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**EVALUATION OF THE EFFECTS OF TREHALOSE ON THE  
AMPLIFICATION OF THE 15 SHORT TANDEM REPEATS LOCI OF THE  
AMPF&STR IDENTIFILER PLUS PCR AMPLIFICATION KIT**

by

**GYEOL YOON**

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Approved by

First Reader

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Robin W. Cotton, Ph.D.  
Associate Professor, Program in Biomedical Forensic Sciences  
Department of Anatomy & Neurobiology

Second Reader

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Sandra Haddad, Ph.D.  
Assistant Professor, Co-Director, Master of Science in Forensics  
Program, Bay Path University

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**ABSTRACT**

It is of great importance to be able to unambiguously interpret deoxyribonucleic acid (DNA) profiles, especially with Low Template (LT) DNA and mixture DNA that may contain major and minor contributors. Reducing stochastic effects, such as heterozygote peak imbalance, dropouts, and stutter artifacts have been studied by scientists in order to improve the evaluation of low quality DNA profile.

There has been much research on a compatible solute, trehalose, in its effectiveness in enhancing the polymerase chain reaction (PCR), especially with GC-rich templates of DNA, and thermal stabilizing *Thermus Aquaticus* (*Taq*) DNA polymerases. Based on previous research, the effect of trehalose on peak heights, peak height ratios, and stutter ratios (n-1) from 15 short tandem repeats (STR) loci of the AmpF $\ell$ STR<sup>®</sup> Identifiler<sup>®</sup> Plus PCR Amplification Kit was evaluated with 0.025ng, 0.05ng, 0.1ng, and 1ng of DNA, through the addition of 0M (control), 0.2M, and 0.4M of trehalose for each quantity of DNA. Although there was an observation regarding changes in average peak heights at 1ng of DNA with the addition of 0.2M, and 0.4M of trehalose, no conclusions could be made with the average peak heights for 0.025ng, 0.05ng, 0.1ng, and 1ng of

DNA. The reason is that the propagation of pipetting error during the preparation of each batch could have contributed to the difference in the amount of DNA between each conditions which can be directly reflected in peak heights. Furthermore, unexpected discrepancy between the average peak heights for 0.1ng of DNA from the first and the second experiments rendered 0.1ng of DNA incompatible for comparison. With regards to average peak height ratios for 0.025ng, 0.05ng, 0.1ng, and 1ng of DNA, and average reverse stutter ratios for 0.1ng, and 1ng of DNA, there were no evidence to suggest that 0.2M or 0.4M of trehalose had any effects. Consistent trends for 0.1ng (Exp. 1 and 2) and 1ng of DNA from a statistical analysis through one-way ANOVA of individual loci, suggested that trehalose may have varying effects on certain loci. However, this observation must be approached with caution as it is uncertain whether unique trends across each data sets for certain loci were observed by chance due to small sample sizes or due to mechanisms of stutters and trehalose that are currently unknown.

Future studies regarding the effect of trehalose on peak heights should be done with more precision through minimizing pipetting error, which can be accomplished by preparing one batch from which aliquots are taken.

The result of the research does not show enough evidence to prove the usefulness of trehalose since the addition of trehalose does not yield consistently higher average peak heights and peak height ratios, and lower average reverse stutter ratios across 15 STR loci. Therefore, our results do not support that 0.2M and 0.4M of trehalose are useful within the parameter of forensic DNA analysis as they do not enhance the polymerase chain reaction (PCR) and improve stochastic effects for DNA profiles.

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## LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CE	Capillary Electrophoresis
CODIS	Combined DNA Index System
°C	Degrees Celsius
dNTP	Deoxynucleotide Triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Etylenediaminetetraacetic acid
LT	Low Template
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
rfu	relative fluorescence unit
STR	Short Tandem Repeat
<i>Taq</i>	<i>Thermus Aquaticus</i>
TE	Tris-EDTA
μL	Microliter

## 1. Introduction

For the past few decades, using specific DNA sequences for the identification of an individual has greatly increased the power of forensic DNA analysis for court. The DNA profile of an individual, which is a comprehensive graphical information of unique DNA sequences at specific loci that an individual possesses, must be unambiguously interpreted. The reason is that it is critical for evaluating the minimum number of contributors, generating conclusions as to whether a person's DNA can or cannot be excluded from the DNA profile, and determining the sex of the contributor(s). Providing expert witness regarding this information can give significant advantage in clarifying the facts during court testimony. However, generating unambiguous DNA profiles can be challenging especially when low template (LT) (>100 pg) is available for amplification<sup>1</sup>. The development and publication of Polymerase Chain Reaction (PCR) in 1985 by Kary Mullis and others (Kary Mullis et al., 1986) greatly advanced the amplification of low amount of DNA. Moreover, the application of polymorphic tetrameric and pentameric STRs in multiplex PCR<sup>3</sup> and the development of capillary electrophoresis (CE) instrumentation made a huge leap towards more time and effort-saving process with a high power of discrimination. Much effort has been put into research in order to generate a good quality DNA profile by improving true allelic peaks generated from LT DNA and reducing stochastic effects such as heterozygous imbalance and drop outs, as well as artifacts such as strand slippage. Compatible solutes, such as trehalose<sup>4</sup>, betaine<sup>5</sup>, sorbitol<sup>6</sup>, etc., have been identified as potential PCR enhancers in past journal articles. These compounds may enhance DNA amplification by lowering the melting temperature

of DNA<sup>4,7</sup> or through the thermostabilization of the *Taq* (*Thermus Aquaticus*) polymerase  
4.

## 1.1 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is an enzymatic process in which specific sequences of DNA are replicated or amplified to yield several million copies of the sequences. Typically, the cycle of PCR involves denaturation of double stranded DNA, annealing of primers to single stranded DNA, and extension to produce a new strand. For the forensic applications, the components of PCR depend on the manufacturer of the kits. However, generally components of PCR include but are not limited to Tris-HCl, magnesium chloride, deoxynucleotide triphosphates (dNTP), thermally stable DNA polymerase, bovine serum albumin (BSA), primers, and template DNA (John M. Butler, 2009).

PCR cycling parameters and components slightly vary by manufacturers, such as Promega Corporation and the AmpF/STR kits from Applied Biosystems. The reason is that each kit uses different primer sequences, which require different hybridization conditions, that result in different annealing rates (John M. Butler, 2009). Prior to the first denaturation, many manufacturers such as those previously mentioned, use an extended preincubation of 95°C (Degree Celcius) for 10 to 11 minutes in order to activate AmpliTaq Gold DNA polymerase. AmpliTaq Gold DNA polymerase is a modified form of *Thermus aquaticus* DNA polymerase that is activated thermally at 95°C, thereby allowing hot-start PCR (John M. Butler, 2009). The use of AmpliTaq Gold DNA

polymerase has greatly benefited the specificity of PCR amplifications as the polymerase becomes selectively activated at a high temperature which prevent the formation of primer dimers and random amplifications due to mispriming (John M. Butler, 2009). A Tris buffer with pH of 8.3 at 25°C will go down to ~pH 6.9 as the temperature increases to 95°C. At pH below 7.0, the chemical modification moieties, or derivatization of the epsilon-amino groups of the lysine residues, fall off, thereby restoring the active site of the polymerase (T. Moretti et al., 1998). Typically, the first step of PCR is denaturation, in which the mixture is heated to ~94°C. During this step, hydrogen bonds that hold double stranded DNA together are broken, thereby yielding two single stranded DNA molecules for each double stranded DNA. Once the double stranded DNA molecules are denatured, the temperature is then reduced to 50-60°C, which results in the forward and reverse primers annealing to specific complementary sequences within the loci (Terence A. Brown, 2014). Each primer has its unique DNA sequence that anneals to complementary bases when the temperature is suitable for annealing to take place. Modification of primer annealing time by several fold has been done in previous studies in an attempt to improve detection of low template DNA or minor component in mixtures<sup>11</sup>. By increasing the annealing time to approximately 20 minutes, more primers are allowed to effectively bind to primer binding sites, thereby improving the amplification efficiency in the early cycles<sup>11</sup>. Upon annealing of primers, the temperature is raised to ~74°C, an optimal working temperature for *Taq* DNA polymerase, which results in extension, or DNA synthesis from the 5' primer to the 3' end. Extension involves *Taq* polymerase inserting complementary bases, or dNTPs (dATP, dTTP, dCTP, and dGTP)

starting from the 3' hydroxyl of each primers and extending throughout the flanking regions and highly polymorphic repeating units of nucleotides, called microsatellites or STR <sup>12</sup>. Each individual has varying number of repeats of STR, which makes amplification of STR a highly powerful discriminating tool among different individuals when multiple STRs from different loci are employed. In addition to the previously mentioned components, magnesium chloride (MgCl<sub>2</sub>) is also an important component during the extension step of PCR for *Taq* DNA polymerase to work. Optimal concentration of Mg<sup>+2</sup> is critical since Mg<sup>+2</sup> serves as a cofactor for *Taq* DNA polymerase to perform replication. Typically, the optimal Mg<sup>+2</sup> concentration depends on concentrations of template DNA primers, dNTPs and chelators, such as ethylenediaminetetraacetic acid (EDTA). The reason is that Mg<sup>+2</sup> not only binds to *Taq* DNA polymerase, but also to template DNA primers, and dNTPs, and is chelated by EDTA <sup>13</sup>. At the end of the first cycle of the PCR, a set of long products, which include the STR regions, are synthesized from each strand, in which 5' ends are identical but 3' ends are randomly terminated by chance (Terence A. Brown, 2014). After the first cycle, the next cycle of denaturation-annealing-extension yields two of the long products from the first cycle, and two short products. Each subsequent cycle increases the number of short products exponentially. Typically, the number of cycles varies between 28 to 34 cycles <sup>1</sup>. The number of cycles can be manipulated by the operator for specific purposes, such as to improve peak heights. Previous research showed that increasing the number of cycles improved peak heights with tradeoffs, such as higher stutter ratios and peak height

imbalance. According to the study, as the number of cycle increases, average peak heights, and the number of alleles detected from low template DNA increase <sup>1</sup>.

## 1.2 Stochastic Effects

Stochastic effects are random variation of results between replicates. Stochastic variation occurs, especially with low amounts of DNA, because of random sampling effects regarding the law of PCR chemistry that occur in the early stage of amplification <sup>14</sup>. A high degree of stochastic variation may result in allele drop in, or drop out, low heterozygote peak height ratios, and high stutter ratios, which can be problematic when deciphering the genotypes of individual contributors from a DNA profile. This issue arises due to drop out of alleles and overlapping peak heights of high stutter peaks and allele peaks due to severe peak height imbalance (John M. Butler, 2015).

Stutter, or strand slippage, is an artifact that is characterized by one or more repeating units of nucleotides above or below an allele. Stutter can occur when the DNA polymerase pauses and dissociates from the template strand during replication. The DNA polymerase attached to a newly synthesized strand may reanneal to the template strand at one repeating unit further or behind. If the DNA polymerase reanneals to the template strand at a position of one repeating unit further, the resulting synthesized strand is one repeating unit shorter than the template strand, and is called a reverse stutter. However, if it reanneals to the template strand at a position of one repeating unit behind, then the resulting strand is one repeating unit longer than the template strand, and is called forward stutter <sup>16</sup>. Most stutter ratios are below 15% of true allele peak height under

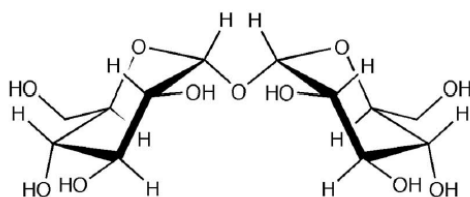
standard PCR conditions for commercially available STR kits, such as Identifiler® Plus<sup>17</sup>. Therefore they can be distinguished from true alleles, except when minor contributors exist in a mixture, in which case minor alleles may look like stutter products of major alleles. This can make interpretation of DNA profiles difficult as stutter products can be misinterpreted as true alleles. A previous study has documented that average stutter ratios of low copy number of DNA samples can be decreased by reducing the annealing/extension temperature to 56°C using Identifiler® and Identifiler® Plus commercial STR kits<sup>18</sup>. Furthermore, another study has documented that specific DNA polymerases, such as *Bacillus stearothermophilus* (*Bst*) DNA polymerase, do not slip due to constitutive strand-displacement activity<sup>19</sup>. From the same study, it was documented that magnesium concentration and ssDNA-binding-protein from *E. coli* also affect slippage of different DNA polymerases<sup>19</sup>. Despite various efforts to reduce stutter, complete elimination of stutter peaks have not been achieved.

Allele dropout is a phenomenon where an allele fails to be amplified due to stochastic effects, leading to no alleles being observed in the electropherogram. Allele dropout is an extreme form of heterozygote peak imbalance, where two alleles from a locus are amplified unequally. Allele dropout and heterozygote peak imbalance are regarded as forensic challenges as they can obscure the interpretation of DNA profiles, especially when distinguishing different genotypes of a mixture DNA profile. When a small amount of DNA is available during the early stage of amplification, primers may not consistently be able to find and hybridize to corresponding primer binding sites at target areas of DNA. Ineffective or inconsistent hybridization of primers to one or both

heterozygote alleles may result in allele or locus drop out, respectively. Also, a previous scholarly article showed that lower average peak heights that could be generated from lower amount of DNA exhibited more variations in heterozygote imbalance <sup>20</sup>, suggesting that a lower amount of DNA could confound the interpretation of DNA profile as lower peak heights could possibly be overlooked as stutter artifacts, alleles from a minor contributor, etc. Very small amounts of DNA, or LT DNA, typically refers to examination of less than 100pg or 15 diploid cells <sup>8</sup>. Amplification of LT DNA can be problematic since the success rate of obtaining good profiles may be low. In order to improve the sensitivity of detection of LT DNA, several approaches have been suggested, which include modifications made during the PCR and/or post-PCR manipulations <sup>21</sup>. Various approaches include increasing PCR cycle number to allow DNA molecules to go through more cycles of amplification, reducing the volume of the PCR to increase the concentration of DNA molecules, post-PCR clean-up to remove ions that compete with DNA during electrokinetic injection, increasing injection time, etc. <sup>21</sup>. Furthermore, Gill et al recommended modifying calculations of likelihood ratio by considering the probability of allele dropout <sup>1</sup>. Despite many approaches to alleviate the issue, ongoing research, and validation studies, a consensus method for LT typing and interpretation has not been proposed.

