Validity and Reliability Considerations for Undergraduate Engineering Education Research Studies Involving Salivary Biomarkers

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***Abstract*—This research-to-practice full paper explores the influence of video-based and paper-based training materials on undergraduate engineering students' development of skills and competencies in biomedical laboratory techniques, from a multimodal perspective. With recent advances in physiological and biological detection methods, scholars increasingly rely on laboratory sensors and techniques to investigate potential connections between academic physiological and psychological stress and performance. For this work, we explored the biological measure of salivary hormonal biomarkers (e.g., alpha-amylase) and their relationships to performance. For this work, we conducted an exploratory study on 7 undergraduate engineering students participating in a summer research experience. These students were provided with pre- and post-quizzes before and after their initial training in laboratory cell culture preservation techniques. During the quiz-taking experience, students responded to quizzes developed by their research advisor, while saliva samples were collected at three points: before, during, and after the quiz. To ensure the validity of the experiment, the researchers engaged in extensive discussions on overcoming the challenges of designing an experimental study that combines biological, physiological, and performance measures. This research-to-practice paper focuses on the considerations necessary for designing a quasi-experimental study that utilizes biometric tools to assess academic performance and stress management among undergraduate engineering students.**

Our findings suggest that both video-based and paper-based training materials significantly impact students' skill acquisition and competency development in biomedical laboratory techniques. We also observed that salivary hormonal biomarkers could be useful indicators of stress and performance during academic tasks. This research provides valuable insights into the interplay between training materials, stress, and performance, highlighting the importance of multimodal approaches in educational research. We offer recommendations for other researchers interested in conducting similar studies in the future. These include strategies for effectively integrating biometric tools into educational research, addressing potential challenges, and ensuring the reliability and validity of the collected data. By sharing these insights, we aim to contribute to the advancement of research methodologies in the field of engineering education.

Keywords—validity, reliability, salivary biomarkers, engineering education research, undergraduate

I. Introduction

Enhancing the undergraduate research training experience for engineering students is a subject of considerable discussion. Little is known about the impacts research training has on students' emotive and cognitive states in near-real-time. One of the challenges to designing such quantitative experimental designs is the issue of validity.

Among the main three types of validity are the following: (1) internal, (2) external and (3) ecological [1] (Table 1). It is important to distinguish between these terms when planning a study's research design to ensure the study's results are trustworthy. Internal validity assesses the research study's design, conduct, and analysis to ensure that the study findings produce credible results that contain minimal systematic bias [1]. External validity examines if a research study can be applied to other contexts. Ecological validity examines if the study findings can be generalized from a research to a practice setting [1]. Furthermore, ecological validity questions if the study findings can be applied to participants with different characteristics compared to those in the are part of a study for a longer period of time [1]. All three validations are based on the judgement of the research team and cannot be calculated through a statistic [1].

TABLE I. Validity Types

Source	*Definition*	*Elements to consider*
Internal	Examines whether the manner in which a study was designed, conducted, and analyzed allows trustworthy answers to the research questions in the study	Improper randomization, missing data, and pipetting technique
External	Examines whether the findings of a study can be generalized to other contexts	Short term studies, limited number of participants, and medical conditions that could affect salivary samples
Ecological	Examines whether the results of a study can be generalized in real-life settings	Applicability to students in different disciplines

This research to practice paper will discuss validity and reliability considerations to design a quasi-experimental study to study undergraduate students via engineering education research

using uses salivary biomarkers to study the associations between academic stress and performance.

II. Background

In recent years, the use of salivary alpha-amylase (sAA) as a biomarker, specifically psychoneuroendocrinological, to track stress-related changes in the sympathetic nervous system (SNS), has become more prevalent [2]. During moments of psychological stress, sAA (a-1,4-a-D-glucan 4-glucanohydrolase; enzyme commission number (EC) 3.2.1.1) is released from the parotid and submandibular salivary glands upon activation of the autonomic nervous system (ANS); thereby, signifying elevated catecholamine activity of the sympathetic nervous system [2][3]. Although both salivary sAA and cortisol act independent of each other, physiological and psychological stress can release both along the hypothalamic-pituitary-adrenal (HPA) axis yielding in adrenal cortex stimulation [3]. After the HPA is activated, cortisol is released into the bloodstream with the highest levels in saliva being detected 25-30 minutes post-HPA activation with the lag time between cortisol levels in plasma and saliva being 1 to 2 minutes [4].

