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An analysis of the accuracy and function of three presumptive methods used in forensic science for the detection of urine

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**AN ANALYSIS OF THE ACCURACY AND FUNCTION OF THREE
PRESUMPTIVE METHODS USED IN FORENSIC SCIENCE FOR THE
DETECTION OF URINE**

by

NANCY VIEN DINH

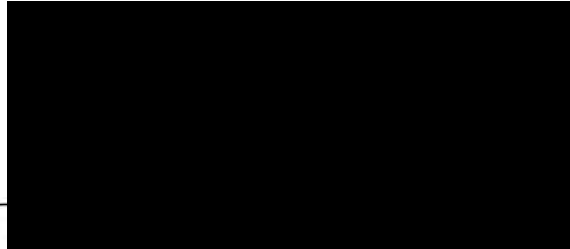
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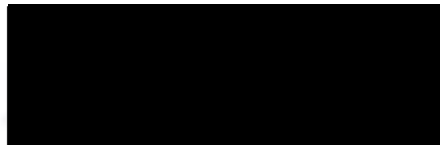
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NANCY VIEN DINH

Boston University School of Medicine, 2012

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Abstract

The presumptive detection of urine from evidence found at a crime scene can assist investigators in determining the events that occurred during the commission of the crime. The Jaffe test is a traditional method which relies on the detection of creatinine, a constituent of urine. In 2009, the Uritrace[®] test device was developed to detect the presence of creatinine in urine. In 2010, the RSID[™]-Urine immunochromatographic card was released as a method for detection of a reportedly more specific component of urine, Tamm-Horsfall protein. The significance of these various techniques lies in their capacity to accurately detect the respective urinary constituents to allow for a presumptive determination of urine.

The objective of this study is to compare the three presumptive tests to determine how effectively and accurately each method could be used to detect

their respective target molecules in urine. Areas of research interest include the area of the stain that is sampled, manipulation of buffer volumes, the level of cross-reactivity with non-urine samples, and the detection of nucleated epithelial cells in aged urine stains. It was discovered that, with regards to the Jaffe and Uritrace[®] methods, the area in which the known urine stain was sampled did not affect the result of the test; however that was not the case for RSID[™]-Urine. Decreasing the extraction volume for Uritrace[®] and RSID[™]-Urine did not inhibit positive results, implicating that it is possible to adequately perform either of the tests at lower levels of dilution. Jaffe and Uritrace[®] were shown to be susceptible to false positive signals, whereas RSID[™]-Urine was not. Nucleated epithelial cells were not detected in any of the aged urine stain samples, suggesting that the persistence of cellular material available for potential downstream DNA testing may be minimal. Photoimaging analysis was also used to assess the ease of interpretation of results using Uritrace[®]. An evaluation of all three methods revealed that although the Jaffe test is not the most specific method, it is the most practical and cost-effective method for the forensic detection of urine.

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List of Abbreviations
(in alphabetical order)

+	positive result
-	negative result
ALS:	alternate light source
Approx.:	approximately
°C:	Celsius degrees
CA:	California
CCR:	creatinine clearance rate
DNA:	deoxyribonucleic acid
ELISA:	enzyme-linked immunosorbent assay
et al:	et alia, "and others"
g:	grams
IL:	Illinois
kDA:	kilodalton
kg:	kilogram
L:	liter
LLC:	limited liability company
µL:	microliters
min:	minute
mg:	milligram
mL:	milliliter

mm:	millimeter
mmol:	millimolar
NY:	New York
ng:	nanograms
nm:	nanometers
NIRCL:	Northeastern Illinois Regional Crime Laboratory
PBS:	phosphate-buffered saline
pH:	potential of hydrogen
®:	registered trademark
rpm:	revolutions per minute
RSID™:	rapid stain identification
THP:	Tamm-Horsfall protein
™:	trademark
TPDFL:	Tulsa Police Department Forensic Laboratory
UV:	ultraviolet

1. INTRODUCTION

1.1 Forensic Relevance of Urine Evidence

The forensic identification of biological fluids at a crime scene can aid in the reconstruction of events. Urine identification, in particular, has played an influential role in the investigation of sexual and physical assault, child/elder abuse, and breaking and entering cases, to name a few.¹⁻³ For example, if a victim claimed that a perpetrator urinated on the victim's clothing after a sexual assault, samples derived from the clothing can be tested to determine whether or not the victim's story can be corroborated.

The detection of urine can also provide support for locating the area in which a crime may have occurred. The sympathetic nervous system, responsible for the "fight-or-flight" mechanism, is activated in circumstances where emotions, such as fear or anger, are aroused. A typical physiological reaction which results in such circumstances is the emptying of the bladder. Furthermore, it is known that upon death, the sphincter muscles in the bladder may relax and, as a result, urine is released.¹ Location and identification of urine stains in this scenario can suggest the general vicinity in which the decedent could have been at the time of death.⁴ The high-profile case surrounding the death of 6-year old child beauty pageant contestant JonBenét Ramsey involved a vast number of evidentiary findings at the scene, including a urine stain on the front of JonBenét's pajama pants. At the crime scene, JonBenét's body was discovered in a supine position, in close proximity to a urine stain on the floor of the basement which had a

pattern consistent with the stain on her pants. Experts were able to identify the urine as postmortem bladder release, allowing investigators to conclude that JonBenét's body had been moved after she had died.⁵

Detection of urine stains on evidence obtained from the scene of the crime can help determine whether the evidence is consistent with any first-hand witness testimonies and can also help investigators postulate a timeline of events which may have taken place during the crime. Though it is unlikely that any criminal case would rely on the rigorous identification of urine alone, the information is still beneficial for understanding what may have happened at the crime scene.⁶ Other areas of forensic science, such as DNA analysis and toxicology, also make use of evidentiary urine samples and will be detailed later in this section.

1.2 Composition of Urine

Urine is naturally produced in the mammalian kidney as a culmination of waste products filtered from blood. The liquid is then stored in the bladder and eventually excreted through the urethra. Figure 1 presents a flowchart of an abbreviated urinary pathway.¹ The average daily output of urine by an adult is approximately 630-2030 mL urine per kilogram body weight per day, but this value can vary based on the amount of liquid intake as well as other dietary conditions.^{6,7} The urinary constituents themselves also depend on these factors, as well as temperature, the individual's health state, and the level of physical

exertion. Huang et al.⁸ demonstrated that an increase in physical activity, such as playing outdoor tennis, can lead to an increase in the efficiency of the body to eliminate waste products and toxic endogenous by-products, like uric acid, via the urinary pathway. This boost in efficiency will inevitably alter the composition of the urine in such a way where, in the absence of exercise, intra-subject comparisons would generate a completely different urinary composition. It has also been previously shown that the composition of urine is highly variable between individuals.^{3,8,9}

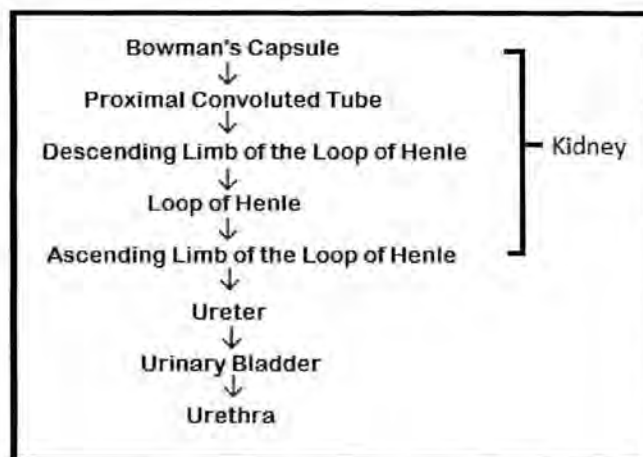


Figure 1: Urinary pathway

Approximately 55-70 grams of a urine void is composed of solids, including epithelial cells from the urinary tract and proteins.¹⁰ Table 1 contains a detailed list of common compounds recovered from urine, as well as where they originate in the body and the range of daily output reported.