### 1.3 Trehalose

Trehalose is a disaccharide molecule in which two D-glucose units are linked together by an  $\alpha,\alpha$ -1,1-glycosidic bond <sup>22</sup> (Fig. 1).



**Figure 1. Chemical structure of trehalose<sup>7</sup>**

It is a moderate amphiphile, meaning that it possesses both hydrophobic moieties from carbon rings and hydrophilic moieties from hydroxyl groups <sup>22</sup>. It is synthesized by a variety of organisms such as bacteria, yeast, fungi, and other eukaryotes to be used as a source of energy and carbon. In yeast and plants, trehalose serves as a signaling molecule to control metabolic pathways and growth. Also, it serves as a compatible solute, meaning that it provides tolerance against environmental stress, such as extreme temperature, oxidation, desiccation, and dehydration, thereby stabilizing cells and cellular components such as protein, membrane, and DNA <sup>22</sup>. Due to various uses of this molecule, trehalose, has been commonly used in pharmaceutical and chemical industries. Previous studies have documented that trehalose can either stabilize or destabilize biomolecules depending on the substrate and the concentration of trehalose. In the presence of high concentration of trehalose, stabilization of double-stranded DNA was observed in a dry state through hydrogen bonding of trehalose with phosphate groups of DNA, which results in phosphate-phosphate repulsion and stabilization of base-stacking <sup>23</sup>. On the other hand, trehalose has shown to destabilize folded proteins <sup>24</sup> and double-stranded DNA <sup>4</sup> in solution. The degree of DNA destabilization induced by the presence of trehalose depends on the length and guanine-cytosine (GC) content of DNA <sup>25</sup>.

Destabilization of GC-rich DNA in the presence of trehalose, thereby enhancing PCR efficiency, was proposed by one study<sup>4</sup>. According to the study, trehalose enhances PCR efficiency by stabilizing *Taq* polymerase, lowering overall melting temperature, promoting single-stranded state of DNA, or eliminating secondary structures that still persist in the single-stranded state which can hinder the elongation step<sup>4</sup>. The interaction of trehalose and DNA at a molecular level has been spotlighted for the past decade by researchers. Based on previous studies, trehalose has been proposed to compete with DNA for water as trehalose provides hydrophilic interfaces to water molecules<sup>26</sup>. This results in sequestering water molecules and promoting dehydration of DNA molecules. Furthermore, hydrophilic interactions between phosphate backbone of DNA and trehalose increases flexibility of the polymer. The proposed mechanism induces destabilization of double-stranded DNA, leading to exposure of the bases (base flipping)<sup>7</sup>. Also, trehalose has been proposed to provide more favorable hydrophobic interactions than water to DNA bases. From a thermodynamic analysis study, trehalose was found to have more effectiveness at bridging interface between hydrophobic surface area of the DNA bases and the surrounding water<sup>7</sup>.

#### 1.4 Objectives

The main objective of this research is to evaluate the effect of trehalose on the amplification by assessing the DNA profiles produced with and without the addition of trehalose. The analysis is focused on evaluating average peak heights, peak height ratios, and stutter ratios. Improving the quality of DNA profile, especially for samples

containing low quantity of DNA or highly degraded DNA, has extensively been researched for the past two decades. Many attempts have been made, such as manipulating the number of cycles and annealing or extension temperatures in order to improve the effectiveness of amplification during PCR. Furthermore, evaluating the amplification through incorporating additives, such as sorbitol, 1,2-propanediol, etc. into the PCR cocktail, has also been studied <sup>6 27 28 29</sup>. There has been an extensive research regarding diverse effects of trehalose on proteins and DNA, and its effect on amplification. However, there are no reported studies evaluating the effect of trehalose using forensic STR kits through assessment of the quality of DNA profiles. We have generated DNA profiles by incorporating trehalose in the amplification using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit. We have evaluated peak height, peak height ratios, and stutter ratios of 15 STR loci from the kit by comparing the samples that contained varying concentrations of trehalose with the samples without trehalose.

## **2. Materials and Methods**

### **2.1 Preparation**

#### *2.1.1 Preparation of Saliva*

Liquid saliva was collected from one male donor into two 1.5 ml collection tube, which were labeled Saliva 1 and Saliva 2. Also, an Extraction Blank was prepared with deionized H<sub>2</sub>O. Saliva 1 and 2, and the Extraction Blank were stored in a freezer at -20°C until further use.

### *2.1.2 Preparation of Trehalose Stock Solution*

Trehalose stock solutions were prepared with D-(+)-trehalose dihydrate (Molecular Weight: 378.33 g/mol) (Sigma-Aldrich, Inc.), and Tris-EDTA (TE) buffer. A trehalose stock solution of 1.2M concentration was prepared by transferring 0.681g of trehalose into a 1.5 ml collection tube. TE Buffer was added slowly into the collection tube containing trehalose until the final volume reached 1.3 ml. The trehalose solution was vortexed for 15 seconds, and was incubated at 56°C in an Isotemp® water bath (Thermo Fisher Scientific Inc., Waltham, MA), until the trehalose was completely dissolved in TE Buffer. TE Buffer was added slowly to the trehalose solution until the final volume of the solution reached 1.5 ml. The solution was vortexed again for 15 seconds to ensure homogeneity of the solution. The trehalose stock solution of 1.2M concentration was stored at -20°C until further use. Following the same procedure for the preparation of 1.2M trehalose stock solution, 1.5M trehalose stock solution was prepared with 1.135g of trehalose and TE Buffer in a 2 ml collection tube at a total volume 2.0 ml.

## 2.2 Extraction

Extraction of DNA from Saliva 1, Saliva 2, and the Extraction Blank was performed using the Qiagen QIAamp® DNA Investigator Kit (Qiagen, Hilden, Germany). Extractions were performed following “Protocol: Isolation of Total DNA from Small Volumes of Blood or Saliva” from QIAamp® DNA Investigator Handbook – June 2012. A fifty microliter from each sample (Saliva 1, Saliva 2, and the Extraction Blank) was transferred into a 1.5 ml microcentrifuge tube. Buffer ATL was added to a

final volume of 100  $\mu$ L. Ten microliters of proteinase K was added, followed by 100  $\mu$ L of Buffer AL. The samples were vortexed for 15 seconds and were incubated overnight at 56°C in an Isotemp® water bath. Fifty microliters of ethanol (96-100%) was added to each tube and vortexed for 15 seconds, followed by 3 minutes of incubation at 15-25°C. The entire lysate from each tube was transferred to 2 ml QIAamp MinElute column. The samples were centrifuged at 8000 rpm in an Eppendorf centrifuge, model #5424 (Eppendorf AG, Hamburg, Germany) for 1 minute. The QIAamp MinElute columns were placed in clean 2 ml collection tubes, and the collection tubes that contained the flow-through were discarded. Five hundred microliters Buffer AW1 was added to each column, followed by centrifugation at 8000 rpm for 1 minute. The columns were placed in clean 2 ml collection tubes, and the collection tubes that contained the flow-through were discarded. Seven hundred microliters of ethanol (96-100%) was added to each column, and the columns were centrifuged at 8000 rpm for 1 minute. The columns were placed in clean 2 ml collection tubes, and the collection tubes that contained the flow-through were discarded. The tubes containing each sample were centrifuged at 14,000 rpm for 3 minutes to dry the membrane. The columns were placed in clean 1.5 ml microcentrifuge tubes, and the collection tubes that contained the flow-through were discarded. The lids of the columns were opened and incubated at room temperature for 10 minutes. Fifty microliters Buffer ATE was applied to the center of the membrane of each column. The lids of the columns were closed and incubated at room temperature for 1 minute, followed by centrifugation at 14,000 rpm for 1 minute. The samples that

contained extracted DNA were labeled Saliva Extract 1, Saliva Extract 2, and Extraction Blank. The samples were stored at -20°C until further use.

### 2.3 Quantification

All of the quantitative polymerase chain reactions (qPCR) were done using the Quantifiler® Duo Quantification kit (Applied Biosystems®, Grand Island, NY) following the Quantifiler® Duo User's Manual (Applied Biosystems®, Grand Island, NY) to prepare the reactions and set up the plates with the exception of noted changes in reaction parameter. As for the instrument, 7500 Real-Time PCR System (Applied Biosystems®, Grand Island, NY) was used to run the reactions.

In order to prepare samples containing trehalose and standard dilution series, an additional volume of 5 µL for trehalose had to be accounted in the reaction. The first set of quantification was done with additional 5 µL of dH<sub>2</sub>O in 25 µL of reaction mix, primer mix, and dilution series of standard DNA along with a regular 25 µL of reaction with dilution series of standard DNA to evaluate whether a total of 30 µL reaction with dilution series of standard DNA produced a straight line of curve when the log of concentration was plotted against cycle threshold. For all of the reactions, data from 'Duo Human' were used to generate standard curves. Target concentrations for each dilution series of standard DNA in a total volume of 25 µL were calculated, which were used to calculate dilution factors in order to generate the same target concentrations for each dilution series of standard DNA in a total volume of 30 µL. Both standard curves generated from 25 µL and 30 µL volumes using dilution series of standard DNA were

generated. Standard curve equations and  $R^2$  values (coefficient of determination) for both volumes were determined;  $R^2$  values were evaluated to determine the strength of linearity of the standard curve<sup>30</sup>. The second set of quantification was done to evaluate the effect of three different concentrations (0.05M, 0.1M, and 0.2M) of trehalose on the standard curve as compared to that of the control (0M of trehalose). The reaction was performed by adding 5  $\mu$ L of 0.3M, 0.6M, and 1.2M of trehalose solutions on each 25  $\mu$ L of regular reaction mix that contained reaction mix, primer mix, and dilution series of standard DNA for the target concentrations of trehalose to be 0.05M, 0.1M, and 0.2M. Both 0.6M and 0.3M trehalose solutions were prepared by making series of twofold dilutions from 1.2M trehalose stock solution. Also, evidence of PCR inhibition due to the presence of trehalose was evaluated by checking whether the cycle threshold ( $C_T$ ) of internal positive controls for each sample fell in the range of 28 to 31; NED<sup>TM</sup>  $C_T$  which falls between 28 and 31 with a variation of approximately 1  $C_T$  across the standard curve is a normal range<sup>30</sup>. The third set of quantification was done with DNA that was extracted from two different saliva samples from the same donor using Qiagen QIAamp® DNA Investigator Kit: saliva extract 1, saliva extract 2, and blank extract. All of the data were exported to Microsoft Excel, and graphs of standard curves were generated using the same software by plotting log of concentration in x-axis and cycle threshold in y-axis.

#### 2.4 Amplification

All of the samples were prepared using the AmpF $\ell$ STR® Identifiler® Plus PCR Amplification Kit (Applied Biosystems®, Grand Island, NY). All reactions were carried

out following the AmpF $\ell$ STR $\text{\textcircled{R}}$  Identifiler $\text{\textcircled{R}}$  Plus PCR Amplification Kit User's Guide <sup>17</sup>. With regards to reagents used for preparation of each sample, 10  $\mu\text{L}$  of Identifiler $\text{\textcircled{R}}$  Plus Master Mix, 5  $\mu\text{L}$  of Identifiler $\text{\textcircled{R}}$  Plus Primer Set, Saliva Extract 1 or 2, TE buffer, and 1.5M trehalose were used. The average DNA quantities from Saliva Extract 1 and 2 were 3.793 ng/ $\mu\text{L}$  and 2.716 ng/ $\mu\text{L}$ , respectively. For the preparation of standard samples, or samples representing 0M of trehalose, trehalose was substituted by TE buffer. Positive control, negative control and reagent blank, were also prepared for each set of PCR. For the positive control, negative control, and reagent blank, 10  $\mu\text{L}$  of 9947A control DNA, TE buffer, and blank extract, respectively, were used. For the first set of PCR reactions, Identifiler $\text{\textcircled{R}}$  Plus Master Mix, Identifiler $\text{\textcircled{R}}$  Plus Primer Set, Saliva Extract 1, and a stock solution of 1.5M of trehalose were combined to prepare a total of six batches, each batch containing 8 replicates of 1ng or 0.1ng of DNA with 0.4M, 0.2M, or 0M trehalose. From hereon, the first set of experiment (Exp. 1) represents samples containing target quantities of 0.1ng and 1ng of DNA, and the second set of experiment (Exp. 2) represents samples containing target quantities of 0.025ng, 0.05ng, and 0.1ng DNA. Dilutions of DNA and trehalose for each batch were done by adding appropriate amount of TE buffer to obtain the correct target quantity of DNA and concentrations of trehalose. The volumes for each reagent, Saliva Extract 1, and 1.5M trehalose stock solution needed for each batch were calculated based on the target quantity and concentrations of DNA and trehalose, respectively, for each replicate. Upon preparation of six batches, 8 aliquots from each batch were distributed to corresponding PCR tubes. The second set of PCR was performed following the same procedure as the first set of PCR, but preparing target

quantities of 0.1ng, 0.05ng, and 0.025ng of DNA using Saliva Extract 2 with the same target concentrations of trehalose for each replicate. For the second set of experiment, a total of nine batches were prepared, and 8 aliquots from each batch were distributed to corresponding PCR tubes. All samples were amplified on GeneAmp® PCR System 9700 (Applied Biosystems®, Grand Island, NY) following the standard 28-PCR-cycle protocol that is specified in AmpFℓSTR® Identifiler® Plus PCR Amplification Kit User's Guide<sup>17</sup>: initial incubation was set at 95°C for 11 minutes; alternating 28 cycles of denaturation and annealing/extension steps were set at 94°C for 20 seconds and 59°C for 3 minutes, respectively; final extension step was set at 60°C for 10 minutes; final hold was set at 4°C. Upon completion of PCR, all samples were stored at -20°C until capillary electrophoresis could be performed.

