Female Degraded 1

BUMF1/ BUDM1= Buccal Fresh Male 1 / Buccal Male Degraded 1

MNFF1 / 1= Menstrual Fresh Female 1 / Menstrual Female Degraded 1

A. Fresh samples: -

Table 6

Well	Flour	Content	Sample	Biomarker	Cq
A01	SYBR	Unkn	BLFF1	*SLC4A1*	23.7
A02	SYBR	Unkn	HKG		23.99
A03	SYBR	Unkn	BLFF2		24.21
A04	SYBR	Unkn	HKG		25.11
A05	SYBR	Unkn	BLFM1		23.1
A06	SYBR	Unkn	HKG		23.8
A07	SYBR	Unkn	BLFM2		22.87
A08	SYBR	Unkn	HKG		23.49
A09	SYBR	Unkn	BUFF1	*HTN3*	18.09
A10	SYBR	Unkn	HKG		18.99
A11	SYBR	Unkn	BUFF2		19.97
A12	SYBR	Unkn	HKG		20
B01	SYBR	Unkn	BUFM1		19.26
B02	SYBR	Unkn	HKG		20.47
B03	SYBR	Unkn	BUFM2		20.61
B04	SYBR	Unkn	HKG		21.43
B05	SYBR	Unkn	MNFF1	*MMP11*	20.01
B06	SYBR	Unkn	HKG		21.42
B07	SYBR	Unkn	MNFF2		25.27
B08	SYBR	Unkn	HKG		25.79
B09	SYBR	Unkn	MNFF3		25.11
B10	SYBR	Unkn	HKG		25.12
B11	SYBR	Unkn	MNFF4		23.03
B12	SYBR	Unkn	HKG		24.24

B. UV degraded samples: -

Table 7

Well	Flour	Content	Sample	Biomarker	Cq
A01	SYBR	Unkn	BLFD1	*SLC4A1*	32.00
A02	SYBR	Unkn	HKG		33.12
A03	SYBR	Unkn	BLFD2		28.43
A04	SYBR	Unkn	HKG		29.66
A05	SYBR	Unkn	BLMD1		30.74
A06	SYBR	Unkn	HKG		30.79
A07	SYBR	Unkn	BLMD2		27.12
A08	SYBR	Unkn	HKG		28.04
A09	SYBR	Unkn	BUFD1	*HTN3*	23.6
A10	SYBR	Unkn	HKG		23.98
A11	SYBR	Unkn	BUFD2		23.35
A12	SYBR	Unkn	HKG		23.94
B01	SYBR	Unkn	BUMD1		30.07
B02	SYBR	Unkn	HKG		31.15
B03	SYBR	Unkn	BUMD2		29.25
B04	SYBR	Unkn	HKG		30.19
B05	SYBR	Unkn	MNFD1	*MMP11*	30.23
B06	SYBR	Unkn	HKG		32.87
B07	SYBR	Unkn	MNFD2		21.21
B08	SYBR	Unkn	HKG		22.98
B09	SYBR	Unkn	MNFD3		24.49
B10	SYBR	Unkn	HKG		29.03
B11	SYBR	Unkn	MNFD4		23.84
B12	SYBR	Unkn	HKG		23.92