In situations of prolonged stress, such as an exam, after the initial increase of salivary cortisol levels 20-30 minutes after exposure to a stressor, the HPA becomes overstimulated and desensitized causing the amount of cortisol secreting to decrease [4]. It is important to note that the concentration of cortisol is dependent on sex, age, and population characteristics [4]. In people who are generally healthy, the average level of salivary cortisol in the morning is 459.6 ± 235.2 nmol/l and in the afternoon, it is 340.5 ± 207.5 nmol/l [5]. Additionally, normal levels of salivary alpha-amylase can range from 40-140 U/L with the geometric mean being 89.14 U/ml at 0900 h and peak levels being 156.87 U/ml at 1625 h [6] [7]. The levels salivary alpha-amylase fluctuate throughout the day's course [7].

From the *psychological* perspective, the term "stress" has two referents: (1) the characteristic of a stimulus, which causes stress (e.g. exams can be described as stressful experiences) and (2) the subjective experience of feeling stress as well as anxiety or worry (e.g., students who experience test anxiety) [8]. Current research does not distinguish if: (1) the target subject of analysis is "academic" or "examination" stress; (2) make a clear distinction between the terms "academic" or "examination", "stress", "anxiety" or "worry"; and (3) if stress is the cause or an effect of the experience [8]. It is known, however, that unattended, sustained levels of stress stemming from academic and examination settings impact students' emotional well-being, health, and performance [8].

Within the context of engineering, the phenomena of both academic and examination stress can be connected to the difficulties that engineering students' when applying learned content throughout complex, ill-structured engineering problems [9]. Multiple academic pressure/support variables have been identified and their correlation with salivary cortisol concentration in undergraduate students has been investigated [10]. These variables include: (1) plans to attend graduate school, (2) pressure from friends and/ or relatives to succeed, (3) beliefs about course examinations not impacting their professional futures, (4) studies and work in groups, (5) the difficulty and challenging nature of their discipline of study, (6) the tendency to be an "overachiever", and (7) the number of hours per week spent studying [10]. From these studies, it can be concluded that: (1) students who reported they spent more hours studying had lower concentrations of salivary cortisol levels and (2) students who reported increased levels of acute perceived stress believe that examinations would have an impact on their future [10].

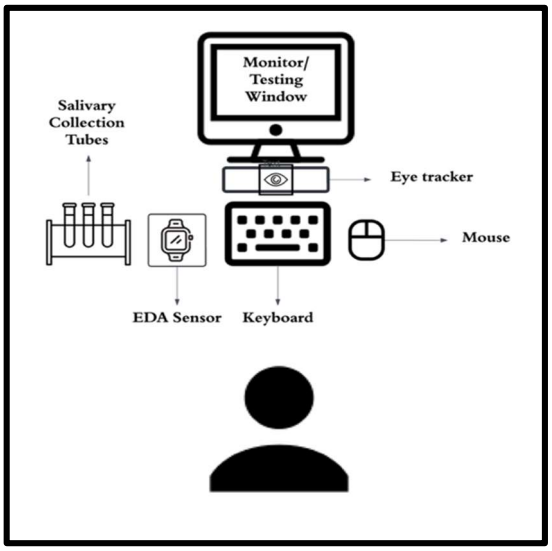
Furthermore, the stress culture of engineering adds to the mental health crisis prevalent in university campuses around the United States [11]. A recent exploratory study conducted at the University of Illinois in 2020 revealed that out of 30 undergraduate engineering students, almost all students indicated that their stress was constant along with reporting hair loss, a physical symptom of stress [11]. It was also found that undergraduate engineering students are undereducated when it comes to mental health with many of them partaking in unhealthy coping mechanisms along with consistently mislabeling stress and anxiety [11]. Although engineering is classified as a "high stress" undergraduate major, assessing how students biologically react to partaking of stressful academic tasks such as an exam, can inform researchers and educators on how to improve the teaching and learning of engineering education for improved student well-being and performance [11].

III. Methods (Challenges)

A. Performance Measure Set-Up

Ensuring an adequate performance measure set-up contributed to the validation of this research study by providing both the researchers and participants with an intuitive researching and testing environment. The research study took place in a laboratory setting rather than a traditional engineering assessment room due to the multiple sensory technologies (e.g. EDA, eye-tracker, and saliva) being utilized to measure participants' physiological reactions [12]. Students were segmented throughout two performance measurement sessions and seated with one empty station in between to give them more space.