## 2.5 Capillary Electrophoresis

Capillary electrophoresis (CE) was performed on ABI 3130 Genetic Analyzer (Applied Biosystems®, Grand Island, NY) following the instructions on AmpFℓSTR® Identifiler® Plus PCR Amplification Kit User's Guide and Applied Biosystems 3130/3130xl Genetic Analyzers Getting Started Guide<sup>17,31</sup>. Each sample included two reagents: 9.5 µL of GeneScan 500 LIZ Size Standard and 0.5 µL Hi-Di deionized formamide. The volume of each reagent was calculated based on the total number of samples. Calculated volumes of the reagents were combined in a 1.5 mL microcentrifuge tube, and were vortexed and spun. Ten microliters of the combined reagents were aliquoted into each well of the 96-well MicroAmp reaction plate, followed by addition of

1  $\mu$ L of each sample corresponding to each well. The plate was vortexed and spun. For the allelic ladder, AmpFISTR® Identifiler® Plus Allelic Ladder was used. The first and second set of experiments included a total of four and five allelic ladders, respectively. The plate was secured with septa, vortexed and spun. The samples were denatured on GeneAmp® PCR System 9700 at 95°C for 3 minutes. Then, the samples were immediately chilled on a metal block at -20°C. In ABI 3130 Genetic Analyzer, POP-4™ (Applied Biosystems®, Grand Island, NY) was used as a polymer solution, 1X Genetic Analyzer Buffer was used to fill anode buffer reservoir and buffer reservoirs, and Milli-Q water was used to fill water reservoirs. For the first set of experiment, samples containing 1ng of DNA were run at 5 seconds injection, and those containing 0.1ng of DNA were run at both 5 and 10 seconds injection. For the second set of experiment, all of the samples were run at 10 seconds injection. All injections were run at a voltage of 3 kilovolts. The profiles that were generated were analyzed using Genemapper® ID Version 3.1 software (Applied Biosystems®, Grand Island, NY). For first and second set of experiments, analytical thresholds of 8 and 3 relative fluorescence units (rfu), respectively, were used. The analytical thresholds (not listed) for each run were decided based on the extent of baseline noise being filtered out by the analytical thresholds without excluding stutter artifacts that were needed for stutter ratio analysis. Data were exported into Microsoft Excel.

## 2.6 Statistical Analysis

Statistical analysis of peak heights, peak height ratios, and reverse stutter ratios from the exported data produced by CE was performed by using Microsoft Excel for statistical calculations and JMP Pro 12 (SAS, Cary, NC) for generation of graphs and statistical analysis. From hereon, the first allele refers to the allele of a lower molecular weight from a heterozygous locus, and a second allele refers to the allele of a higher molecular weight. During the analysis, it was determined that 0.1ng of DNA at 5 seconds injection (1<sup>st</sup> Exp.) was proportionally similar to that of 10 seconds injection (1<sup>st</sup> Exp.), therefore, the data regarding 0.1ng of DNA at 5 seconds injection was replaced by that of 10 seconds injection. Also, a previous validation study has shown that two different injection times (4 seconds and 10 seconds) and kit lots yielded a relatively smaller variance of peak heights of an allele compared to that resulting from capillary changes, and variation in amplification<sup>32</sup>, suggesting that variation in proportion of average peak heights between 5 seconds and 10 seconds injections is unlikely to be observed. For the analysis of PHR, all of the heterozygous loci were used and PHR was calculated by dividing the peak height of the allele with the smaller rfu by the peak height of the allele with the larger rfu. Reverse stutter artifacts, or true allele number minus 1 repeating unit, were used for analysis of stutter ratios. Stutter ratios were calculated by dividing the rfu of reverse stutter artifacts of the true allele by the rfu of the true allele. Second alleles of heterozygous loci, in which the alleles were two repeating units higher than first alleles were not used for analysis of stutter ratios due to reverse stutter products of the second alleles possibly containing forward stutter products of the first alleles. For the second set

of experiment, only reverse stutter ratios from 0.1ng of DNA (10 seconds injection time) were analyzed because most of the reverse stutter artifacts from 0.05ng and 0.025ng DNA could not be distinguished from the baseline noise. Using JMP Pro 12, evaluation of average, standard errors, lower 95% percentile of the data, and upper 95% percentile of the data was performed on each amount of DNA treated with 0M, 0.2M, or 0.4M of trehalose for the analysis of average peak heights, PHR, and stutter ratios from the first and second set of experiments. In addition, two independent samples t-test was performed with an  $\alpha$  level of 0.05, computing the significance of difference in means of each pair of different concentrations of trehalose. Probabilities of obtaining p-values for two independent samples t-test below an  $\alpha$  level of 0.05 were highlighted by red, indicating that the results are significantly different. Those that were slightly above 0.05 but below 0.1 were highlighted by blue. For average peak heights and stutter ratios, two independent samples t-test was performed on each allele, and each selected reverse stutter position of an allele, respectively. Due to the unexpected discrepancy in peak heights between 0.1ng of DNA at 10 seconds injection from the first experiment and those from the second experiment, analysis of average peak heights for 0.1ng of DNA for the purpose of comparing those average peak heights with average peak heights from other quantities of DNA or with each other, was precluded. In order to better visually understand the pattern for differences of average peak heights and stutter ratios for each locus between 0.4M or 0.2M of trehalose and 0M of trehalose, differences of average peak heights and average stutter ratios of each locus between 0.4M or 0.2M of trehalose and 0M trehalose were computed across 15 loci. This was not performed on peak height

ratios, as it was determined that differences of average peak height ratios of each locus for all quantities of DNA were randomly distributed. For the evaluation of average PHR across 15 loci, line graphs representing each amount of DNA treated with 0M, 0.2M, and 0.4M of trehalose were generated in one figure and were compared. For the evaluation of average stutter ratio, line graphs representing each amount of DNA treated with 0M, 0.2M, and 0.4M of trehalose were generated for each quantity of DNA.

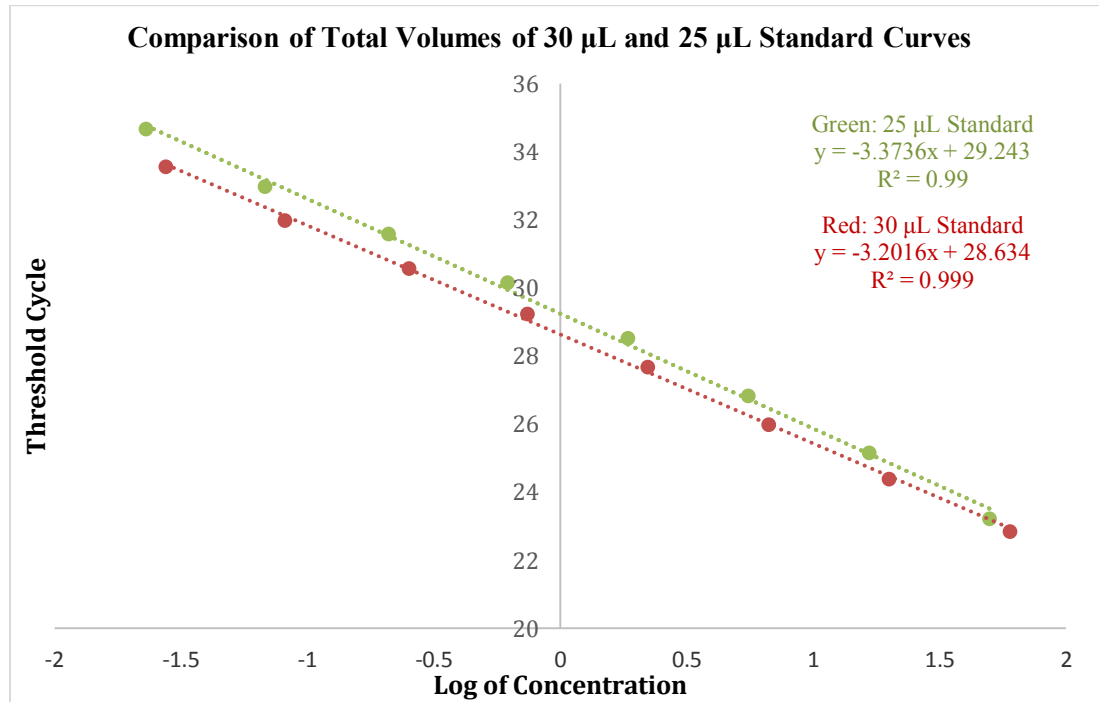
### **3. Results and Discussions**

#### **3.1 Quantitative PCR Experiment**

##### *3.1.1 Validation of 30 $\mu$ L Total Volume qPCR*

In order to include the extra volume needed to add the trehalose into each qPCR sample, a preliminary validation experiment comparing standard curves of Quant Duo generated from 25  $\mu$ L and 30  $\mu$ L total reaction volumes was done. The standard curve dilution series was amplified in both 25  $\mu$ L and 30  $\mu$ L volumes. The reason for this preliminary experiment was to evaluate whether an overload of 5  $\mu$ L for a typical 25  $\mu$ L volume could affect standard curve linearity through the assessment of coefficient of determination, or  $R^2$  value. The strength of linearity of the standard curve through measuring  $R^2$  value is important in this case, since any stray data points generated that deviate from the established standard curve when amplifications are done with and without trehalose may indicate effects of trehalose on PCR. Standard curves for both volumes were prepared by plotting a log of concentration on the x-axis and cycle

threshold ( $C_T$ ) on the y-axis, and the equations for two different total volumes were generated (Fig. 2).



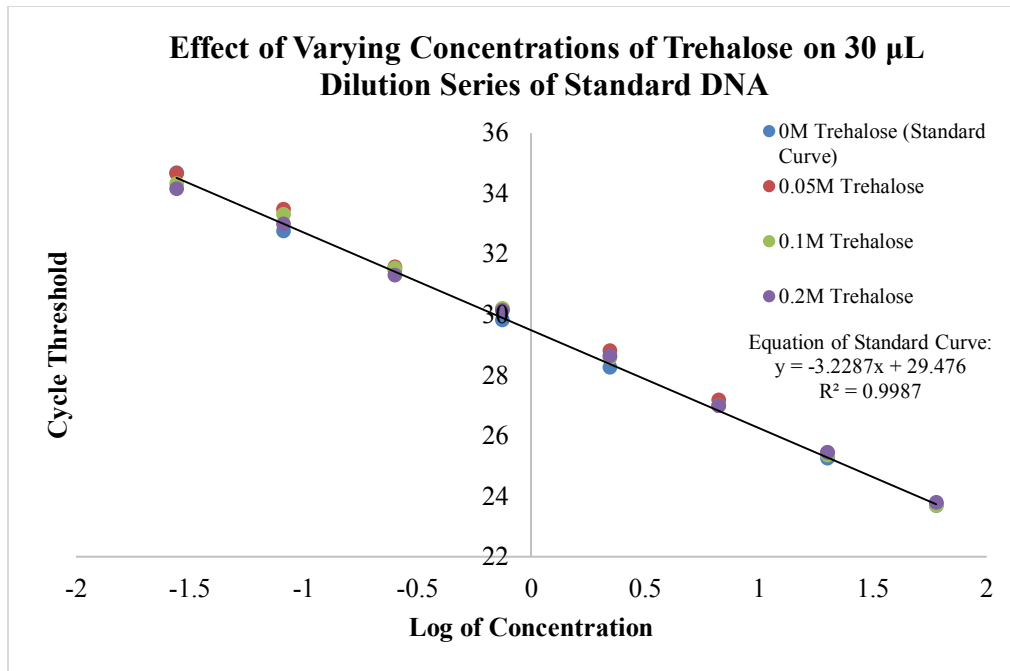
**Figure 2. Comparison of standard curves generated from a 25 µL (green) and a 30 µL (red) dilution series of standard DNA generated by qPCR using 7500 Real-Time PCR System**

The results (Fig. 2) show that a standard curve generated from 25 µL volume of dilution series of standard DNA represented by green yielded an equation that was slightly different from that of 30 µL. The standard curve created from 30 µL volume is shifted slightly downward than that created from 25 µL volume, and the magnitude of slope of 30 µL volume is slightly less than that of 25 µL volume. The  $R^2$  value, or coefficient of determination for 30 µL dilution series of standard DNA is 0.999, indicating that the linear model fits the data well. The strong  $R^2$  value for 30 µL dilution series of standard DNA suggests that additional 5 µL total volume does not have an effect

in lowering the  $R^2$  value. Therefore, adding 5  $\mu\text{L}$  of trehalose on 25  $\mu\text{L}$  of total volume is permissive for the evaluation of positive effects of trehalose.

### *3.1.2 Effect of Varying Concentrations of Trehalose on Standard Curves*

After determining that a standard curve generated from 30  $\mu\text{L}$  dilution series of standard DNA was reasonable to use, the next set of reactions were performed with four 30  $\mu\text{L}$  dilution series of standard DNA, containing 0M (control), 0.05M, 0.1M, and 0.2M of trehalose. This experiment was performed in order to observe any deviations in standard curves generated from dilution series of standard DNA with and without trehalose. The result (Fig. 3) indicate that no significant difference is observed within the standard curves representing varying concentrations of trehalose as compared to the control. It is not clear whether slight variations in data points are due to normal variations in PCR chemistry, sampling, or the effect of trehalose. Therefore, the results suggested that further experiments using a standard PCR kit are needed for an in depth evaluation. Prior to further experiment, an evaluation of the Internal Positive Control (IPC) was performed in order to assess whether using a standard PCR kit would be inhibited by the addition of up to 0.2M trehalose.