C. Humidity degraded samples: -

Table 8

Well	Flour	Content	Sample	Biomarker	Cq
A01	SYBR	Unkn	BLFD1	*SLC4A1*	22.16
A02	SYBR	Unkn	HKG		22.84
A03	SYBR	Unkn	BLFD2		22.67
A04	SYBR	Unkn	HKG		23.89
A05	SYBR	Unkn	BLMD1		24.32
A06	SYBR	Unkn	HKG		27.30
A07	SYBR	Unkn	BLMD2		21.68
A08	SYBR	Unkn	HKG		23.23
A09	SYBR	Unkn	BUFD1	*HTN3*	27.80
A10	SYBR	Unkn	HKG		27.85
A11	SYBR	Unkn	BUFD2		22.10
A12	SYBR	Unkn	HKG		24.98
B01	SYBR	Unkn	BUMD1		24.99
B02	SYBR	Unkn	HKG		26.90
B03	SYBR	Unkn	BUMD2		21.13
B04	SYBR	Unkn	HKG		22.05
B05	SYBR	Unkn	MNFD1	*MMP11*	26.12
B06	SYBR	Unkn	HKG		28.70
B07	SYBR	Unkn	MNFD2		22.84
B08	SYBR	Unkn	HKG		24.60
B09	SYBR	Unkn	MNFD3		23.91
B10	SYBR	Unkn	HKG		24.00
B11	SYBR	Unkn	MNFD4		26.93
B12	SYBR	Unkn	HKG		27.10

40°C induced heat degraded samples: -

D. 40°C induced heat degraded samples

Table 9

Well	Flour	Content	Sample	Biomarker	Cq
A01	SYBR	Unkn	BLFD1	*SLC4A1*	23.70
A02	SYBR	Unkn	HKG		24.96
A03	SYBR	Unkn	BLFD2		30.67
A04	SYBR	Unkn	HKG		31.89
A05	SYBR	Unkn	BLMD1		23.00
A06	SYBR	Unkn	HKG		24.81
A07	SYBR	Unkn	BLMD2		22.34
A08	SYBR	Unkn	HKG		23.19
A09	SYBR	Unkn	BUFD1	*HTN3*	28.40
A10	SYBR	Unkn	HKG		29.45
A11	SYBR	Unkn	BUFD2		22.12
A12	SYBR	Unkn	HKG		26.47
B01	SYBR	Unkn	BUMD1		30.30
B02	SYBR	Unkn	HKG		31.03
B03	SYBR	Unkn	BUMD2		33.00
B04	SYBR	Unkn	HKG		33.02
B05	SYBR	Unkn	MNFD1	*MMP11*	25.13
B06	SYBR	Unkn	HKG		26.27
B07	SYBR	Unkn	MNFD2		27.90
B08	SYBR	Unkn	HKG		27.99
B09	SYBR	Unkn	MNFD3		30.82
B10	SYBR	Unkn	HKG		32.87
B11	SYBR	Unkn	MNFD4		31.96
B12	SYBR	Unkn	HKG		32.13

Gene expression data: -

A. UV degraded samples:

Table 10

Biomarker	Sample code	C_T Value degraded	*UBE2G2* (HKG) C_T Value degraded	C_T Value Fresh	*UBE2G2* C_T Value Fresh	Fresh Samples Gene Expression	ΔC_T degraded	ΔC_T Fresh	$\Delta\Delta C_T$	Fold difference $2^{-\Delta\Delta CT}$	Average
SLC4A1	BLFD1	32.00	33.12	23.7	23.99	1.2	-1.12	-0.29	-0.83	1.77	1.55
	BLFD2	28.43	29.66	24.21	25.11		-1.23	-0.9	-0.94	1.91	
	BLMD1	30.74	30.79	23.1	23.8		-0.32	-0.7	0.38	1.30	
	BLMD2	27.12	28.04	22.87	23.49		-0.92	-0.62	-0.3	1.23	
HTN3	BUFD1	23.60	23.98	18.09	18.99	1.7	-0.38	-0.9	0.52	0.69	0.83
	BUFD2	23.35	23.94	19.97	20.00		-0.59	-0.03	-0.56	0.67	
	BUMD1	30.07	31.15	19.26	20.47		-1.08	-1.21	0.13	0.91	
	BUMD2	29.25	30.19	20.61	21.43		-0.94	-0.82	-0.12	1.08	
MMP11	MNFD1	30.23	32.87	20.01	21.42	1.9	-2.64	-1.41	-1.23	2.34	1.76
	MNFD2	21.21	22.98	25.27	25.79		-1.77	-0.52	-1.25	2.37	
	MNFD3	24.49	25.03	25.11	25.12		-0.54	-0.01	-0.53	1.44	
	MNFD4	22.84	23.92	23.03	24.24		-1.08	-1.21	0.13	0.91	