1.3 The Detection of Urine

The majority of forensic urine detection tests are adaptations of pre-existing tests used in clinical settings. For example, indican levels were first intended as a diagnostic test for measuring bacterium activity in the intestine and small bowel.^{12,13} Creatinine levels were historically observed as clinical assessments of kidney function and glomerular filtration rate.^{14,15} The goals of these initial applications were to quantitatively gauge biological activity in the urogenital pathway and to use that information to evaluate the wellness or disease state of an individual. The presumptive detection of urine in forensic science departs tremendously from this goal, as it stringently relies on the accuracy of the identification of singular urinary constituents, independent of the individual completely.

Given such rhythmic behavior between and within subjects, designing presumptive tests for urine based on the detection of any particular component can pose many challenges for forensic analysis. The introduction of screening tests in forensic analyses paved way for continuous reevaluation and optimization of the techniques to cater to the interests of the field as well as address the inter- and intra-subject variation. For example, urine is seldom found

5

Substance	Source	Urine (mg/kg/day)	Serum/Plasma (mg/kg/day)	Saliva (mg/kg/day)	Semen (mg/kg/day)	Sweat (mg/kg/day)
Tamm-Horsfall protein	(Kidney) Thick ascending limb of loop of Henle	~ 100	Low amounts	Low amounts	--	--
Uric acid	Purine metabolism	~0.56-2.1	~0.16-0.39	~0.05-0.87	~0.6	~0.007-0.025
Urea	Oxidation of amino acids or ammonia via urea cycle	~140-350	~1.6-3.5	~0-1.81	~7.2	~1.2-5.7
Creatinine	Muscle/liver/kidney	~10.5-21	~0.06	~0.028-0.046	--	~0.01-0.13
Sodium	Must be ingested	~2.53	~3.23	--	--	--
Indican	Converted from tryptophan	0.35-1.4	0.0095-0.0105	--	--	--
Phosphate	Must be ingested	~7-10.5	~0.24-0.376	~0.74-2.11	~1.1	<0.001

Table 1: Common compounds recovered and historically tested for in urine.^{6,10,11}
 (Calculations are relative to a 70 kg individual excreting 1 L of urine per day)

in a liquid state at a crime scene. Therefore, a carefully designed technique combining sensitivity and specificity aspects must be developed almost primarily for the detection of urine in the form of dried stains.¹⁶ As a consequence, a variety of confounding variables are introduced, including substrate interference, dispersion of the urine and its constituents on the substrate, and difficulty locating the stain.

Prior to using urine identification assays, potential urine stains must be recognized or located. Urine is known to have a characteristic odor, though its detection is subjective and not the most practical.¹⁷ Small stains or aged urine stains may no longer have a perceivable odor due to the limited surface distribution of scent and natural diffusion into air. Aside from odor, urine is also associated with a distinct range of colors. Urine color can be attributed to the pigment urochrome.¹⁸ The color spans from a straw/pale yellow to a deep brown, the latter suggesting possible endocrine dysfunction or disease.¹

Dehydration contributes greatly to the observed color of urine. The more dehydrated an individual, the less dilute his or her urine and consequently, the higher the accumulation of toxins, bacteria, and other miscellany within a single urine void. Diet can influence the color of urine as well. For example, some fruits and vegetables can temporarily change the color of urine to red whereas some drugs can cause urine to turn blue or green.¹⁸ Additionally, color varies somewhat between urine voids, regardless if the same foods were consumed prior to urination.^{19,20} Urine may thus be difficult to locate given such varying

appearances. Using color as a basis for urine detection can also be problematic when the stain is located on a substrate or surface that is not white, as it can be difficult to spot or accurately assess with the naked eye. Moreover, a faint or small urine stain can sometimes be hard to visualize on a white background.

A common technique for visually enhancing biological stains is the use of an alternate light source (ALS) or ultraviolet (UV) light.¹⁰ It has been reported that urine stains can be visualized under excitation wavelengths of 415nm, 450nm, and 505nm with yellow, orange, and red barrier filters, respectively.²¹ Seidl et al.²² demonstrated that using the Lumatec[®] Superlite 400 ALS with wavelength of 415nm and an orange barrier filter, fluorescence was observed in nonporous surfaces like tile and formica up to a maximum urine dilution of 1:1000, whereas cotton fabric and carpet only exhibited fluorescence up to maximum dilutions of 1:100 and 1:10, respectively. Wawryk et al.²³ utilized a Polilight[®] with a wavelength of 450nm and an orange barrier filter to enhance the visualization of a neat urine stain on human skin, though the stain failed to fluoresce after the first day.

The amount of fluorescence emitted is dependent on the quality of the stain with regards to initial concentration, extent of absorption into the substrate, as well as the presence of microorganisms which can degrade fluorescence-producing proteins in the urine.^{4,10,17,24} Substrates which are highly absorbent or have inherent fluorescence are challenging for locating urine stains. Highly absorbent substrates can absorb the urine stain before it is dry and subsequently

mask its detection due to its absence on the surface of the material.²⁴ Substrates which exhibit fluorescence can result in a decrease in contrast between the background substrate and the urine stain, making the stain difficult or impossible to visualize.^{21,25}

A number of biological fluids can produce stains which fluoresce in the same manner as urine. The appearance of each type of stain is dependent on the specimen quality, degree of contrast against a substrate, and type of ALS employed to visualize the stain.^{23,24,25} For example, under UV light, a saliva stain can appear bluish-white; however, against the same background, other stains such as weakly fluorescing semen stains can appear the same or similar.¹¹ Non-biological stains, such as tea, brighteners in detergents, and fabric conditioners, can also exhibit fluorescence using an ALS.^{21,26} Fluorescence detection is often effective for locating a stain but caution must be taken when determining the type of fluid observed.

1.4 Creatinine

Creatinine (Figure 2) is a catabolic breakdown product of the organic acid creatine phosphate, a molecule produced primarily by amino acids of the liver and kidney to supply the body's muscles with energy. Creatinine levels in urine are historically used as an index of kidney health by calculating the extent of creatinine clearance as a measure of glomerular filtration rate.^{6,15} The creatinine clearance rate (CCR) refers to a clinical computation used to evaluate renal

function. CCR is a function of the volume of the urine sample and the amount of creatinine in urine relative to the amount in serum.²⁷

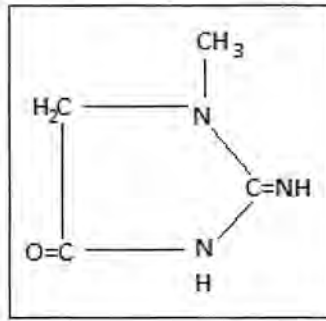


Figure 2: Creatinine ($C_4H_7N_3O_2$).²⁸

The CCR equation reinforces the fact that creatinine is also present in other body fluids. Creatinine is present in serum, saliva, and sweat at an average rate of 0.06, 0.028-0.046, and 0.01-0.13 mg per kg body weight per day, respectively.^{6,10} Despite its presence in multiple body fluids, creatinine is a choice candidate for urine detection because it exhibits its highest concentration in urine. Huang et al.⁸ assessed creatinine levels in sixteen males and found that creatinine was excreted in urine at a mean rate of 21.5 ± 7.4 mmol/L, as compared to 0.031 ± 0.017 mmol/L in sweat and 0.102 ± 0.025 mmol/L in saliva. However, the relative standard deviation of mean urinary creatinine excretion was 34.4%, indicating a substantial amount of variation between the sixteen individuals. On the contrary, Greenblatt et al.⁹ examined creatinine levels in urine from eight male subjects throughout the course of a 24-hour period and found that within-subject analysis followed a roughly normal distribution with standard

deviations ranging from 10.5 to 14.4%. In the same study, it was shown that considerable between-subject variation of creatinine excretion levels in urine existed among the eight individuals. Greenblatt et al. attributed the within-subject variation to body weight and surface area differences. Results from both studies demonstrate that creatinine levels are variable across reportedly healthy subjects and are present in other body fluids. These two elements make the optimization of a urine identification technique which relies on the detection of creatinine in forensic investigations challenging.