**Figure 3. Comparison of dilution series of standard DNA, each sample containing 0M (blue), 0.05M (red), 0.1M (green), and 0.2M (purple) of trehalose using 7500 Real-Time PCR System. A standard curve for 0M trehalose (control) and its equation are displayed on the graph.**

### 3.1.3 Evaluation of Internal Positive Control (IPC)

In order to ensure that trehalose does not have any inhibitory effects on the amplification of DNA, cycle thresholds for Quant Duo IPCs for samples that contained trehalose were evaluated. Cycle thresholds for Duo IPCs that fell between 28 and 31 would be regarded as having no significant inhibitory effects, and would suggest that the study could be proceed forward to evaluate effects on the amplification. However, those that fell above 31 would be regarded as having some inhibitory effects; inhibitory effects will reduce the efficiency of the amplification to undergo more cycles to reach 31 rfu. As shown in Table 1, all quantities of samples, each with 0M, 0.05M, 0.1M, and 0.2M of

trehalose resulted in cycle thresholds for Duo IPCs falling between 28 and 31, suggesting no significant inhibitory effects by trehalose. Therefore, further PCR experiment for in depth analysis of peak heights, PHRs, and stutter ratios was performed.

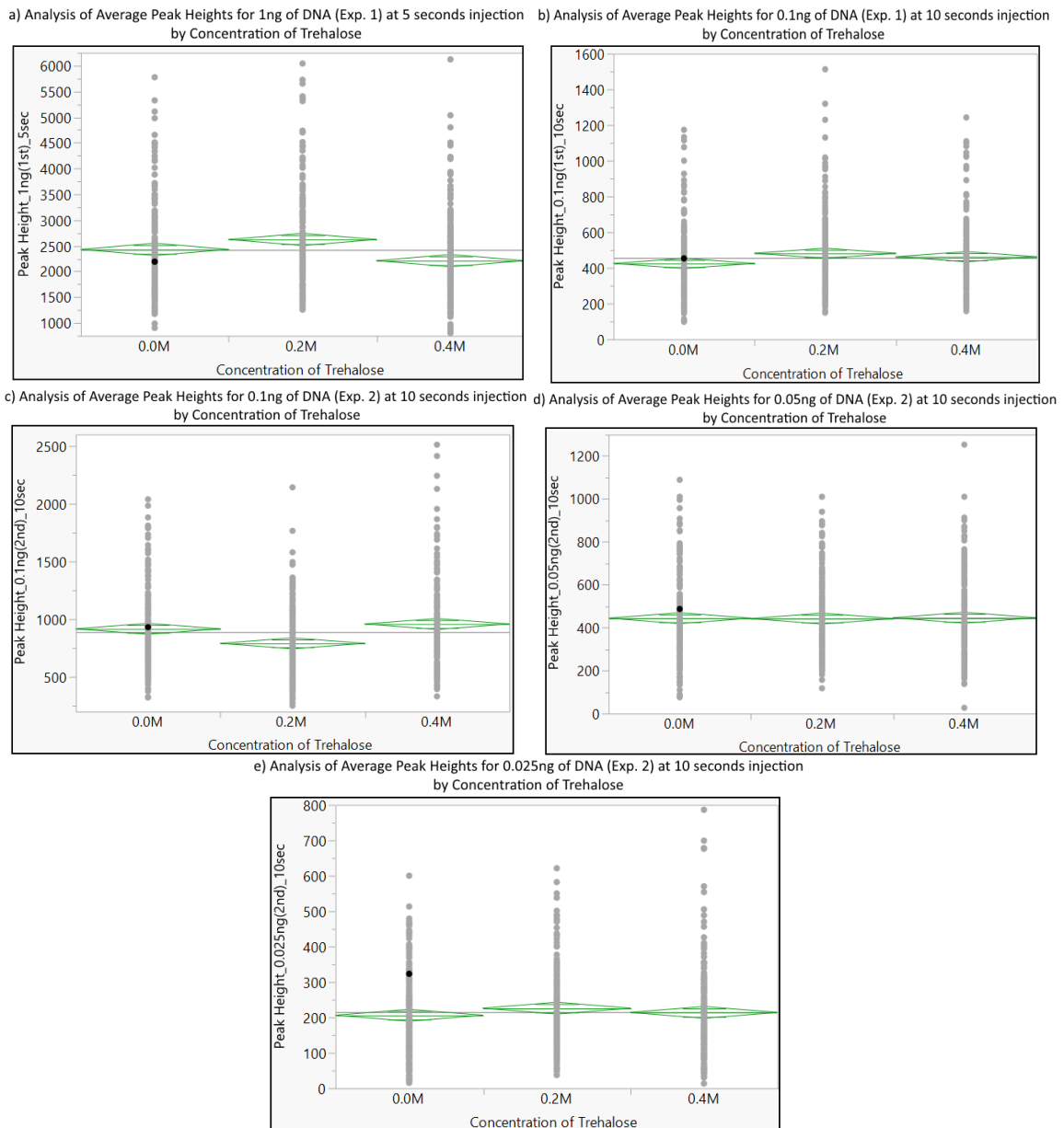
**Table 1. Table of Cycle Threshold Values of Duo IPCs across All Quantities of DNA**

<b>Quantity of DNA (ng)</b>	<b>0M Trehalose (standard)</b>	<b>0.05M Trehalose</b>	<b>0.1M Trehalose</b>	<b>0.2M Trehalose</b>
60	29.79873	29.68786	29.36344	29.29164
20.04	29.77715	29.50356	29.46134	30.07108
6.672	29.68786	29.36344	29.29164	29.6254
2.22	29.50356	29.46134	30.07108	29.55034
0.744	29.36344	29.29164	29.6254	29.24001
0.252	29.46134	30.07108	29.55034	29.3578
0.0816	29.2046	29.6254	29.24001	29.40236
0.0276	29.29164	29.55034	29.3578	29.51367
Average	29.51104	29.56933	29.49513	29.50654

### 3.2 Evaluating the Effect of Varying Concentrations of Trehalose on Peak Heights of Different Quantities of DNA

The effect of 0.4M, 0.2M, and 0M of trehalose on average peak heights for 0.025ng, 0.05ng, 0.1ng, and 1ng of DNA was evaluated. Eight replicates for each condition were amplified with Identifiler® Plus PCR Amplification Kit. Mean, standard error, lower 95% percentile of the data, and higher 95% percentile of the data of each condition combining all of the data points from each profile were generated (Fig. 4; Table 2). For the result of 0.1ng of DNA from the first experiment, a 10 seconds injection time was used instead of a 5 seconds injection time. Also, the result of a 0.1ng of target DNA at a 10 seconds injection time from the second experiment was used. Two independent

samples t-test was performed on average peak heights for pairs of different concentrations of trehalose per quantity of DNA in order to evaluate the statistical significance of difference in average peak heights (Table 3), and the p-values were generated. The results of these comparisons are displayed in Table 2 and 3.



**Figure 4. Average peak heights for 0M (control), 0.2M, and 0.4M of trehalose in (a) 1ng (1<sup>st</sup> Exp.; 5 seconds injection), (b) 0.1ng (1<sup>st</sup> Exp.; 10 seconds injection), (c) 0.1ng (2<sup>nd</sup> run; 10 seconds injection), (d) 0.05ng (2<sup>nd</sup> run; 10 seconds injection), (e) 0.025ng (2<sup>nd</sup> run; 10 seconds injection) of DNA amplified with Identifiler® Plus**

**Table 2. Summary of Average Peak Heights, Standard Error, Lower 95%, Upper 95% for each Condition Amplified with Identifiler® Plus**

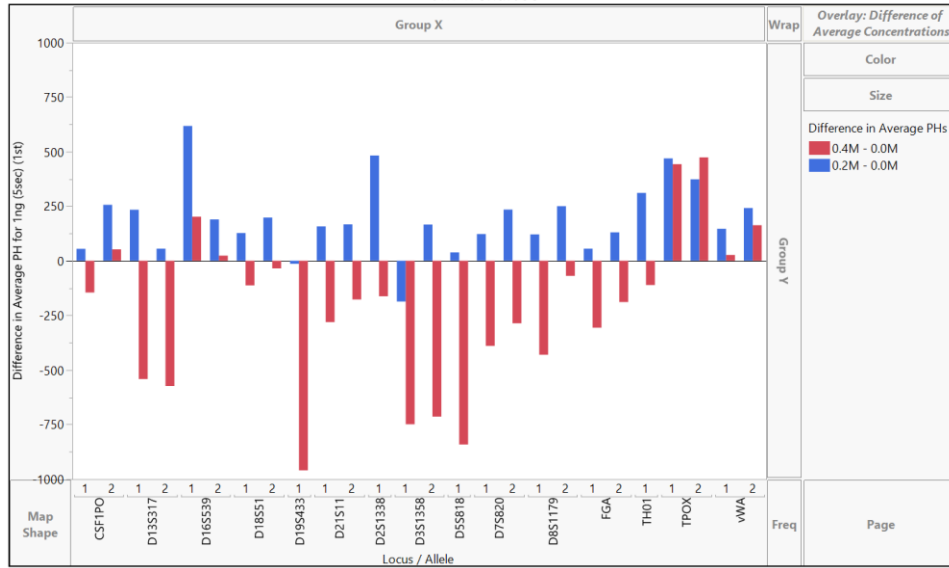
Experiment	Quantity of DNA	Injection Time	# of Data Points	Concentration of Trehalose	Mean (rfu)	Standard Error	Lower 95%	Upper 95%
1	1 ng	5 sec.	208	0M	2429.38	62.556	2309.8	2549.0
	1 ng	5 sec.	208	0.2M	2621.79	63.629	2502.2	2741.4
	1 ng	5 sec.	208	0.4M	2210.55	56.306	2090.9	2330.2
	0.1 ng	10 sec.	208	0M	424.918	13.754	396.44	453.40
	0.1 ng	10 sec.	208	0.2M	481.933	15.540	453.46	510.41
	0.1 ng	10 sec.	208	0.4M	462.418	14.149	433.94	490.90
2	0.1 ng	10 sec.	208	0M	916.524	23.789	869.10	963.9
	0.1 ng	10 sec.	208	0.2M	790.572	22.153	743.15	838.0
	0.1 ng	10 sec.	208	0.4M	958.332	26.316	910.91	1005.8
	0.05 ng	10 sec.	208	0M	443.942	13.701	418.29	469.60
	0.05 ng	10 sec.	208	0.2M	442.168	12.427	416.52	467.82
	0.05 ng	10 sec.	208	0.4M	446.034	13.030	420.38	471.69
	0.025 ng	10 sec.	208	0M	206.014	8.2678	189.27	222.76
	0.025 ng	10 sec.	208	0.2M	225.995	8.3344	209.25	242.74
	0.025 ng	10 sec.	208	0.4M	214.087	8.9872	197.30	230.87

**Table 3. Summary of Two Independent Samples t-test for Experiments 1 and 2 Comparing the Difference in Average Peak Heights at Each Concentration of Trehalose Per Quantity of DNA Amplified with Identifiler® Plus**

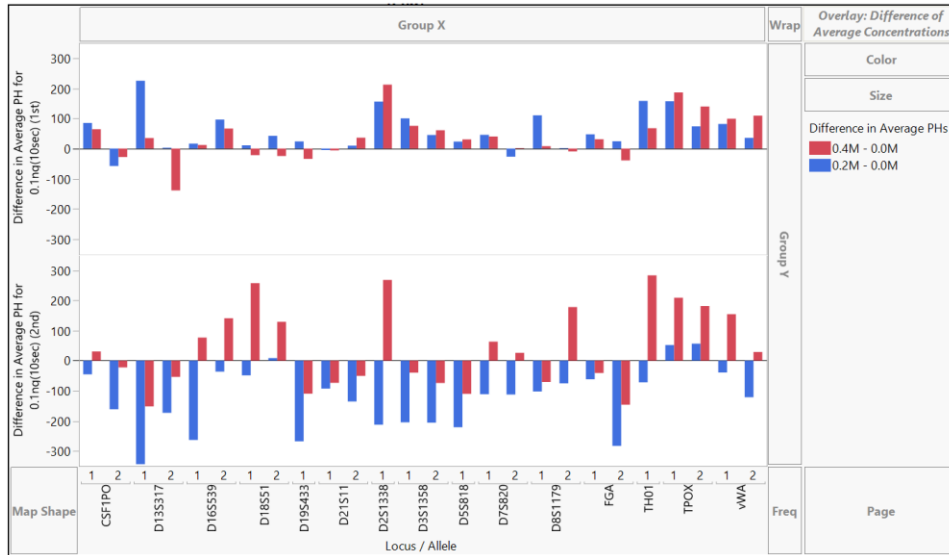
Experiment	Quantity of DNA	Injection Time	# of Data Points	Ordered Differences	p-Value
1	1 ng	5 sec.	208	0.4M and 0M	0.0113
	1 ng	5 sec.	208	0.2M and 0M	0.0259
	0.1 ng	10 sec.	208	0.4M and 0M	0.0679
	0.1 ng	10 sec.	208	0.2M and 0M	0.0056
2	0.1 ng	10 sec.	208	0.4M and 0M	0.2213
	0.1 ng	10 sec.	208	0.2M and 0M	0.0002
	0.05 ng	10 sec.	208	0.4M and 0M	0.9099
	0.05 ng	10 sec.	208	0.2M and 0M	0.9235
	0.025 ng	10 sec.	208	0.4M and 0M	0.5040
	0.025 ng	10 sec.	208	0.2M and 0M	0.0981

For better visualizations of the results, bar graphs (Fig. 5) showing differences in average peak heights across 15 loci for 0.4M and 0M trehalose, as well as those for 0.2M and 0M trehalose, were generated with JMP pro 12. A total of 26 alleles were evaluated for average peak heights.