FIGURE I. Performance Measurement Set-Up



From Figure 1, the design of the performance measure set-up can be observed to be composed of: (1) a monitor/ testing window, (2) salivary collection tubes, (3) an EDA sensor, (4) an eye-tracker, and (5) a keyboard and mouse set.

The performance measurement set-up was constructed in such a way that participants have ample space to: (1) engage in the examination experience in a comfortable manner; (2) set-up and use all the technologies provided; and (3) provide salivary samples. It is important to note that the way the performance measure lab setup, as illustrated in Figure 1, favors people who are right-hand dominant. However, the set up can be easily reconfigured to accommodate a person who is left-hand dominant by switching the placement of the mouse, EDA sensor, and salivary collection tubes. The EDA sensor is utilized to identify changes in emotional or cognitive states through sympathetic, physiological arousal [13]. It was used in combination with salivary biomarkers (e.g. alpha-amylase and cortisol) to analyze ANS metabolic outputs (e.g. sweaty palms) which can be an indicator of stress [13]. In addition to that, eye-trackers were utilized to assess attentional processes affected by stress and to identify points of stress within the assessment itself, potentially informing better assessment design to reduce engineering student stress [14]. Disposal of the salivary collection methods was also considered by providing participants with disposable tools that they could place inside small disposal bins found at their station after use. After the participant completed the research study, the researcher disposed of the bin via the biohazard waste bin as per University of Florida Environmental Health & Safety (EH&S) guidelines.

Furthermore, the ecological validity of the set-up was ensured by providing students with any testing materials they need for the duration of the assessment [15]. This included scratch paper, formula sheets, and writing materials [16]. Providing these testing materials allowed students to assist in designing a near real-time authentic examination experience where students could work out problems as needed [15].

B. Financial

The selection of salivary alpha-amylase immunoassay kits by a supplier (e.g., Tecan, Salimetrics) is a complex process that requires a thorough cost analysis. Alpha-amylase immunoassay kits are upwards of $210 USD, as quoted by a Tecan representative, making it a financial challenge to perform a study with a large sample size [17]. These 96-well plates often come equipped with lyophilized stock standards, controls, a sample buffer, and a substrate solution.

The immunoassay manufacturer Tecan (used for our study) provided five standards and two controls which calculates to 21 wells being used as a reference measure for calibration. Since each saliva sample needs to be completed in triplicates, a total of 25 saliva samples could be analyzed per kit. However, the quantities of the materials supplied were often not enough to run the analysis on a full 96-well plate. The quantity of the supplies included in the immunoassay kit does not account for human errors such as a pipetting error, dropping a bottle, or wanting to rerun an experiment.

Additionally, immunoassay manufacturers typically do not sell stop, start, or stock solutions separately requiring the purchase only full kits. When taking into considering the purchase of an extra kit to ensure enough supplies, the batch numbers also need to match to minimize quality control errors. This can add an unneeded layer of additional sample validation. The exact quantities of supplies provided in the immunoassay kits could be attributed to the pipetting system utilized by the manufacture to calculate the quantity of supplies needed to perform a full 96-well immunoassay. The pipetting system is most likely different than the one utilized in our laboratory; thereby, having a different rate of absorption than our pipettes.

Insufficient quantity of supplies can later cause a dilemma for the researcher while performing the alpha-amylase test procedure. The researchers are tasked to ask themselves if they should risk the reliability and validity of the samples because there was not enough material supplied by the manufacture to properly run the experiment or if they should open another salivary immunoassay kit to complete the experiment. The choice may require additional considerations such as making sure the batch numbers are the same, that the calibration curve is within the same slope and trend as the previous kit and properly adjusting concentration calculations by kit and tracking used in each kit. The researcher may also want to repeat a few samples by kits to ensure that there are no statistically significant differences between kits and calibration curve and sample concentrations.

Analyzing the results of the alpha-amylase immunoassays in-house due to financial constraints can pose an additional layer of additional challenge as both purchasing an absorbance reader versus sending the saliva samples for analysis via a third-party vendor can entail additional ethical considerations such making sure salivary samples are properly disposed of per EH&S guidelines. It is important to discuss these processes with a vendor before sending salivary samples to them for analysis. [18]. For our study, participant saliva samples were properly inactivated by adding 1 part concentrated (8.25%) bleach to 13 parts liquid and mixing well, letting the solution sit for 30 minutes, and pouring the solution down the drain under running water per our institutional EH&S guidelines [18]. After that, it was packaged for biomedical waste disposal pick-up by placing it inside a red bag lined biomedical waste fiberboard box [18].