1.5 Tamm-Horsfall Protein

Tamm-Horsfall protein (THP), formerly known as uromodulin, is a high molecular weight protein (~90 kDA) expressed on the endothelium as a renal epithelial component of the thick ascending limb of the loop of Henle.² It is the most abundant protein in normal human urine and is primarily excreted in large groups of individual molecules, or aggregates.²⁹ Its localization in the kidney makes THP an attractive candidate for the forensic detection of urine. The THP molecule is composed of approximately 30% carbohydrates by weight, the majority of which are essentially N-linked sugars.³⁰ THP molecules consist of an extensive network of intrachain disulfide bonds. Disulfide bonds are strong links formed by the sulfur moieties of two molecules sharing electrons in a mutual covalent bond. These bonds increase the stability and rigidity of the molecule.³¹ THP's function is still unclear, but it has been implicated as a combatant of

microbial infections in the urinary tract and bladder²⁹⁻³¹, a quantity-based index for acute renal failure and kidney stone formation^{32,33}, and a messenger that recruits leukocytes in the course of an inflammatory response.³⁴

Johnstone et al.³² suggested that a leak may exist in the urinary tract, leading to a detectable concentration of THP in serum. In this study evaluating the utility of THP serum levels as markers for determining urinary tract obstruction, the authors calculated a range of serum-THP concentration values (1.26-61.9 ng/mL) in 13 healthy individuals, demonstrating the amount of variance that occurs between individuals.

THP is a forensically relevant molecule in that it is relatively specific for human urine. THP can also be found at low levels in human serum and saliva.³² Akutsu et al.² performed a study using enzyme-linked immunosorbent assay (ELISA) to measure the relative concentration of THP with respect to other body fluids. In this study, THP was much more abundant in urine than any other body fluid; however, once the dilution factor reached 1:1000, the amount of THP in plasma levels surpassed that of urine, indicating that the detection of THP in plasma is less affected by the diluting process than THP in urine. Nonetheless, THP is still considered to be less prevalent in other body fluids, thus making it the choice molecule for presumptive forensic detection of urine. However, like many urinary constituents, levels of THP can vary from individual to individual as well as within the same individual.

1.6 Urine in Forensic Toxicology

Collection of urine specimens for the analysis of controlled substances is a popular method used in forensic toxicology due to its non-invasiveness as well as the accuracy with which urine can be used to establish the general amount and the time elapsed since intake of a drug.³⁵ Urine screening is a standard prerequisite for prospective federal employees in the United States, a requirement set forth by the Substance Abuse and Mental Health Services Administration division of the Human and Health Services.³⁶ Many tests involve the use of colorimetric spot test reagents to presumptively determine the presence of a drug or toxin in the urine. It is not uncommon for individuals to substitute or adulterate their urine samples to avoid failing a screening test; however, both actions would lead to serious legal consequences.³⁷ Substitution of a urine sample entails an individual submitting a sample that visually mimics urine or one which contains another individual's urine sample. Adulterating a urine sample involves the addition of a chemical or substance which could possibly inhibit the detection of a drug in the urine sample; examples of such substances are bleach and vinegar.³⁸ If successful, both substitution and adulteration of urine samples can result in a negative drug test.

Results from toxicology testing on urine stain samples can be beneficial for both ante- and post-mortem (before and after death) forensic investigations. One study on the persistence and recovery of forensic drug urine specimens from specialized paper demonstrated that drugs could still be sufficiently

extracted and identified from a urine stain.³⁹ Such a finding may suggest that in a sexual assault case, detection of drugs from urine stains found on the victim's underwear can demonstrate that the victim may have been under the influence of drugs during the commission of the crime, and thus could not defend him or herself.³⁵ Previous studies have shown that use of specialized kits for extraction of urine from a substrate, followed by separation by chromatography were successful techniques for detecting drugs in urine stains as old as 30 days.^{35,39}

1.7 Urine as a Source of DNA

The ability to link a specific individual to a urine stain found at a crime scene can play a valuable role in a forensic investigation. Urine is cited as one of the most challenging biological matrices for obtaining individualizing evidence, namely nuclear DNA.^{16,40,41,42} A suspected urine stain is typically subjected to presumptive testing with methods such as the Jaffe test for creatinine, followed by DNA testing. Numerous analytical techniques have been developed to isolate DNA from urine stains and have demonstrated success. However, due to the limited quantity and quality of DNA present in urine stains, procedures for extracting and obtaining interpretable genetic profiles from urine stains can be taxing and time-consuming as a result of the extra steps required to process degraded or low quantity DNA.^{37,43} In addition to the scarcity of DNA present in evidentiary urine stains, it has been reported that epithelial cells and other components which house DNA tend to be randomly distributed within a stain. In

some cases, separate stains created by sequential drops from the same urine void can result in varying levels of DNA.⁴⁰

Sources of DNA in urine include white blood cells and nucleated epithelial cells. White blood cells, or leukocytes, are usually found at a concentration of 200-500 cells/mL of urine.⁴⁴ The different types of epithelial cells include renal, transitional, and squamous. A number of features determine their classification, such as size, morphology, and area of origin.⁴⁵ Reportedly, normal human urine typically contains no more than 400 epithelial cells/mL of urine.⁴⁷ It is also important to note that numerous studies have found that female urine tends to contain more epithelial cells, and consequently DNA material, than male urine.^{16,40,42,47,48,49}

Apart from the difficulties of DNA testing which are inherent to urine specimens, forensic scientists must also find methods for coping with other external challenges, such as storage, temperature, and the age of the stain. Vu et al.⁴⁷ found that in comparing the amount of DNA in fresh liquid urine as compared to liquid urine frozen at -20°C for 24 hours, the quantity of DNA decreased sharply regardless of gender, though genetic profiles were still developed for these degraded samples. Research by van der Hel et al.⁵⁰ demonstrated in a population-based study of 15-25 year old frozen liquid urine samples that the ability to generate a DNA profile from these aged samples varied, but ultimately achieved a genotype success rate of 89.3%. Despite the outcomes reported, there is still a lack of research on the persistence of nuclear

material in urinary stains with regards to these external challenges. However, when taking into account the impact storage conditions and age have on the accessibility of DNA in liquid urine, it is reasonable to believe that the chances of obtaining a genetic profile from an aged urine stain under the same conditions may be minimal. Attempting to analyze a stain from a crime scene which may be potentially degraded due to storage conditions, age, and environmental insult might not provide researchers with useful information beyond presumptive testing, assuming the appropriate target molecules have not degraded.

A vast number of obstacles do exist in attempting to generate a DNA profile from a urine stain; however, with improved technology and with the introduction of automated techniques to the laboratory, the analysis of urine stains may become more attractive and influential in the forensic field.

1.8 Purpose of Study

Many of the drawbacks which accompany traditional identification methods for urinary components stem from a lack of specificity with regards to species and physiological origin. Moreover, due to the lack of specificity, determination of an appropriate threshold of sensitivity is problematic because all urinary components tested for are not unique to urine and can be found at various levels in other body fluids of many different species.^{3,4,6,15} The purpose of this study is to compare two relatively novel techniques – the Uritrace[®] and RSID[™]-Urine assays – and a traditional technique still used *in crime labs* today –

the Jaffe test – to evaluate the efficacy and value that each test contributes to the identification of urine stains in a forensic setting.

2. MATERIALS AND METHODS

2.1 Reagents

The following reagents used were acquired from Fisher Scientific, LLC (Rochester, NY): hematoxylin, eosin Y, picric acid solution saturated, and 5% sodium hydroxide solution (2.5 g sodium hydroxide pellets dissolved in 47.5 mL of distilled water).