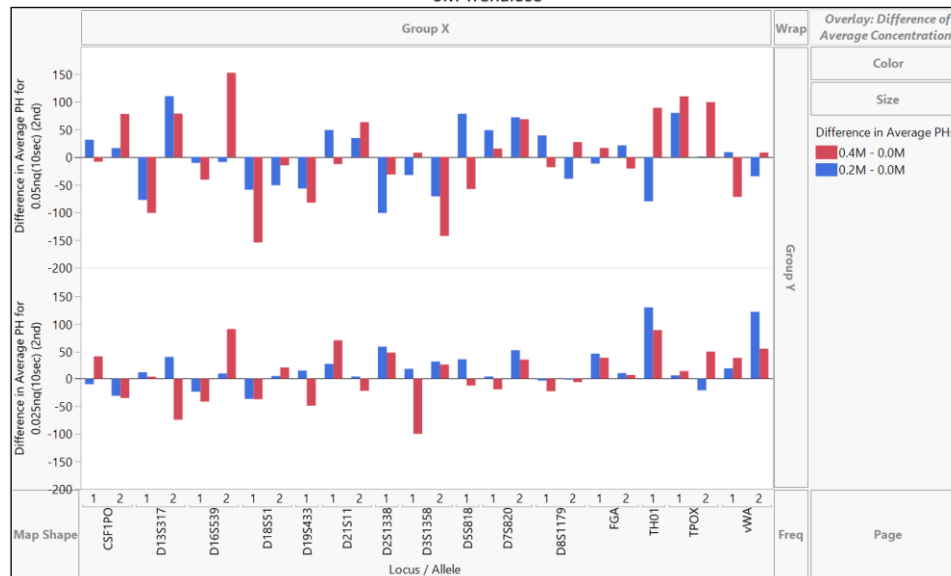
a) Difference in Average Peak Heights of 1.0ng DNA for 0.4M and 0.2M Trehalose as Compared to Those of 0M Trehalose



b) Difference in Average Peak Heights of 0.1ng DNA for 0.4M and 0.2M Trehalose as Compared to Those of 0M Trehalose



c) Difference in Average Peak Heights of 0.05ng and 0.025ng for 0.4M and 0.2M Trehalose as Compared to Those of 0M Trehalose



**Figure 5. Difference in average peak heights for 0.4M and 0.2M trehalose as compared to those for 0M trehalose at (a) 1ng (1<sup>st</sup> Exp.; 5 seconds injection), (b) 0.1ng (1<sup>st</sup> Exp.; 10 seconds injection) from the top and 0.1ng (2<sup>nd</sup> Exp.; 10 seconds injection) from the, and (c) 0.05ng (2<sup>nd</sup> Exp.; 10 seconds injection) from the top and 0.025ng (2<sup>nd</sup> Exp.; 10 seconds injection) from the bottom. The bar graphs were generated by JMP Pro 12.**

The statistical analysis for 1ng of DNA comparing average peak heights using 0M, 0.2M, and 0.4M of trehalose (Figure 4; Table 2; Table 3), shows that average peak height for 0.2M of trehalose is approximately 7.9% higher than those of the control (0M trehalose). On the other hand, it shows that average peak height for 0.4M of trehalose is approximately 9.0% lower than those of the control. An exception to this observation is both alleles of TPOX locus, in which addition of 0.4M of trehalose resulted in higher average peak heights followed by 0.2M of trehalose as compared to those of the control. The bar graph for 1ng of DNA that shows a general trend in average peak heights across 15 STR loci for 0.2M and 0.4M of trehalose shows higher overall average peak heights

for 0.2M of trehalose and lower average peak heights for 0.4M of trehalose compared to the control (Fig. 5). For 0.1ng of DNA at 10 seconds injection time from the first experiment, the additions of 0.2M and 0.4M of trehalose show higher overall average peak heights as compared to those of the control (Fig. 5; Table 2 and 3).

In contrast to the result from the first experiment, 0.1ng of DNA at 10 seconds injection time from the second experiment shows an unexpected discrepancy in the results. The addition of 0.2M of trehalose shows lower overall average peak heights, an observation that was the opposite from those of the first run. One of the possible sources of error that could have caused discrepancy in the results for 0.1ng of DNA between the first and the second experiments is the difference in the actual quantities of DNA that were amplified between the first and the second experiments, since two different DNA extracts were used. It is evident from the data that average peak heights from 0.1ng of DNA for 0M of trehalose at 10 seconds injection (2<sup>nd</sup> experiment) are approximately 2 folds higher than those from the first experiment. During the collection of saliva and the extraction of DNA, two separate DNA extracts (Saliva Extract 1 and 2) from the same donor were yielded, which resulted in different quantities of DNA measured by qPCR (the average DNA quantities from DNA extract 1 and 2 were 3.793 ng/ $\mu$ L and 2.716 ng/ $\mu$ L, respectively). The average DNA quantities for both extracts calculated from a standard curve may have not been accurate to calculate correct dilution factors for specific target amount of DNA to end up in each amplification tube. Also, the result suggested that peak heights in experiment 2 increased proportionally with increasing DNA amounts, whereas those of the experiment 1 did not show a proportional increase.

Therefore, the results from the 0.1ng of DNA reactions in experiment 1 are outliers, which can't be compared to those from experiment 2. This discrepancy of the two results due to outliers from experiment 1, suggests that no conclusions can be made regarding average peak heights for 0.1ng of DNA. A statistical analysis of average peak heights and the bar graphs for LT DNA, or 0.025ng and 0.05ng of DNA with 0.2M and 0.4M of trehalose show no specific patterns or effects (Fig. 5; Table 2 and 3).

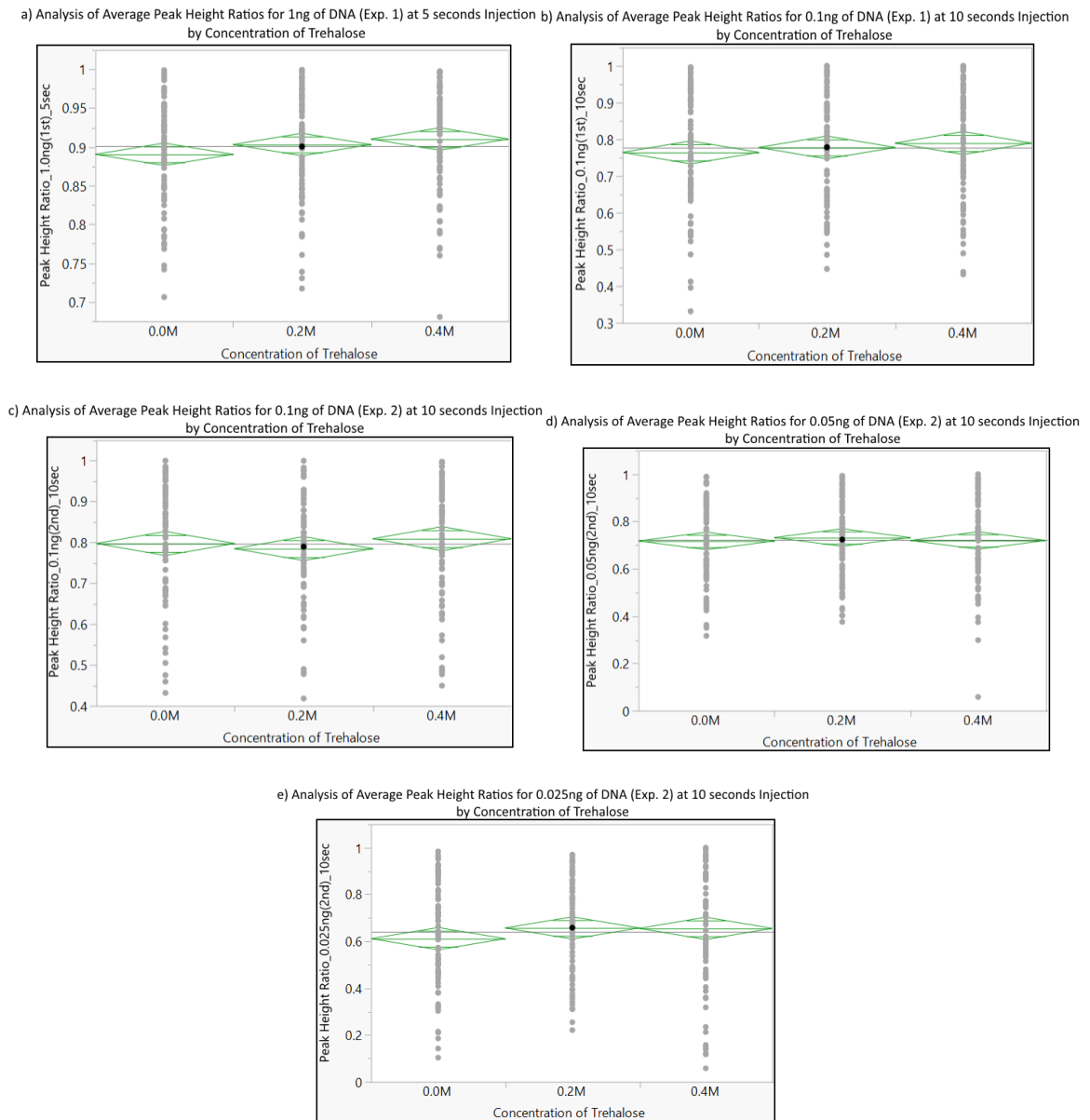
The overall result showed that there was a lack of evidence that both 0.2M and 0.4M of trehalose produced reproducible effects on average peak heights for 0.025ng, 0.05ng, 0.1ng, and 1ng of DNA. Due to the lack of consistent results, it was concluded that the significant differences in average peak heights for 0.2M and 0.4M of trehalose compared to those of the control at 1ng of DNA may have resulted from the propagation of pipetting error during the preparation of each batch, which may have contributed to unequal concentrations of DNA in each batch. Instead of aliquoting replicates of samples containing DNA from one batch, each batch was prepared separately through the addition of each components, and aliquoted from thereon. Introduction of pipetting error during the addition of each components into each batch and aliquoting 8 replicates from each batch could have resulted in different amount of DNA in each conditions which are directly reflected in average peak heights. For future studies, it may be necessary to proceed the experiment by preparing aliquots from one batch that contains DNA, TE buffer, and master mix, followed by the addition of different concentrations of trehalose in each aliquots, in order to minimize pipetting error. Also, it may be of interest to evaluate the total GC contents within each locus for a particular STR amplification kit

including primer binding regions, and flanking regions to evaluate whether there is an association between GC contents and concentrations of trehalose resulting in higher or lower amplification efficiency and average peak heights as previous study by Andrej-Nikolai Spiess, et al. <sup>4</sup> showed. The result from Spiess study showed that increasing the PCR efficiency could be achieved with higher GC contents of templates by adding high concentrations of trehalose. Evaluation of GC contents for each locus may reveal whether higher average peak heights observed at TPOX across all quantities of DNA with the addition of 0.4M of trehalose was significant.

### 3.3 Evaluating the Effect of Varying Concentrations of Trehalose on Peak Height Ratios

The effect of 0M, 0.2M, and 0.4M of trehalose on average peak height ratios for 0.025ng, 0.05ng, 0.1ng, and 1ng of DNA were evaluated and compared. For each condition, a statistical analysis was done (8 replicates of heterozygous loci of the AmpF $\ell$ STR $\text{\textcircled{R}}$  Identifiler $\text{\textcircled{R}}$  Plus PCR Amplification Kit) to evaluate average peak height ratios, standard error, lower 95% percentile of the data, and upper 95% percentile of the data (Fig. 6, Table 4). Like the evaluation of average peak heights, 0.1ng of DNA with 10 seconds injection time for both the first and the second experiments were performed. Two independent samples t-test was performed on each pair of conditions per quantity of DNA, and p-values were generated to evaluate the significance (Table 5). Average peak height ratios across all heterozygous loci for 0.025ng (2<sup>nd</sup> Exp.; 10 seconds injection), 0.1ng (2<sup>nd</sup> Exp.; 10 seconds injection), and 1ng (1<sup>st</sup> Exp.; 5 seconds injection) with 0M, 0.2M, and 0.4M of trehalose are shown in the line graph (Fig. 7). The line graph shows

that average peak height ratios for 0.025ng, 0.1ng, and 1ng of DNA, are around 0.6, 0.8, and 0.9, respectively, with no specific differences between each concentration of trehalose for each amount of DNA.



**Figure 6. Average peak height ratios for 0M (control), 0.2M, and 0.4M of trehalose in (a) 1ng (1<sup>st</sup> Exp.; 5 seconds injection), (b) 0.1ng (1<sup>st</sup> Exp.; 10 seconds injection), (c)**

0.1ng (2<sup>nd</sup> run; 10 seconds injection), (d) 0.05ng (2<sup>nd</sup> run; 10 seconds injection), (e) 0.025ng (2<sup>nd</sup> run; 10 seconds injection) of DNA amplified with Identifiler® Plus

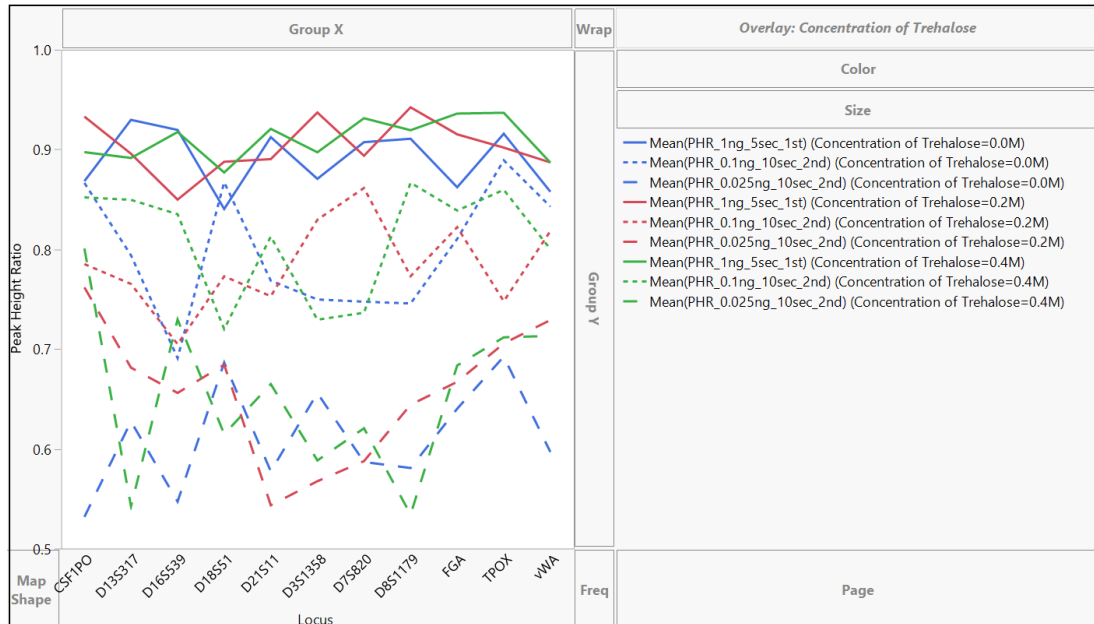
**Table 4. Summary of Average Peak Height Ratios, Standard error, Lower 95%, Upper 95% for Each Condition Amplified with Identifiler® Plus**