C. Experiment and Saliva Protocol

Creating an ecological balance for the collection of salivary alpha-amylase and cortisol proved to be a challenge due to their stability. Although both salivary biomarkers have proven to be reliable for the analysis of biobehavioral research, their stability is dependent on their half-life [19]. Salivary alpha-amylase levels follows a diurnal rhythm with a noticeable decrease within 60 minutes after humans awake and a steady increase throughout the rest of the day [7]. During periods of physical and psychological stress such as exercise or written examinations, alpha-amylase rises but begins to return to baseline levels after 5 minutes and is at baseline level after 10 minutes [20]. Salivary cortisol, on the other hand, has a longer half-life beginning to decrease after 20 minutes post physical or psychological stress and returning to pre-stressor levels 30-40 minutes post-stress [21]. Taking the half-lives both alpha-amylase and cortisol into consideration, the researchers needed to ensure that the timing of the salivary sample collection

This material is based upon work supported in part by the National Science Foundation (NSF) No. EED-1661100/1661177. See acknowledgment.

aligned with the research design of the study to maintain its ecological validity. Therefore, since the duration of the assessment was 1.5 hours, salivary samples were collected 10-minutes prior to the start of the assessment, upon completion of the second question, upon completion of the fourth question, and 10-minutes post-study completion.

Incorporating both salivary cortisol, a hormone, and salivary alpha-amylase, an enzyme, into the same research study required for researchers to think about the stability of both and select an analytical technique to evaluate them. There are three methods to analytically quantify salivary samples: (1) separation methods, (2) electrochemical methods, and (3) immunological methods [19]. By keeping in mind, the primary objective of the research study which was to analyze the assessment experience and performance of undergraduate engineering students, immunological methods were selected as we wanted to identify and quantify enzymes and hormones utilizing enzyme-linked immunosorbent assays (ELISA) [19].

D. Saliva Collection Method

Simulating a true undergraduate performance measure experience while simultaneously conducting a research study proved to be a challenge because of the participant saliva sampling and collection methods. The research study took place in a laboratory setting that was not reflective of a traditional classroom setting (impacting ecological validity); therefore, this could cause participants to feel uncomfortable. Additionally, there were multiple sensory technologies (e.g. eye trackers and EDA sensors) assessing them alongside the periodic salivary samples they had to provide throughout the performance measure experience. Therefore, multiple factors regarding the saliva collection methods had to be considered. These factors include: (1) saliva flow rate, (2) time consumption, (3) cost, (4) sample volume, (5) invasiveness, (6) area of mouth to be analyzed, and (7) capability of collecting biomarkers of interest [19].

In total, there are 3 areas of the mouth, saliva glands, that are suitable for saliva collection: (1) parotid, (2) submandibular and sublingual, and (3) minor glands [19]. The parotid gland is the largest of the three salivary glands and is in the retromandibular fossa which is below and in front of each ear [22]. The submandibular and sublingual salivary glands are located under the mandibula and under the tongue, respectively [22]. Salivary alpha-amylase is produced in the parotid and submandibular glands while cortisol is produced in the adrenal glands [2][3]. Each salivary gland contributes a different amount to the overall saliva production with the parotid gland producing 20%, the submandibular producing 65%, the sublingual producing 2-8%, and the minor glands producing 10% [19]. Therefore, it is important to know which salivary gland secretes the biomarker of interest. Furthermore, since salivary alpha-amylase is regulated by the sympathetic branch of the ANS in response to stress, compared to cortisol, which is produced through the HPA axis, reflecting both endocrine and autonomic responses, salivary alpha-amylase serves as a more direct marker of sympathetic nervous system activity during stress [2][4]. Knowing what the target salivary gland is assists in making a well-informed decision that is both beneficial for the research team because they will acquire quality samples and for the participants because they will a more accurate and less invasive salivary collection process will be used for collection.