2.2 Sample Collection

2.2.1 Urine Samples

Eight volunteers donated freshly voided urine samples. The subjects consisted of 4 male and 4 female subjects who were 24-27 and 19-24 years of age, respectively. Urine specimens were collected in clean sample cups labeled with an assigned sample number, as well as the following information: gender, age, and time of sample submission. All sample volumes were documented. Within one hour of receipt, 1 mL of each urine sample present was immediately spotted onto a 4in. x 4in. cotton cloth. One mL of distilled water was spotted onto a cotton cloth as a negative control.

2.2.2 Non-Urine Samples

A volume of 1 mL of each beverage – Lemon-lime Powerade[®], Nantucket Nectars[®] Lemonade, Red Bull[®], and Coors[®] Light beer – was spotted onto a 4in. x 4in. cotton cloth. All samples were left at room temperature to dry before packaging to transport to an alternate laboratory for testing.

The serum samples used were obtained from three of the volunteers. Fresh blood samples were obtained through a finger prick method using a safety engineered capillary blood sampling device (BD Genie™ Lancet, Franklin Lakes, NJ) whereby volunteers deposited their blood samples directly into a microcentrifuge tube. The samples were then centrifuged using an Eppendorf[®] (Hauppauge, NY) Centrifuge 5415D for 8 minutes at 13,000 revolutions per minute (rpm) to separate the serum from the cellular portion of each sample tube. The aqueous portion, or supernatant, of each sample was decanted and placed in new microcentrifuge tubes and spun once more for 2 minutes at 13,000 rpm. The supernatants were decanted and the entire volume of each sample (which did not exceed 260 µL for any of the samples) was spotted onto a cotton cloth. The samples were allowed to dry for one week at room temperature before testing.

2.3 Part I: Sensitivity and Localization of Urinary Components

Figure 3 represents a simplified flowchart of the Part I procedure.

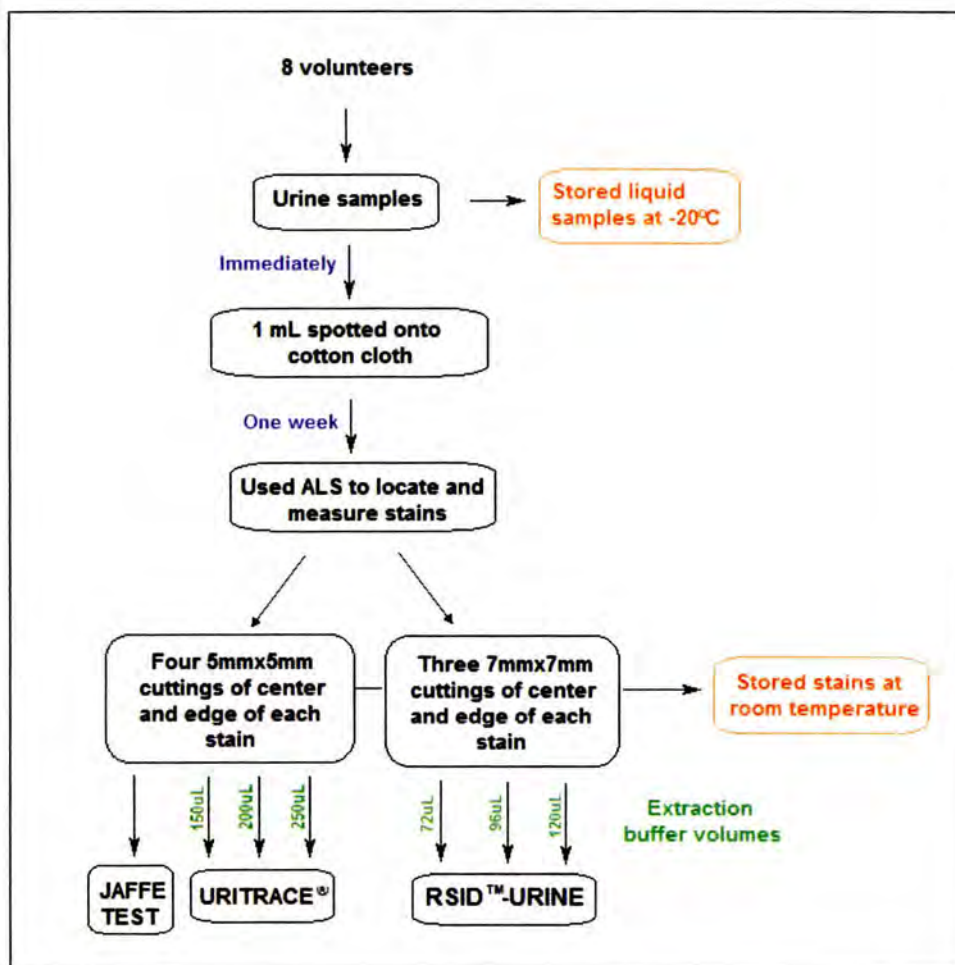
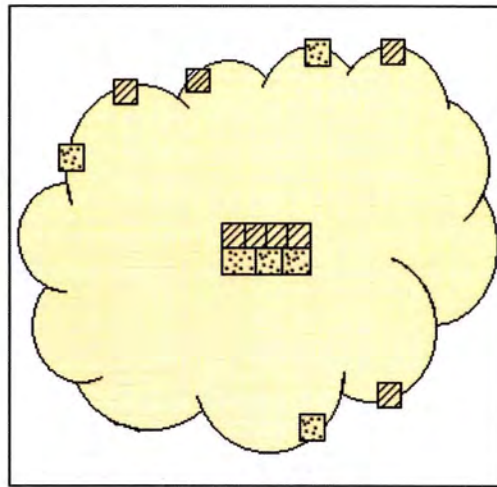


Figure 3: Scheme of sample collection and Part I sample preparation procedure.

A 450 nm (blue) Crimelite® (Foster and Freeman, Sterling, VA) alternate light source (ALS) with orange barrier filter goggles was used in order to measure the length and width of the stains. After an estimate of the dimensions was determined, the center of the stain was deduced using the midpoints of the parameters. Three 5mm x 5mm cuttings were obtained from the center of each

stain and placed into 150 μ L, 200 μ L, and 250 μ L volumes of distilled water to be extracted for analysis using Uritrace[®] (Abacus Diagnostics, West Hills, CA). One 5mm x 5mm cutting was obtained from the center of each stain and placed into a spot plate in preparation for the Jaffe test. Three 7mm x 7mm cuttings were obtained from the center of each stain and placed into 72 μ L, 96 μ L, and 120 μ L volumes of RSID[™]-Urine extraction buffer to be extracted for analysis using RSID[™]-Urine (Independent Forensics, Lombard, IL). This procedure was repeated for the edge of the stain (Figure 4). The sample cutting from the edge of the stain encompassed a portion of the fluorescing region as well as the border of the stain and the area immediately adjacent to the border that did not appear to fluoresce. The urine stains were then stored at room temperature for seven days prior to extraction.

The Jaffe test was performed by addition of a drop of saturated aqueous picric acid followed immediately by a drop of 5% sodium hydroxide to the sample stain. A color change from yellow to orange occurring within a 15 minute period was denoted a positive result. In addition, any change in intensity from the bright yellow of the picric acid to a deeper yellow/light orange was considered to be a weak positive reaction. No color change within the 15-minute window was reported as a negative result. Figure 5 presents an example of these three results.