Experiment	Quantity of DNA	Injection Time	# of Data Points	Concentration of Trehalose	Mean	Standard Error	Lower 95%	Upper 95%
1	1 ng	5 sec.	88	0M	0.8904	0.00726	0.8759	0.9049
	1 ng	5 sec.	88	0.2M	0.9030	0.00771	0.8886	0.9175
	1 ng	5 sec.	88	0.4M	0.9101	0.00710	0.8956	0.9246
	0.1 ng	10 sec.	88	0M	0.7704	0.0159	0.7391	0.8016
	0.1 ng	10 sec.	88	0.2M	0.7779	0.0160	0.7567	0.8091
	0.1 ng	10 sec.	88	0.4M	0.7940	0.0157	0.7628	0.8253
2	0.1 ng	10 sec.	88	0M	0.7644	0.0152	0.7332	0.7957
	0.1 ng	10 sec.	88	0.2M	0.7778	0.0145	0.7466	0.8091
	0.1 ng	10 sec.	88	0.4M	0.7898	0.0152	0.7586	0.8211
	0.05 ng	10 sec.	88	0M	0.7974	0.0185	0.7680	0.8269
	0.05 ng	10 sec.	88	0.2M	0.7848	0.0181	0.7554	0.8143
	0.05 ng	10 sec.	88	0.4M	0.8091	0.0197	0.7796	0.8385
	0.025 ng	10 sec.	88	0M	0.7178	0.0235	0.6808	0.7548
	0.025 ng	10 sec.	88	0.2M	0.7323	0.0220	0.6953	0.7693
	0.025 ng	10 sec.	88	0.4M	0.7198	0.0270	0.6828	0.7568

**Table 5. Summary of Two Independent Samples t-test for Experiments 1 and 2 Comparing the Difference in Average Peak Height Ratios at Each Concentration of Trehalose Per Quantity of DNA Amplified with Identifiler® Plus**

Experiment	Quantity of DNA	Injection Time	# of Data Points	Ordered Differences	p-Value
1	1 ng	5 sec.	88	0.4M and 0M	0.0592
	1 ng	5 sec.	88	0.2M and 0M	0.2254
	0.1 ng	10 sec.	88	0.4M and 0M	0.2591
	0.1 ng	10 sec.	88	0.2M and 0M	0.5511
2	0.1 ng	10 sec.	88	0.4M and 0M	0.5821
	0.1 ng	10 sec.	88	0.2M and 0M	0.5512
	0.05 ng	10 sec.	88	0.4M and 0M	0.9405
	0.05 ng	10 sec.	88	0.2M and 0M	0.5856
	0.025 ng	10 sec.	88	0.4M and 0M	0.1985
	0.025 ng	10 sec.	88	0.2M and 0M	0.1802

Average Peak Height Ratios Across Heterozygous Loci for 1ng(1st; 5sec), 0.1ng(2nd; 10sec), and 0.025ng(2nd; 10sec) of DNA



**Figure 7. Line graph of average peak height ratios across all heterozygous loci for 1ng of DNA at 5 seconds injection time from the first experiment (regular lines), 0.1ng at 10 seconds injection from the second experiment (dotted lines), and 0.025ng at 10 seconds injection from the second experiment (break lines) with 0M (blue), 0.2M (red), and 0.4M (green) of trehalose generated from JMP Pro 12**

The result of statistical analysis across all quantities of DNA suggest that variations in average peak height ratios for each concentration of trehalose are more likely to be seen by chance (Fig. 6, Table 4 and 5). Also, the result of two independent samples t-test indicate that there is less likely to be a statistical significance in the difference of average peak height ratios between 0M and 0.4M of trehalose as well as 0M and 0.2M of trehalose. Therefore, there was no evidence to suggest that the addition of trehalose produced any reproducible effects on average peak height ratios. Furthermore, the result of average peak height ratios across all heterozygous loci for 0.025ng, 0.1ng, and 1ng of DNA with 0.4M, 0.2M, and 0M of trehalose suggest that average peak height

ratios decrease as the amount of DNA input decreases (Fig. 7). Also, variations in average peak height ratios across the loci decrease as the amount of input of DNA increases. This observation complies with many previous observations, which demonstrated that variation in heterozygote peak imbalance increased as average peak heights decreased<sup>20</sup>. However, this result does not show any patterns and differences between average peak height ratios yielded from 0M, 0.2M, and 0.4M of trehalose for each quantity of DNA. Average peak height ratios for each condition suggests random variations with approximately similar variances among three different concentrations of trehalose per quantity of DNA. The observation suggesting no evidence of association shown from Figure 7 is supported by p-values from Table 5, where all of the p-values are above the critical value, 0.05. The lowest p-value yielded, 0.0592, for the difference of average peak height ratios for 0.4M and 0M of trehalose at 1ng of DNA may have been due to small number of heterozygote loci and sample size. Further experiments may be needed with a larger sample size and more heterozygote loci in order to verify if this low p-value occurred by chance.

### 3.4 Evaluating the Effect of Varying Concentrations of Trehalose on Reverse Stutter Ratios

The effect of 0M, 0.2M, and 0.4M of trehalose on average reverse stutter ratios for 1ng, and 0.1ng of DNA was evaluated. For each condition, statistical analysis was performed on reverse stutters of the first and the second alleles (data not shown). Only reverse stutter ratios for the second alleles that are more than 2 units larger than the first

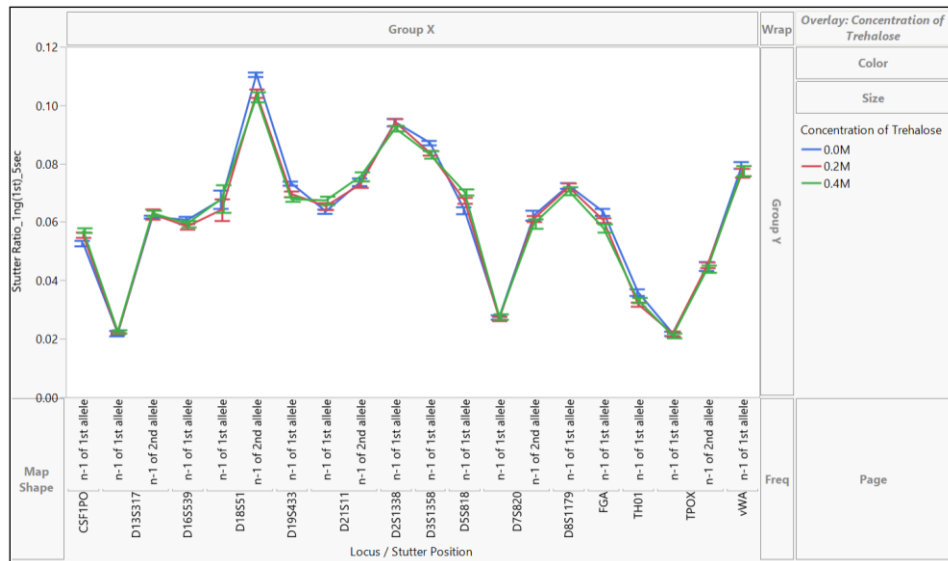
alleles were chosen for analysis. A total of 20 reverse stutter ratios was evaluated for statistical analysis. The stutter ratio analysis was not performed on 0.025ng and 0.05ng of DNA since the reverse stutter artifacts were hard to distinguish from the baseline noise. As with average peak heights and average peak height ratios, a statistical analysis was performed on two different injection times (5 seconds and 10 seconds) for 0.1ng of DNA, and that of 5 seconds injection time was omitted for the same reason. Also, 0.1ng of DNA with 10 seconds injection time for both the first and the second set of experiments were performed. The figures and tables for the statistical analysis and two independent samples t-test are not shown as it was determined that the data was not meaningful to demonstrate the difference in average reverse stutter ratios for each conditions.

#### *3.4.1 Evaluating Line Graphs of Average Reverse Stutter Ratios for Each Conditions*

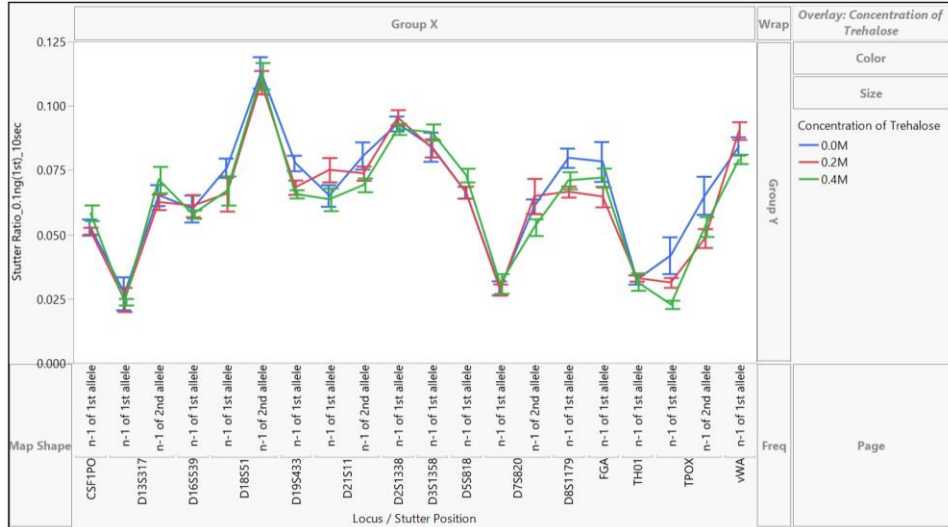
Line graphs of average reverse stutter ratios for 20 alleles throughout 15 STR loci for 0.1ng (Exp. 1 and 2) and 1ng (Exp. 1) of DNA were generated by JMP Pro 12 (Fig. 8). The line graphs for each condition were compared within (with line graphs of each concentration of trehalose per quantity of DNA) and with other line graphs of other quantities of DNA to evaluate the variations of the line graphs and standard errors. For each line graph representing each quantity of DNA, 3 different concentrations are shown (0M in blue, 0.2M in red, and 0.4M in green). The results of three line graphs show a similar pattern of average stutter ratios throughout all of the loci. The line graph representing 1ng of DNA show that all three concentrations of trehalose yielded very similar patterns of average reverse stutter ratios with relatively minimal variations as

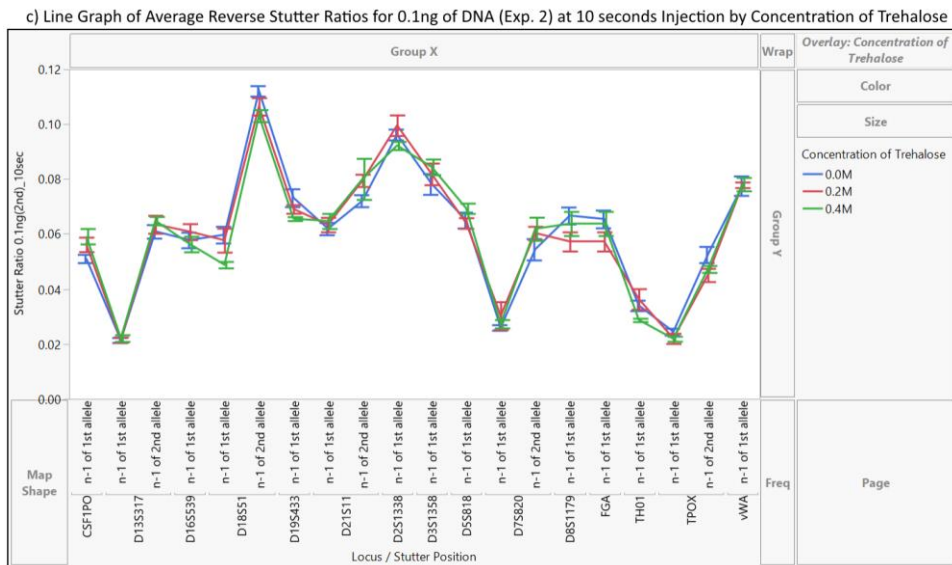
compared to those of 0.1ng of DNA (Exp. 1) and 0.1ng of DNA (Exp. 2). Also, the standard errors for each average reverse stutter ratios from the line graph of 1ng of DNA show relatively smaller and consistent standard errors as compared to those of 0.1ng of DNA from the 1<sup>st</sup> and 2<sup>nd</sup> experiments. Between the line graphs of 0.1ng of DNA for the 1<sup>st</sup> and 2<sup>nd</sup> experiments, that of the 2<sup>nd</sup> experiment shows relatively smaller variations and standard errors between each line representing different concentration of trehalose. However, it is not possible to visually determine which concentration of trehalose within the experiment is relatively lower or higher in average reverse stutter ratios across 15 STR loci than the other two concentrations.

a) Line Graph of Average Reverse Stutter Ratios for 1ng of DNA (Exp. 1) at 5 seconds Injection by Concentration of Trehalose



b) Line Graph of Average Reverse Stutter Ratios for 0.1ng of DNA (Exp. 1) at 10 seconds Injection by Concentration of Trehalose