B. Humidity degraded samples:

Table 11

Biomarker	Sample code	C_T Value degraded	*UBE2G2* (HKG) C_T Value degraded	C_T Value Fresh	*UBE2G2* C_T Value Fresh	Fresh Samples Gene Expression	ΔC_T degraded	ΔC_T Fresh	$\Delta\Delta C_T$	Fold difference $2^{-\Delta\Delta CT}$	Average
SLC4A1	BLFD1	22.16	22.84	23.7	23.99	1.2	-0.68	-0.29	-0.39	1.3	2.325
	BLFD2	22.67	23.89	24.21	25.11		-1.22	-0.9	-0.32	1.2	
	BLMD1	24.32	27.30	23.1	23.8		-2.98	-0.7	-2.28	4.9	
	BLMD2	21.68	23.23	22.87	23.49		-1.55	-0.62	-0.93	1.9	
HTN3	BUFD1	27.80	27.85	18.09	18.99	1.7	-0.05	-0.9	0.85	0.6	2.6
	BUFD2	22.10	24.98	19.97	20.00		-2.88	-0.03	-2.85	7.2	
	BUMD1	24.99	26.90	19.26	20.47		-1.91	-1.21	-0.7	1.6	
	BUMD2	21.13	22.05	20.61	21.43		-0.92	-0.82	-0.1	1.0	
MMP11	MNFD1	26.12	28.70	20.01	21.42	1.9	-2.58	-1.41	-1.17	2.3	1.925
	MNFD2	22.84	24.60	25.27	25.79		-1.76	-0.55	-1.21	2.3	
	MNFD3	23.91	24.00	25.11	25.12		-0.09	-0.01	-0.08	1.1	
	MNFD4	26.93	27.10	23.03	24.24		-0.17	-1.21	1.04	2.1	

C. 40°C induced heat degraded samples

Table 12

Biomarker	Sample code	C_T Value degraded	*UBE2G2* (HKG) C_T Value degraded	C_T Value Fresh	*UBE2G2* (HKG) C_T Value Fresh	Fresh Samples Gene Expression	ΔC_T degraded	ΔC_T Fresh	$\Delta\Delta C_T$	Fold difference $2^{-\Delta\Delta}$ C_T	Average
SLC4A1	BLFD1	23.70	24.96	23.7	23.99	1.2	-1.26	-0.29	-0.97	2.0	1.475
	BLFD2	30.67	31.89	24.21	25.11		-1.22	-0.9	-0.32	1.2	
	BLMD1	23.00	24.81	23.1	23.8		-1.81	-0.7	-1.11	2.1	
	BLMD2	22.34	23.19	22.87	23.49		0.05	-0.62	0.67	0.6	
HTN3	BUFD1	28.40	29.45	18.09	18.99	1.7	-1.05	-0.9	-0.15	1.1	1.5
	BUFD2	22.12	24.00	19.97	20.00		-1.88	-0.03	-1.85	3.6	
	BUMD1	30.30	31.03	19.26	20.47		-0.73	-1.21	0.48	0.7	
	BUMD2	33.00	33.02	20.61	21.43		-0.02	-0.82	0.8	0.6	
MMP11	MNFD1	25.13	26.27	20.01	21.42	1.9	-1.14	-1.41	0.27	0.8	1.725
	MNFD2	27.90	27.99	25.27	25.79		-0.09	-0.52	0.43	0.7	
	MNFD3	30.82	32.87	25.11	25.12		-2.00	-0.01	-1.99	3.9	
	MNFD4	31.13	32.96	23.03	24.24		-1.83	-1.21	-0.62	1.5	