 = 7mmx7mm cutting
 = 5mmx5mm cutting

Figure 4: Urine stain on cotton cloth with the each size cutting indicated.
(not to scale)

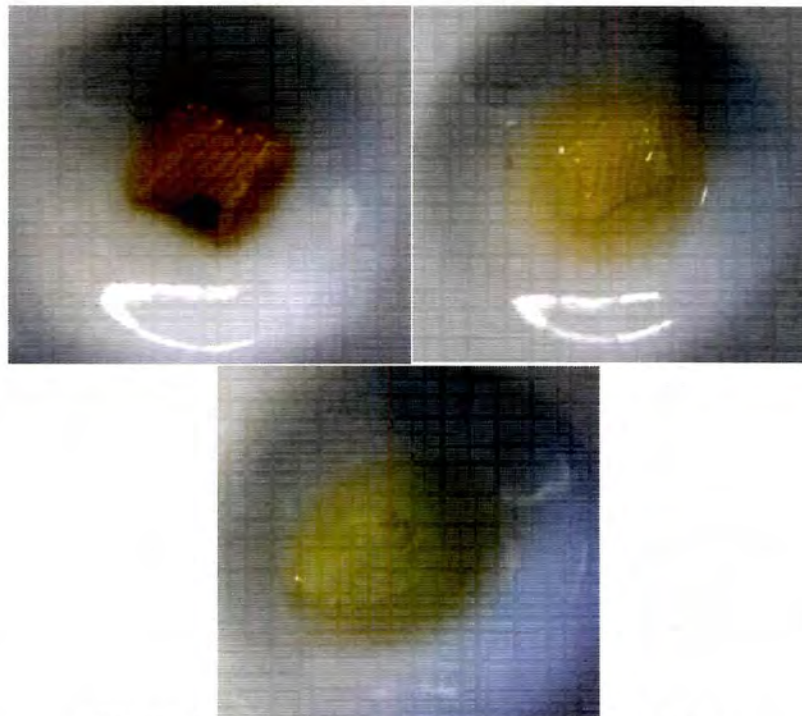
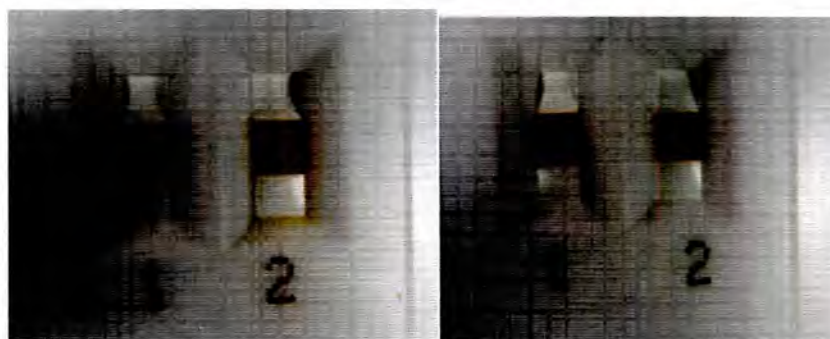


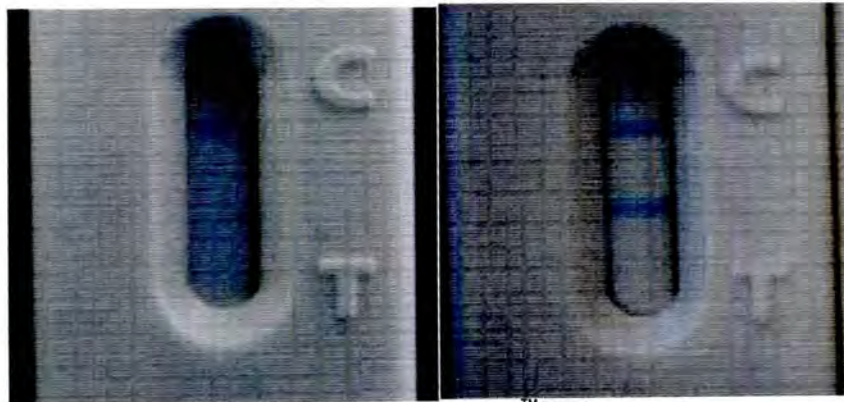
Figure 5: Example images of Jaffe test results.
Top left - positive; top right - weak positive; bottom - negative

For the Uritrace[®] sample cuttings, the extractions were carried out per the manufacturer's instructions.⁵¹ Two extraction liquid volumes in addition to the manufacturer's recommended volume of 250 μ L were also assessed (150 μ L and 200 μ L). Each cutting was extracted for 2 hours using distilled water in a microcentrifuge tube. At the end of the extraction period, 100 μ L of each urine stain extract was pipetted into Area 1 of the Uritrace[®] reagent strip. A volume of 100 μ L of distilled water was pipetted into Area 2 as the internal negative control.⁵¹ If the color change in Area 1 was more intense, or a deeper orange, than Area 2, the test was considered positive. If Area 2 was more intense than area 1, the test was negative. In order to be denoted a positive result, the color change had to occur within 10 minutes. Negative results were reported only if Area 2 appeared more intense than area 1 after 10 minutes. An example of the Uritrace[®] test results can be seen in Figure 6 below.



*Figure 6: Example images of results of Uritrace[®] test.
Area 1 is the urine stain extract and Area 2 is the negative control.
From left to right: Positive outcome, negative outcome.*

For the RSID™-Urine test, a 7mmx7mm cutting of the edge and center of each urine stain was placed in a microcentrifuge tube and extracted for approximately 2 hours in RSID™-Urine extraction buffer in accordance with the manufacturer's instructions. After the extraction period, 100µL of each extract was pipetted directly into the sample well of the RSID™-Urine cartridge. After a 15-minute interval, the results were assessed. If one blue line occurred at the control line only, the test was considered negative. If two blue lines occurred at the control and test lines, the test was denoted positive. An example of each of these results is demonstrated in Figure 7.



*Figure 7: Results of RSID™-Urine test.
From left to right- negative outcome, positive outcome*

Results from the Jaffe, Uritrace®, and RSID™-Urine tests were photographed using a Canon Powershot Elph 300HS digital camera under portrait and macro picture settings.

2.4 Part II: False Positives for Creatinine and THP

The analysis of potential false positives for the Jaffe test and Uritrace[®] included the following beverages: Lemon-lime Powerade[®], Nantucket Nectars[®] Lemonade, Red Bull[®], and Coors[®] Light beer. After each sample was spotted, an ALS was used to locate and measure the stains. A cutting measuring 5mm x 5mm was obtained from the center and edge of each sample stain. These stains subsequently underwent analysis following both the Jaffe test and Uritrace[®] protocols outlined previously.

An area of 7mm x 7mm was sampled from each of the three serum stains obtained. The cuttings then underwent analysis following the RSID[™]-Urine protocol outlined previously.

Jaffe test results and assay cards were photographed with a Canon Powershot Elph 300HS digital camera using portrait and macro picture settings.

2.5 Part III: Analysis of Urinary Sediment from Aged Stains with Different Storage Conditions

The frozen liquid urine samples were removed from 20°C after 3 months and were thawed at room temperature. The samples were then gently mixed with a spatula to homogenize the crystallized sediment and the aqueous portion of the specimen. A volume of 1mL of each sample was pipetted onto a cotton cloth and dried overnight at room temperature. These stains were designated Set A. Urine stains stored at room temperature for 3 months were designated Set B. An ALS

(CrimeLite[®], 450 nm blue light with orange barrier filter goggles) was used to assess the size of Set A stains. A banding pattern revealed a central stain which could be distinguished from the overall urine stain. Figure 8 provides an example of such a stain.



Figure 8: Frozen urine spotted on cotton cloth exhibiting banding pattern with central stain and overall stain (Sample 11312-08).