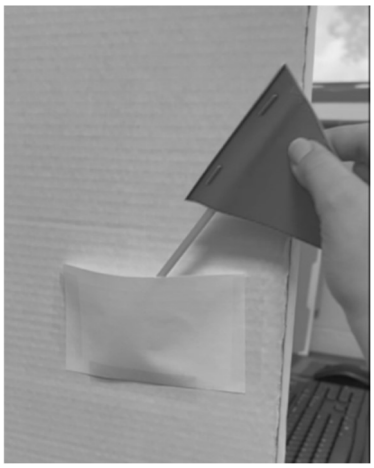
Prior to discussing salivary flow rates as it relates to saliva collection, the comparison of unstimulated versus stimulated salivary flow must first be made clear. Stimulated saliva collection occurs as a reaction to eating food; therefore, in a laboratory setting simulated saliva collection methods utilize masticatory and gustatory methods such as chewing paraffin wax or sour candy drops, respectively, to enable saliva secretion [19]. Unstimulated saliva collection does not utilize any external stimuli to enable saliva secretion [19]. The flow of saliva is under the control of the parasympathetic and sympathetic nervous system, which controls the quantity of saliva released and the composition to indicate the body's physical and psychological condition [19]. Therefore, it is important to take into consideration factors that can affect flow rate so that the research results are accurate and reflect the effect of the external stimulus presented to students during the examination [19]. Factors that affect unstimulated salivary flow rate include: (1) hydration, (2) exposure to light, (3) olfactory stimuli, and (4) body positioning during saliva collection time [19]. Comparatively, factors that affect stimulated salivary flow rates include: (1) mastication because it increases protein composition and salivary pH and (2) gustatory stimuli because they increase saliva secretion and flow rate [19]. A high salivary flow rate is ideal in research setting as it signifies faster collection times; thereby, reducing the amount of time a participant takes to provide a sample versus engaged in the study. Additionally, the specimen collection procedures for our respective alpha-amylase and cortisol kits instructed participants not to eat, drink, chew gum, or brush teeth for 30 minutes before sampling. Both the salivary alpha-amylase and cortisol protocols allowed for participants to enable salivary flow using paraffin. Thus, it will be important to document participants' daily habits. Furthermore, per our salivary alpha-amylase and cortisol ELISA kit instructions, a minimum of 0.5 mL of saliva should be collected per sample. Therefore, collection methods that are more likely to yield a higher or unaffected flow rate are ideal because sufficient saliva samples are required to conduct an analysis on the participants' samples.

Depending on the aim of the research study and the biomarker in question, there are a variety of different salivary collection methods available. These include: (1) passive drooling and draining, (2) spitting, (3) swabbing, (4) dried salivary substrates, and (5) chemical stimulation [19]. When taking cost into consideration during the planning of the research design, it is important to note that some methods require: (1) skilled personnel, (2) customized devices, and (3) complex procedures which can increase the cost of conducting a research study [19]. The passive drool and draining along with the spitting method do not require skilled personnel; therefore, they are the most cost-effective saliva collection methods apart from swabbing [19]. Furthermore, the passive drool and draining, spitting, and swabbing collection methods are noninvasive so they will minimally distract participants during the study which will allow them to fully engage in the study. These collection methods also do not require skilled personnel, which allows the participant an easy way to provide the saliva sample themselves; thereby, more closely reflecting an engineering examination

experience. Invasive techniques such as cannulation and Lashley cup are dangerous and can cause damage to salivary glands as these collection methods are complex and require skilled personnel [19].

E. Systematized Data Collection System

FIGURE II. SALIVA COLLECTION FLAG SYSTEM



The collection of participant salivary samples during the examination experience needed to be completed in a minimally disruptive manner to ensure the ecological balance of all sensory technology while the participant was taking the exam. A flagging system was created as a form of non-verbal communication between the participant and the researcher so as to signify that a salivary sample was ready to be retrieved from their station (see Figure 2). The flagging system was composed of a small red flag that the participant placed inside the pocket of the station divider when their salivary sample was ready to be collected. This system allowed for both the researcher and the participant to have a mutual, non-verbal understanding during the examination experience; thereby, causing minimal interference and maintaining a quite assessment environment that simulates a real one.

Throughout the duration of the 1.5-hour experiment, each participant provided a total of 4 salivary samples. The first sample was collected 10-minutes prior to the start of the assessment, the second was upon completion of the second assessment question, the third was upon the completion of the fourth assessment question, and the fourth was 10-minutes post-study completion. To avoid inflicting confusion upon the participant during the study and to the researcher during data analysis, all 4 salivary collection tubes were labeled with colored stickers to differentiate the points in time at which they were collected. This system ensured that participants provided all 4 samples as it was easy to take a quick inventory by looking at the samples collected. The salivary collection tubes themselves were also marked with blue tape to denote the quantity of saliva that needed to be provided by the participant. This also ensured that the required data collection quantity, 0.5 mL, was collected for proper data analysis.