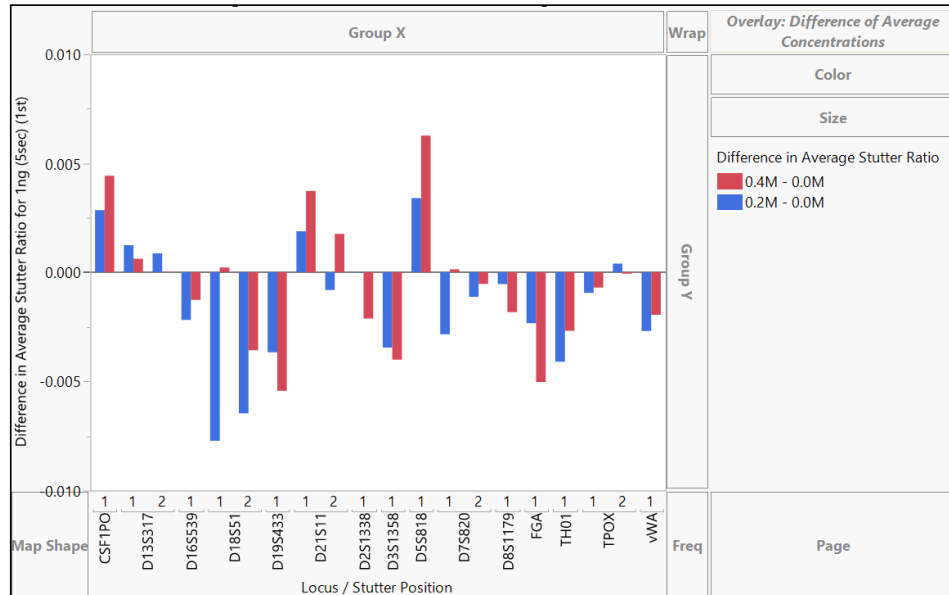




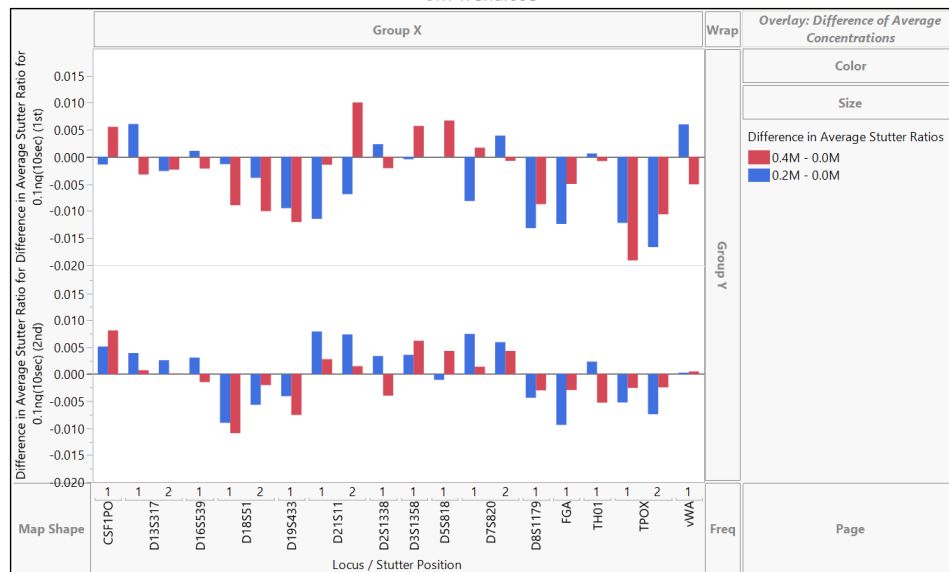
**Figure 8. Line graphs of average reverse stutter ratios for (a) 1ng (1<sup>st</sup> experiment; 5 seconds injection), (b) 0.1ng (1<sup>st</sup> experiment; 10 seconds injection), (c) 0.1ng (2<sup>nd</sup> experiment; 10 seconds injection) with 0M (blue), 0.2M (red), and 0.4M (green) of trehalose generated from JMP Pro 12**

Bar graphs (Fig. 9) showing differences in average reverse stutter ratios across 15 STR loci between those of 0.4M and 0M trehalose (control), as well as those of 0.2M and the control, were generated with JMP Pro 12. A total of 20 average reverse stutter ratios throughout 15 STR loci were evaluated.

a) Difference in Average Stutter Ratios of 1.0ng DNA for 0.4M and 0.2M Trehalose as Compared to Those of 0M Trehalose



b) Difference in Average Stutter Ratios of 0.1ng DNA for 0.4M and 0.2M Trehalose as Compared to Those of 0M Trehalose



**Figure 9. Difference in average reverse stutter ratios for 0.4M and 0.2M of trehalose as compared to those for 0M trehalose (control) for (a) 1ng of DNA, and (b) 0.1ng of DNA from first (top) and second (bottom) experiments.**

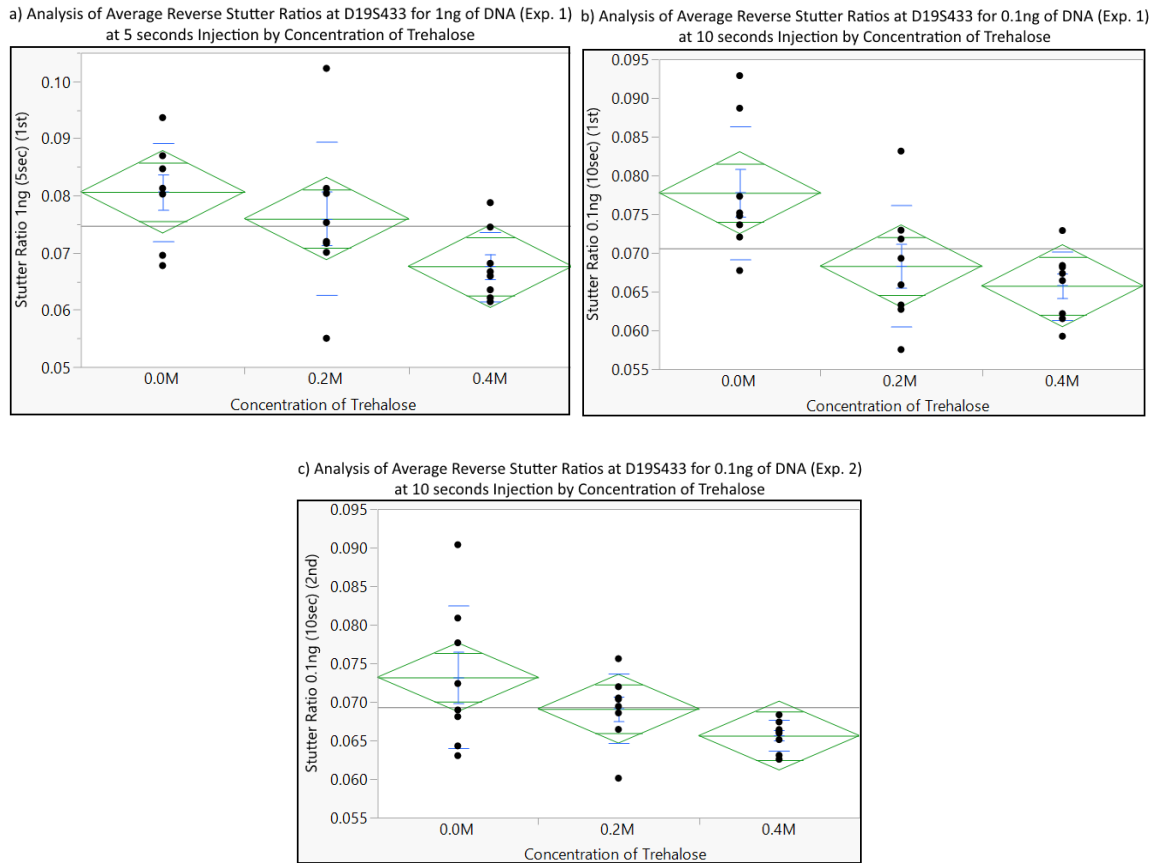
Similarity in unique pattern of average reverse stutter ratios throughout 15 loci between each line graphs (Fig. 8) suggests that stutter ratios depend on number of alleles,

locus, whether there is an interruption in core sequence<sup>33 34</sup>, size of the repeating units of alleles<sup>35</sup>, AT contents<sup>16</sup>, and other factors. Increasing variations between average reverse stutter ratios for different trehalose concentrations and standard errors from 1ng to 0.1ng of DNA support the fact that amplification of LT DNA can lead to stochastic variation in stutter ratios<sup>33</sup>. This suggests that smaller stochastic variations between average reverse stutter ratios for different trehalose concentrations for 0.1ng of DNA (Exp. 2) at 10 seconds injection time has comparably larger target amount of DNA than that of 0.1ng of DNA (Exp. 1), as supported by the comparison of the average peak heights for both of the experiments. There was no evident observations regarding the effects of 0.4M, and 0.2M of trehalose on average reverse stutter ratios on 20 alleles for 0.1ng (1<sup>st</sup> and 2<sup>nd</sup> experiments) and 1ng of DNA. This is suggested by the close patterns, small variations, and standard errors within the line graphs for 1ng of DNA with three different concentrations of trehalose as well as the fact that variations within each line graph from 0.1ng of DNA (1<sup>st</sup> and 2<sup>nd</sup> experiments) could have been due to relatively larger stochastic variation in reverse stutter ratios. Therefore, there was no evidence whether average reverse stutter ratios yielded by 0.4M of trehalose was relatively lower for 0.1ng (1<sup>st</sup> and 2<sup>nd</sup> experiments) and 1ng of DNA. The results from the line graphs are supported by the statistical analysis comparing average reverse stutter ratios for each condition, and bar graphs (Fig. 9) showing difference in average reverse stutter ratios across 15 STR loci for 1ng at 5 seconds injection and 0.1ng at 10 seconds injection (1<sup>st</sup> and 2<sup>nd</sup> experiments). The result from the statistical analysis (data not shown) suggests that differences in average reverse stutter ratios between each concentration per quantity of DNA are likely

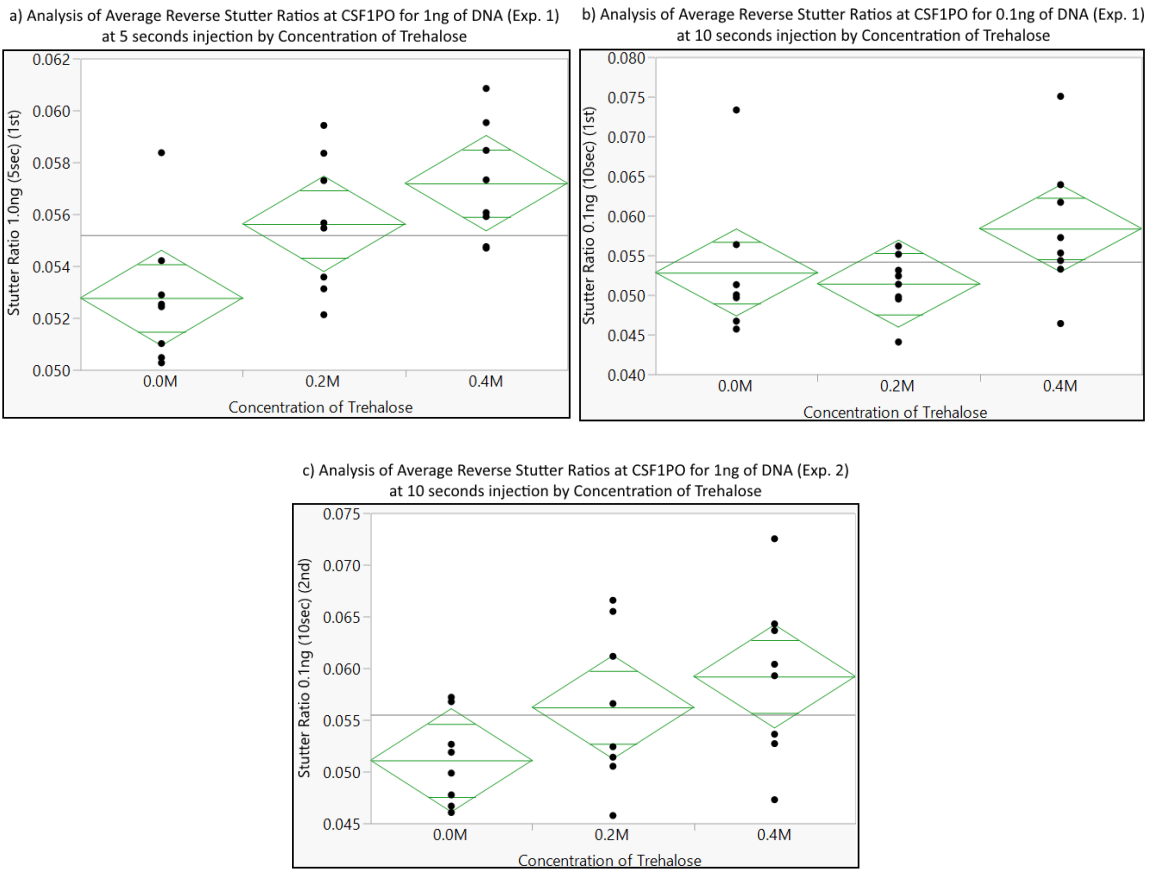
to be seen by chance. This statistical result supports the result from the line graphs as irreproducible patterns for different concentrations of trehalose from the line graphs were observed. Also, the result from the bar graphs supports that of the line graphs as both results suggest that it is not clear whether there is a pattern as to whether 0.4M of trehalose or 0.2M of trehalose produces a consistent difference in average stutter ratios across all 15 STR loci. These results indicate that there is no evidence that 0.4M and 0.2M of trehalose could have reproducible effects on average reverse stutter ratios for 0.1ng and 1ng of DNA.