Several cuttings measuring 7mm x 7mm were obtained from each of the stains. For Set A, a cutting was obtained from the center of the stain, the perimeter of the central stain, and the perimeter of the overall stain; for Set B, a cutting was obtained from the center and the edge of the stain. The cuttings were placed into 2mL-capacity microcentrifuge tubes and approximately 250 μ L of phosphate-buffered saline (PBS) was added to each tube using a modified version of the extraction and centrifugation methods described by Johnson et al.⁴⁰ The samples were spun for 30 minutes and then subjected to a piggy-back

spin, where the cutting was removed from the extract, placed in a Spin-eZe[®] (Fitzco Inc., Spring Park, MN) basket, and spun for another 5 minutes. The supernatant was then removed and discarded. A volume of 20 μ L of each sample was pipetted onto a labeled glass microscope slide, then subsequently allowed to dry before undergoing cytological staining. One drop of hematoxylin was added to the slide and allowed to absorb for approximately 3 minutes, staining any nuclear material blue-purple. After approximately 3 minutes, the hematoxylin was rinsed away with a gentle stream of methanol until the stream ran clear. A drop of eosin Y was then added to the slide and allowed to absorb for approximately 2 minutes. Non-nuclear material, such as the cytoplasm, stained pink. After 2 minutes, the eosin Y was rinsed away with a gentle stream of methanol until the stream ran clear. After a short drying period, the slides were then examined under 400X magnification and the number of cells viewed was recorded. This procedure was repeated for all sample extracts.

A fresh buccal swab also underwent the same procedure to confirm that the extraction technique utilized was sufficiently eluting cells from the cotton cloth into the buffer. A cotton cloth spotted with distilled water served as a negative control.

2.6 Part IV: ImageJ Analysis of Uritrace[®] Results




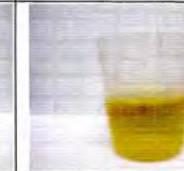
Four stains were produced on cotton cloths using two fresh and two frozen urine samples. The stains were extracted according to the manufacturer's

instructions for Uritrace[®] to serve as positive controls.⁵¹ Five negative controls using distilled water were also tested using the Uritrace[®] cards. The results of the Uritrace[®] test were subjected to a blind verification study, wherein three analysts independently examined the results and recorded their observations. Photographs of the test strips were taken in an MK Photo-eBox[™] (MK Digital Direct Lighting Systems, San Diego, CA) with an Olympus SP500-UZ digital camera using program (P) mode with SQ2 normal image quality (1024x768 pixels). ImageJ, a Java-based imaging program developed by National Institutes of Health⁵², was used to analyze the integrated density of the pixels in each sample well.

3. RESULTS

3.1 Subject profiles and samples

The samples submitted as well as the approximate volumes are documented in Table 2. Individuals reported no health conditions such as drug use or metabolic diseases.

Sample →	11312-011	11312-012	11312-013	11312-014
				
Approx. Volume	55mL	52mL	61mL	50mL



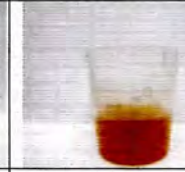
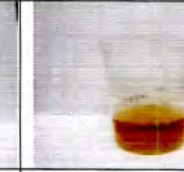
Sample →	11312-05	11312-06	11312-07	11312-08
				
Approx. Volume	38mL	80mL	45mL	27mL

Table 2: Eight samples submitted for testing and the corresponding volumes.

3.2 Part I: Sensitivity and Localization of Urinary Components

3.2.1 Jaffe Test

Positive results were observed for both cutting locations across all samples, with the exception of the center cutting for sample 11312-05 (Table 3). The speed at which a positive result was obtained was approximately the same between cuttings within each individual.

Sample	Jaffe Result	
	Center Cutting	Edge Cutting
11312-01	+ (< 6 min)	+ (< 6 min)
11312-02	Weak + (< 10 min)	Weak + (< 10 min)
11312-03	+ (< 6 min)	+ (< 6 min)
11312-04	Weak + (< 10 min)	Weak + (< 10 min)
11312-05	-	Weak + (< 10 min)
11312-06	+ (< 6 min)	+ (< 6 min)
11312-07	+ (< 6 min)	+ (< 6 min)
11312-08	+ (< 6 min)	+ (< 6 min)
Distilled H ₂ O (Negative control)	-	-

Table 3: Results of Jaffe test on center and edge cuttings of each stain.
+ = positive, weak + = weak positive, - = negative

3.2.2 Uritrace[®]

Table 4 presents the results using three different volumes of buffer for extraction of urinary stains.

Uritrace® Results						
Sample	Buffer Volume					
	150µL- Center Cutting	150µL- Edge Cutting	200µL- Center Cutting	200µL- Edge Cutting	250µL- Center Cutting	250µL- Edge Cutting
11312-01	+	+	+	+	-	+
11312-02	+	+	-	+	-	+
11312-03	+	+	+	-	+	-
11312-04	+	-	+	+	+	+
11312-05	-	+	+	-	+	-
11312-06	+	+	-	+	-	-
11312-07	+	+	+	+	-	-
11312-08	-	+	-	+	-	-

Table 4: Results of Uritrace® test on center and edge cuttings of each stain.

+ = positive; weak + = weak positive; - = negative

highlighted results = samples where negative results were positive at a higher dilution.

3.2.3 RSID™-Urine

Analysis of the samples using RSID™-Urine revealed positive results for all center cuttings, regardless of extraction buffer volume.

	RSID™-Urine Results					
Sample	Buffer Volume					
	72µL- Center Cutting	72µL- Edge Cutting	96µL- Center Cutting	96µL- Edge Cutting	120µL- Center Cutting	120µL- Edge Cutting
11312-01	+	-	+	-	+	-
11312-02	+	-	+	-	+	-
11312-03	+	-	+	-	+	-
11312-04	+	-	+	-	+	-
11312-05	+	-	+	-	+	-
11312-06	+	-	+	-	+	-
11312-07	+	-	+	-	+	-
11312-08	+	-	+	-	+	-
Distilled H ₂ O (negative control)	-	-	-	-	-	-

Table 5: Results of RSID™-Urine test on center and edge cuttings of each stain.
+ = positive; - = negative

3.3 Part II: False Positives for Creatinine and THP

3.3.1 Creatinine

Positive results were observed for all beverages utilizing the Jaffe and Urित्रace® tests for urine, with a higher prevalence in edge cuttings for the Jaffe test compared to center cuttings for Urित्रace® (Table 6).

Sample	Jaffe Results		Uritrace [®] Results	
	Center	Edge	Center	Edge
Lemon-Lime Powerade [®]	-	weak +	-	+
Nantucket Nectars [®] Lemonade	-	+	+	-
Red Bull [®] Energy Drink	+	+	+	+
Coors [®] Light Beer	weak +	weak +	+	-
Neat Urine (positive control)	+	+	+	+
Distilled H ₂ O (negative control)	-	-	-	-

Table 6: Results from center and edge cuttings of stains produced by potential false positive substances using Jaffe and Uritrace[®] tests.
 + = positive; weak + = weak positive; - = negative

3.3.2 Tamm-Horsfall Protein

Analysis of stains produced by all three serum samples (A, B, and C) generated negative results for all center and edge cuttings (Table 7).

Sample	RSID™-Urine Results	
	Center	Edge
Serum A	-	-
Serum B	-	-
Serum C	-	-
Neat Urine (positive control)	+	+
Distilled H ₂ O (negative control)	-	-

*Table 7: Results from center and edge cuttings of stains produced by serum samples using RSID™-Urine.
+ = positive; - = negative*

3.4 Part III: Analysis of Urinary Sediment from Aged Stains with Different Storage Conditions

Microscopic analysis of urinary stains stored at room temperature and stains produced from liquid samples stored at -20°C over approximately three months revealed substrate debris and possible unidentified components of the urinary sediment; however, no intact cellular material was visualized. A minimum of 15 nucleated epithelial cells was observed per field when observed under 400x magnification in the buccal (control) sample.

3.5 Part IV: ImageJ Analysis of Uritrace[®] Results

The Uritrace[®] samples which were utilized for the blind verification study are shown below (Figure 9).