Timing the collection of the salivary samples during the examination was also a consideration the researchers needed to account for. While participants must not be disturbed frequently during the examination, an ecological balance between the number of times a participant provides a sample and the onset/offset of the salivary biomarkers needs to be reached [16]. It has been shown that during undergraduate student examination experiences, cortisol levels decrease from the beginning to the mid-point of the examination experience but then increase after the mid-point as time progresses [23]. Therefore, traditionally, a pre-pre-mid-post-post salivary collection sequence is implemented to account for the time lags needed for salivary biomarkers to onset [16]. However, in this study, we collected salivary samples ten minutes prior to the study beginning, after the second question, after the fourth exam question, and ten minutes post-study because of the small window of time of 1.5 hours.

F. Reliability

Inter- and intra-assay coefficients of variability (CV) define the precision of the immunoassay results [24] While inter-assay CV describes the plate-to-plate consistency calculated from the average values of the high and low controls, the intra-assay CV describes the average value calculated for all the duplicated of an individual CV throughout multiple immunoassay plates [24]. Ideal intra-assay CV values are calculated to be less than 10%, while inter-assay CV values are acceptable if calculated to be less than 15% [24]. Intra-assay CVs of more than >10% describe poor pipetting techniques, especially when performing saliva which has a high viscosity [24]. Inter- and intra-assay CV values are considered to be a form of reliability as intra-assay CV indicates that the assay produces consistent results within a single run, whereas inter-assay CV values indicate that the produces consistent results across different runs to further demonstrate reliability [25]. Therefore, the results of the analytical data correlates to the criteria utilized to validate the methods [25]. The analytical methods are validated by assessing the fundamental parameters of an immunoassay which include: (1) accuracy, (2) precision, (3) selectivity, (4) reproducibility, and (5) stability [25]. When performing an immunoassay, proper pipetting techniques are critical for the reliability of the analysis results, especially when handling highly viscous samples such as saliva [26]. It is recommended to practice “reverse” pipetting instead of “forward” pipetting as it can improve accuracy and reduce pipetting errors [26]. To avoid inaccurate conclusions, the correct pipetting technique must be used as errors in pipetting can lead to variability in the sample volume, impacting the immunoassay’s sensitivity and specificity [26].

IV. DISCUSSION

This research to practice paper meticulously explored the use of salivary biomarkers, specifically, salivary alpha-amylase and cortisol, as indicators of physiological and psychological stress in undergraduate engineering students during an assessment. The integration of these biological with academic performance offers a wholistic approach to understanding how students react under stressful situations (e.g. engineering assessments). Although the research study was conducted in a laboratory setting, the performance set-up was strategically designed to simulate a traditional engineering assessments environment. Thereby, enhancing the ecological validity of the research study itself but also allowing for the accurate collection and

measurement of biometric data in a controlled setting. An important consideration of the performance measurement set-up was ensuring that each participant had sufficient space to comfortably utilize the necessary technologies without feeling overcrowded as this could influence stress levels and, consequently salivary biomarker results.

Furthermore, a significant challenge encountered was the cost associated with the immunoassay kits, priced at over $210 USD each. This could pose a limitation on the number of participants a study could afford to include, thus, affecting the ecological validity and generalizability of the findings. Additionally, the immunoassay kits provided a limited quantity of supplies which required precise management of resources to ensure that enough data could be collected for a reliable analysis. This included planning for potential errors or the need to rerun assays, which could further strain the budget.

Accurately timing the collection of salivary samples was vital due to the fluctuations in salivary biomarker levels in response to stress. Researchers designed a protocol requiring samples to be collected at specific times to collect salivary samples at ideal biomarker concentrations and ensure they aligned with the stressors introduced by the assessment. The precision of salivary sample collection and timing presented a logistical challenge when it came to synchronizing the participants' responses and the assessment administration.

The analysis of salivary biomarker samples required proper handling to prevent contamination and degradation. The limited quantities of supplies provided in the immunoassay kits added to the necessity of adhering to accurate pipetting practices to ensure the inter- and intra-assay coefficients of variability reflected a study with integrity and reliability of its results.

ACKNOWLEDGMENT

This material is based upon work supported in part by the National Science Foundation (NSF) No. EED-1661100/1661177. Any opinions, findings, and conclusions or recommendations expressed in this material do not necessarily reflect those of NSF. We thank Tecan Trading AG for assisting in the development of the salivary immunoassay protocols. We thank Dr. Jing Pan (University of Florida) for providing equipment support. We express our gratitude to our team, the TWISTER Lab (University of Florida) for their collaborative efforts in brainstorming the protocol and research design of this study.

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