#### *3.4.2 Evaluating the Effect of Varying Concentrations of Trehalose on Reverse Stutter Ratios of Individual Loci*

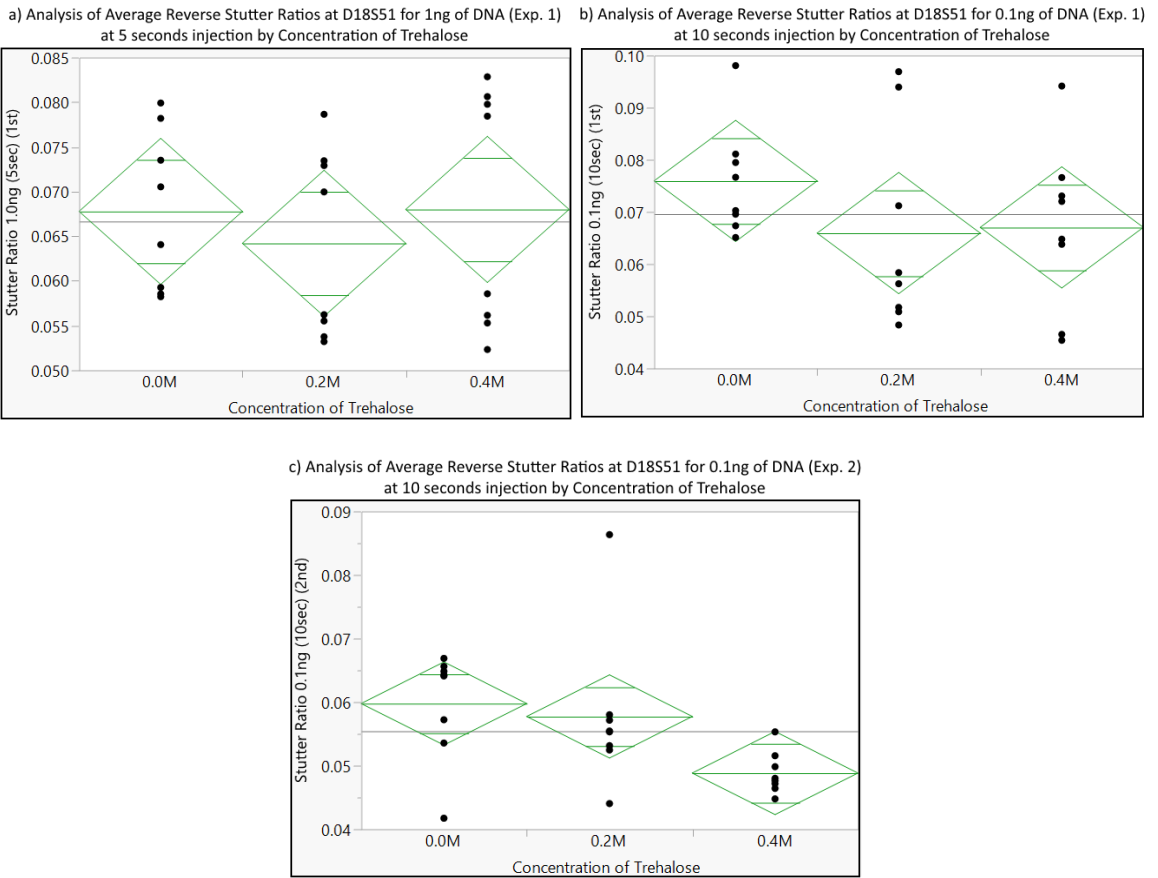
Further statistical analysis of individual locus was conducted in order to evaluate the possibility of effects of 0.2M and 0.4M of trehalose on reverse stutter ratios for each locus for 0.1 and 1ng of DNA. A statistical analysis was performed on each loci, and the two loci, D19S433 and CSF1PO, that show notable results of decreasing and increasing average stutter ratios, respectively, with the addition of trehalose are shown (Fig. 10, and 11). In addition, D18S51 that show no significant difference in average stutter ratios for each conditions is shown (Fig. 12). Two independent samples t-test (Table 6) was also performed on average stutter ratios for each conditions for D19S433, CSF1PO, and D18S51.



**Figure 10. Statistical analysis evaluating the effect of 0M (control), 0.2M, and 0.4M of trehalose on average reverse stutter ratios for (a) 1.0ng (1<sup>st</sup> experiment; 5 seconds injection), (b) 0.1ng (1<sup>st</sup> experiment; 10 seconds injection), and (c) 0.1ng (2<sup>nd</sup> experiment; 10 seconds injection) of DNA for D19S433 amplified with Identifiler® Plus**



**Figure 11. Statistical analysis evaluating the effect of 0M (control), 0.2M, and 0.4M of trehalose on average reverse stutter ratios for (a) 1.0ng (1<sup>st</sup> experiment; 5 seconds injection), (b) 0.1ng (1<sup>st</sup> experiment; 10 seconds injection), and (c) 0.1ng (2<sup>nd</sup> experiment; 10 seconds injection) of DNA for CSF1PO amplified with Identifiler® Plus**



**Figure 12. Statistical analysis evaluating the effect of 0M (control), 0.2M, and 0.4M of trehalose on average reverse stutter ratios for (a) 1.0ng (1<sup>st</sup> experiment; 5 seconds injection), (b) 0.1ng (1<sup>st</sup> experiment; 10 seconds injection), and (c) 0.1ng (2<sup>nd</sup> experiment; 10 seconds injection) of DNA for D18S51 amplified with Identifiler® Plus**

**Table 6. Summary of Two Independent Samples t-test Comparing the Average Reverse Stutter Ratios at Each Concentration of Trehalose Per Quantity of DNA (0.1ng and 1ng) at D19S433, CSF1PO, and D18S51 Amplified with Identifiler® Plus and Generated by JMP 12 Pro**

Experiment	Quantity of DNA	Injection Time	Locus	Allele	# of Data Points	Ordered Differences	p-Value
1	1 ng	5 sec.	D19S433			0.4M and 0M	0.0148
						0.2M and 0M	0.3511
			CSF1PO			0.4M and 0M	0.0262
						0.2M and 0M	0.1452
			D18S51			0.4M and 0M	0.9689
						0.2M and 0M	0.5274
	0.1 ng	10 sec.	D19S433			0.4M and 0M	0.0031
						0.2M and 0M	0.0157
			CSF1PO			0.4M and 0M	0.1509
						0.2M and 0M	0.7111
			D18S51			0.4M and 0M	0.2724
						0.2M and 0M	0.2194
2	0.1 ng	10 sec.	D19S433	0.4M and 0M	0.0208		
				0.2M and 0M	0.1918		
			CSF1PO	0.4M and 0M	0.0019		
				0.2M and 0M	0.0329		
			D18S51	0.4M and 0M	0.0228		
				0.2M and 0M	0.0586		

The result from the statistical analysis and two independent samples t-test that evaluate the effects of 0M (control), 0.2M, and 0.4M of trehalose on average reverse stutter ratios for each locus show varying responses. The result of one-way ANOVA of a locus, D19S433, shows a trend of decreasing average stutter ratios with increasing concentration of trehalose (Fig. 10). This observation of the trend is consistent throughout 0.1ng (Exp. 1), 0.1ng (Exp. 2), and 1ng (Exp. 1). Results showing similar trends were also observed in D8S1179 and TPOX.

On the other hand, CSF1PO shows an opposite trend of increasing average stutter ratios with increasing concentration of trehalose, which is consistent throughout each conditions (Fig. 11). A similar trend was also observed in D5S818, but with less statistical significance.

Most of the individual loci showed no or weakly inconsistent trends. An example of a locus, D18S51, shows no trends for each conditions (Fig. 12). The observation of specific trends for each locus that were consistent throughout each conditions prompted us to evaluate the repeating sequences and types of sequences for each locus (data not shown). The reason was that much previous articles regarding the effect of trehalose on the PCR have shown that the addition of trehalose in a PCR samples had a more effective amplification, especially for GC-rich templates and larger size of DNA<sup>4 7 25</sup>. Therefore, evaluating the GC contents of repeating sequences and types of sequences could be correlated with unique trends for certain individual loci. However, no evident correlations could be found (data not shown) as the GC percentages for loci with repeating sequences showing trends of decreasing average stutter ratios varied from 25% to 58.3%, and types of sequences varied from simple to compound repeats. Observation of specific trends in average stutter ratios for each conditions at certain individual loci can suggest two possibilities. First, the observed effect of trehalose on stutter ratios for certain loci may be irreproducible, or may have occurred by chance due to small sample size. Second, the observed effect may be real, but we lack the in-depth knowledge regarding the mechanisms for stutters and trehalose. This uncertainty could be compensated by future research through elucidating deeper mechanisms regarding stutters and trehalose, evaluating the effect of wider variations of concentrations of trehalose on each locus with larger data sets, and also performing the trehalose experiment with various DNA profiles from multiple donors to observe varying responses on different alleles of the same locus.

#### 4. Conclusions

In conclusion, the effect of 0.2M and 0.4M of trehalose on average peak heights, peak height ratios for 0.025ng, 0.05ng, 0.1ng, and 1ng of DNA and average reverse stutter ratios for 0.1ng and 1ng of DNA from 15 STR loci of the Identifiler® Plus PCR Amplification Kit was evaluated. For the evaluation of average peak heights, the result showed that there were no consistent trend across the quantities of DNA, suggesting that difference in average peak heights for each concentration of trehalose at 0.1ng and 1ng of DNA are likely to be the result of difference in actual quantities of DNA. This error may have resulted from the propagation of pipetting error since each batch for each conditions was prepared separately, and 8 replicates were aliquoted from thereon. The unexpected discrepancies between the first and the second experiments of 0.1ng of DNA further suggest that the propagation of pipetting error from two different quantities of DNA may have resulted in drastic difference in the actual quantities of DNA between the first and the second experiments. Therefore, no conclusions could be made regarding the effect of trehalose on the average peak heights. With regards to overall average peak height ratios for 0.025ng, 0.05ng, 0.1ng, and 1ng of DNA and average reverse stutter ratios for 0.1ng and 1ng of DNA, there was not enough evidence to suggest that either concentration of trehalose had an effect on these parameters. One-way ANOVA evaluating varying effects of trehalose on average reverse stutter ratios for each conditions at individual loci suggested that trehalose may have varying effects on reverse stutters of certain loci. However, it is unclear as to whether the unique trends are coincidental due to small sample sizes, or real due to unknown factors.

In a practical sense, the result of the experiment has suggested that there is not enough evidence that trehalose is useful for forensic application and as a means to improve stochastic effects and generating better quality DNA profiles. The reason is that there is no evidence of trehalose affecting peak heights, peak height ratios, and reverse stutter ratios, especially for LT DNA, where stochastic effects are more prominent. Also, there is no evidence of trehalose affecting the reverse stutter ratios consistently across all of the loci. Further research could be done by employing a more precise method for reducing variations in actual amount of DNA for each conditions, incorporating other molecules of interest (i.e. 1,2-propanediol, betaine, heparin, etc.), using larger range of concentration of trehalose, and larger data set to evaluate the effect of trehalose on stochastic effects for the analysis of DNA profile.

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# Curriculum Vitae

## Gyeol Yoon

(Born 1989)

#B3 901 Beacon Street, Boston MA 02215

Cell: 570-452-1579

yoony8917@bu.edu

### Professional Overview

- Able to think in different perspectives, be flexible, and adjust to different environments and situations through acquiring bicultural properties
- Likes to take challenge, highly dedicated and disciplined, developed through military experience
- Proficient in organizing and planning ahead of time
- Good at analyzing and paying attention to small details
- Laboratory skills developed during undergraduate and graduate research and courses

### Education

- **Master of Sciences: Biomedical Forensic Sciences**, September 2014 to present (anticipated graduation date: August, 2016)  
**Boston University School of Medicine** - Boston, MA, U.S.  
**Thesis Title:** Evaluation of the Effects of Trehalose on the Amplification of the 15 Short Tandem Repeats Loci of the AmpF $\ell$ STR $\text{\textcircled{R}}$  Identifiler $\text{\textcircled{R}}$  Plus PCR Amplification Kit (Thesis advisor: Dr. Robin Cotton, Ph.D)
- **Bachelor of Science : Biochemistry**, 2014  
**Bucknell University** - Lewisburg, PA, U.S.

### Relevant Experiences

- **Assistant Undergraduate Researcher**  
May 2013 to April 2014  
**Bucknell University Chemistry Department** - Lewisburg, PA
  - Project involved inducing a production of an electron transporting protein, flavodoxin, from the bacteria, *Heliobacterium Modesticaldum*, and purifying a protein, flavodoxin from the bacteria.
  - Job entailed growing bacterium in a media in an anoxic chamber, and using techniques such as sonication, ultracentrifugation, Ultra Violet Spectroscopy, and ion exchange chromatography in order to isolate particular components from the bacteria.

- **Chemical Scout**

October 2010 to August 2012

**The Armed Force CBR Defense Command, Korea Chemical Special Force** - Seoul, S. Korea

- Job entailed performing various field works, such as conducting reconnaissance, identification, sampling, and maintaining chain of custody of chemical warfare agents on site for counterterrorism operation and for defense against North Korea's attack.
- Trained to use equipment such as Mass Mobilizer 1, Korea Chemical Agent Monitor-2, and Automatic Portable Detector 2000, which are similar to Gas Chromatography/Mass Spectrometry, and Time of Flight.

- **Lab Assistant (Intern)**

June 2009 to August 2009

**Kyunghee University** - Suwon, S. Korea

- The research project involved extracting and modifying a biological component, Saponin, from red ginseng.
- Job entailed using techniques such as pipetting, centrifugation, and SDS-PAGE.

**Relevant Course Works**

**Undergraduate School Course Works**

- Cell Biology
- Molecular Biology
- Biochemistry
- Genetics
- Organic/Inorganic Chemistry
- Analytical Chemistry
- Immunology
- Virology
- Biochemical Methods

**Graduate School Course Works**

- Crime Scene Investigation
- Pattern Analysis
- Criminal Law I
- Criminal Law II – Mock Court
- Forensic DNA Analysis
- Forensic Biology
- Forensic Chemistry
- Forensic Pathology
- Forensic Toxicology
- Forensic DNA Laboratory
- Forensic Instrumental Analysis
- Forensic Biology Laboratory
- Advanced DNA Analysis
- Trace Evidence Analysis
- Elementary Biostatistics

## **Skills Summary**

- Ultra Violet Visible Light Spectroscopy
- Quantitative Polymerase Chain Reaction (qPCR)
- Polymerase Chain Reaction (PCR)
- Protein and DNA Extraction (sonication, enzyme extraction, solid-phase extraction)
- Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
- Enzyme Linked Immunosorbent Assay (ELISA)
- Forensic DNA analysis (GeneMapper®)
- Western Blotting
- Southern Blotting
- Capillary Electrophoresis (CE)
- Ion Exchange Chromatography
- Liquid Chromatography
- Gas Chromatography
- Mass Spectrometry

## **Languages**

- Knowledge of Korean, English, and Japanese; most fluent in Korean and English.

## **Address**

- 901 Beacon Street, B3, Boston, MA, 02215