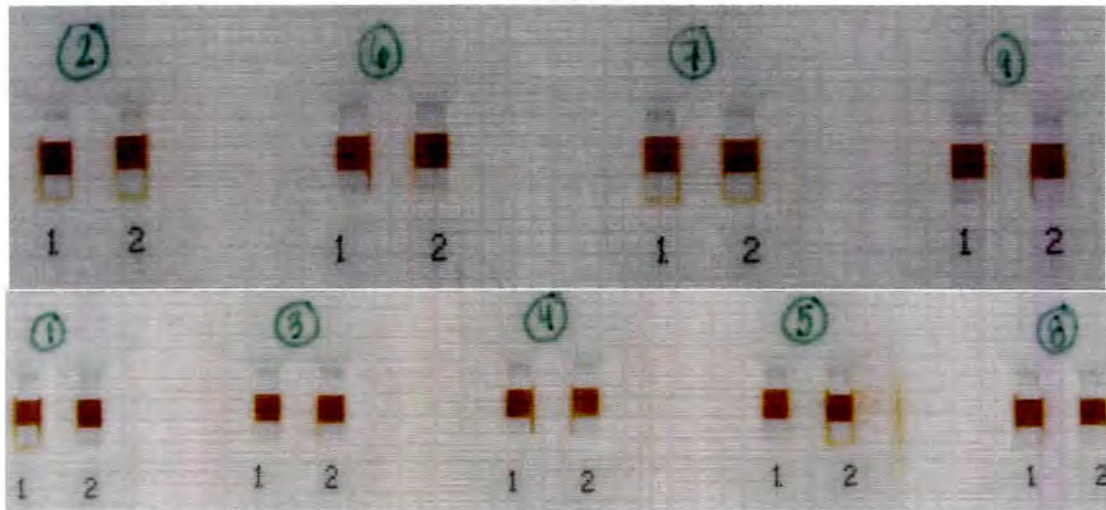


Figure 9: Uritrace[®] results for ImageJ and blind verification study. Top row- Positive controls using neat urine; Bottom row- Negative controls using distilled water.

The results of the blind verification study are shown below in Table 8.

Samples →	1	2	3	4	5	6	7	8	9
Sample	Water	Urine stain	Water	Water	Water	Urine stain	Urine stain	Water	Urine stain
Analyst A Response	-	+	-	+?	-	-?	-?	-?	+
Analyst B Response	-	+	-	+	-	+	+	-	+
Analyst C Response	-	+	-	-?	-	-	-	-	+

Table 8: Results of blind verification study on nine Uritrace[®] samples.
 + = positive; - = negative
 ? = analysts were not confident in their interpretation of the results.

Analysis of integrated densities using the ImageJ software is shown in Table 9. The colored regions of Areas 1 and 2 of each sample were measured using the “Analyze → Measure” command, in which a table with integrated densities was generated. The integrated density pixels were recorded in the table below and the difference between the two areas was taken by subtracting the integrated density of Area 2 from the integrated density of Area 1.

Sample	Expected Uritrace [®] Result	Area	Integrated Density (in pixels)	Difference (Area 1 – Area 2)
1	Negative	1	146,408	-22,864
		2	169,272	
2	Positive	1	129,850	17,263
		2	112,587	
3	Negative	1	169,616	7,716
		2	161,900	
4	Negative	1	98,471	-4,897
		2	103,368	
5	Negative	1	102,124	1,244
		2	94,314	
6	Positive	1	138,081	-577
		2	138,658	
7	Positive	1	195,015	22,118
		2	172,897	
8	Negative	1	192,533	3,774
		2	188,759	
9	Positive	1	199,076	7,503
		2	191,573	

Table 9: ImageJ integrated densities for nine Uritrace[®] samples.

For the negative results, the differences in the integrated densities ranged from -22,864 pixels to 7,810 pixels, with a mean difference value of $-1,692.2 \pm$

5,775.82 pixels. Positive results ranged from -577 pixels to 22,118 pixels, with a mean difference value of $11,576 \pm 5,064$ pixels. Table 10 shows the range, mean, and standard deviation of the integrated density values for each of the two areas on the card.

	<i>Range of Integrated Density Values</i>		<i>Mean of Integrated Density Values \pm 1 Standard Deviation</i>	
	<i>Expected Positives</i>	<i>Expected Negatives</i>	<i>Expected Positives</i>	<i>Expected Negatives</i>
<i>Area 1 (sample)</i>	69,226 (129,850-199,076)	94,062 (98,471-192,533)	165,505.5 \pm 18,305.8	141,830.4 \pm 18,466.7
<i>Area 2 (negative control)</i>	78,986 (112,587-191,573)	94,445 (94,314-188,759)	153,928.8 \pm 17,605.0	143,522.6 \pm 18,816.2

Table 10: Descriptive statistics for the expected positive and negative results for Areas 1 and 2.

4. DISCUSSION

4.1 Part I: Sensitivity and Localization of Urinary Components

4.1.1 Jaffe Test

The Jaffe Test is a presumptive colorimetric test in which picric acid, a chemical with a bright yellow hue, and sodium hydroxide react with creatinine in an alkaline environment to produce creatinine picrate, a red/orange-colored product.³¹ The formation of the creatinine picrate complex is depicted in Figure 10.^{10,28,54}

The reaction taking place in Figure 10 is a simple first-order reaction dependent on the ability of sodium hydroxide to hydrogen bond and in effect,

allow the creatinine anion to bind to the picrate anion.^{14,54} A first-order reaction is defined as one where product formation (in this case the creation of creatinine picrate) relies on the concentration of one reactant. The sodium hydroxide creates an alkaline environment, vital for any interaction to occur between picric acid and creatinine because sodium hydroxide dictates the speed and amount of creatinine picrate produced.⁵⁴

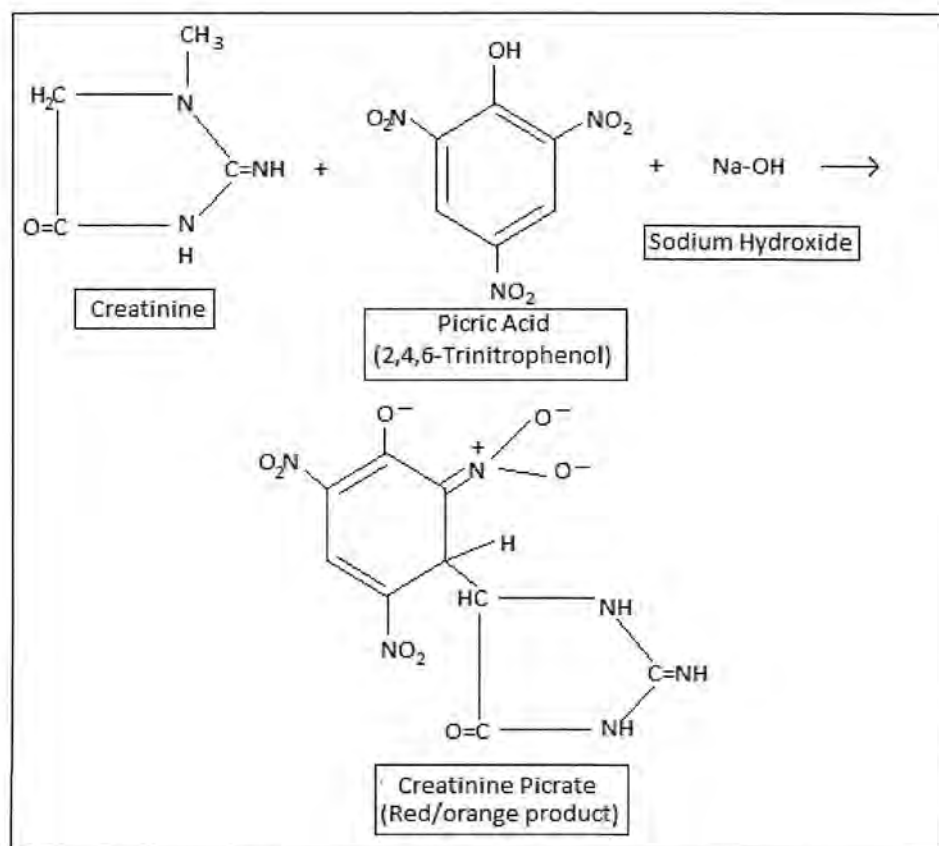


Figure 10: The formation of creatinine picrate complex.

In a forensic laboratory, the Jaffe test is typically carried out with the successive addition of two reagents, picric acid and sodium hydroxide, to a sample of a suspected urine stain in a spot plate. If creatinine is present in the stain, it complexes with the picric acid to form a chromophore (colored product). This technique can be applied directly to cuttings and therefore is advantageous in cases where urine stains are dilute and extraction of the stain into solution would dilute the stain to a potentially undetectable concentration.

The present study examined the ability of the Jaffe test to yield reproducible results between the urine voids of eight subjects and attempted to determine if creatinine is localized to any particular region of a stain. The results from Table 3 showed positive results throughout all samples, regardless of the location of the cutting, with the exception of the center cutting for sample 11312-05. A side-by-side comparison of the liquid urine samples and the Jaffe test results reveals a potential association between the intensity of the color of the urine void (Table 2) and the speed and intensity of the color development of the samples using the Jaffe test. Urine voids that appeared more yellow than orange (in effect, more light than dark) seemed to coincide with weak positive and negative Jaffe results. This trend may have occurred due to the theory that dehydration leads to an increased accumulation of other components of urine, including proteins and other biological elements, in addition to a darker appearance of the urine void.¹⁸ In essence, the urine voids in this study which appeared darker may have had a higher concentration of creatinine (as well as

other urinary components), leading to increased interactions with picric acid, and therefore a more intense color change. In addition, a higher concentration of creatinine would also be directly proportional to the speed at which the color development occurs due to an increased opportunity for interaction.

4.1.2 Uritrace[®]

Like other urinary constituents, creatinine levels can vary substantially between individuals and between urinary voids from the same individual. Creatinine is present in urine at much higher levels than other body fluids; therefore manipulation of sensitivity thresholds can lead to more accurate urine identification. However, due to varying creatinine levels within and between individuals, false negatives (or Type I errors) can occur in samples containing low amounts of creatinine. To control for such errors, adjustments to extraction buffer volume may be a more effective way of improving the accuracy of creatinine detection in urine, while consequently increasing the sensitivity of the test.

The Uritrace[®] test is designed to qualitatively detect creatinine in suspected urine stains. The test comes in the form of a plastic cartridge which contains two chemically-impregnated reagent strips along which the samples migrate laterally and independently. The chemical composition of the reagent strip is proprietary, though a representative of the manufacturer stated that an optimized form of picric acid is used (personal communication). Two areas labeled 1 and 2 are situated on the card to designate where the test sample and

negative control are deposited. When performing a test, the resulting color change on the reagent strips is typically light to dark orange. A difference in intensity between the colors in the two lanes is what determines the outcome of the test.

Results from this study were obtained for Uritrace[®] buffer volumes of 150 μ L, 200 μ L, and 250 μ L. The frequency of positive results decreased as the extraction buffer volume increased to that of the manufacturer's recommended volume of 250 μ L. Extractions using 150 μ L of buffer produced 13/16 total positive Uritrace[®] results, while samples using 200 μ L and 250 μ L of buffer produced 8/16 and 6/16 positive results, respectively. This implies that using a smaller volume of extraction buffer does not inhibit the urine stain extract from traveling up the reagent strip and can still effectively extract enough creatinine from the urine stain to elicit a positive reaction. For both the 150 μ L and 200 μ L buffer extractions, positive results were observed more often with edge cuttings than center cuttings, whereas for the 250 μ L buffer extraction, both center and edge cuttings exhibited the same number of positive results. No trends could otherwise be detected in the results. In sum, creatinine does not appear to preferentially travel to the periphery of the stain or remain concentrated in the center of the stain.

The Northeastern Illinois Regional Crime Laboratory (NIRCL) performed a similar study using one sample of urine to assess whether the location of the cutting obtained from the stain had an influence on the readings produced by the

Uritrace[®] card. Using the manufacturer's recommended extraction buffer volume of 250 μ L, positive results were obtained with both center and perimeter cuttings. NIRCL concluded that the test's performance is unaffected by the area of the stain in which a cutting was collected for analysis. The data from the present study is in agreement; however, the research leading up to this conclusion differs as a result of the greater sample size.⁵⁶

It is of interest to note that some samples exhibited negative results with 200 μ L of extraction buffer, but positive results with 250 μ L of buffer. For example 11312-02, an edge cutting diluted in 200 μ L produced a negative result whereas an edge cutting diluted in 250 μ L produced a positive result. This pattern also occurred for the center cuttings of 11312-04 and 11312-05. Theoretically, the more diluted a stain, the less likely the target molecule is detected due to its limited presence in the solution. Therefore, likely explanations for these unusual outcomes could be that human error may have been introduced at the level of extraction or application to the Uritrace[®] card, or an uneven distribution of creatinine may exist, even within the same area of the stain.

4.1.3 RSID[™]-Urine

Rapid Stain Identification[™]-Urine is a lateral-flow immunochromatographic strip test designed to detect the presence of the Tamm-Horsfall protein (THP). It is reported to detect as little as 5 μ L of human urine, but no definitive level of sensitivity has been determined due to the variability of THP concentration

between individuals.⁵² Its mechanism is based on antibody-antigen binding using rabbit polyclonal anti-human THP antibodies.

For the preparation of urine stain samples for RSID™-Urine, the manufacturer suggests a 1-2 hour extraction period for extraction. In this study, the samples were given the maximum 2 hours for extraction, where the urine samples were placed in a microcentrifuge tube with the provided extraction buffer. The extraction buffer is designed to efficiently extract THP from the stain and to maintain the extract at the appropriate pH in order to facilitate proper functioning of the strip test. After the extraction period, approximately 100µL of each urine stain extract was added to the sample well of the strip. The strip itself is composed of overlapping components (conjugate pad, membrane, and wick). These three components are assembled in such a way that the sample fluid can be transported from the pad to the membrane and eventually be retained on the wick. Both the conjugate pad and the membrane are pre-dispersed with blue latex beads conjugated to mobile rabbit anti-human THP polyclonal antibodies. The extraction buffer acts as a diluent and dissolves these latex beads as it diffuses up the membrane. When an extract containing urine is added to the sample well, the polyclonal antibodies in the membrane will selectively bind to the THP in the sample. This bound complex will migrate up the membrane to the test region of the strip, where a set of immobile rabbit anti-human THP antibodies will capture the complex, creating a blue line. Uncomplexed blue latex bead-conjugated rabbit antibodies will move past the test region to the control line and

bind to the anti-rabbit immunoglobulin present in the control region. A blue line in the test region within 15 minutes indicates the presence of human THP, while a blue line in the control region is indicative of a properly working test strip. If functioning properly, the test strip will always produce a line in the control region.⁵² These principles were applied to the urine sample extracts analyzed in this study.

Analysis was performed using 72 μ L, 96 μ L, and 120 μ L of extraction buffer. It was observed that all center cuttings, irrespective of extraction buffer volume, produced positive results. These results indicate that as little as 60% of the manufacturer's recommended volume of extraction buffer is capable of drawing the sample up the test strip and is sufficient for generating accurate positive results. Additionally, negative results were produced by all edge cuttings, suggesting that the THP molecule is centrally located within the center of the urine stain. Knowledge of where THP is preferentially deposited within a dried urine stain can aid analysts who are using RSID™-Urine in selecting the most probative area in which to test.

4.2 Part II: False Positives for Creatinine and THP

4.2.1 Jaffe and Uritrace® tests: Glucose and other sugars

The Jaffe test is known to give positive results with substances other than urinary creatinine. Picric acid has been shown to have a weak interaction with glucose and other sugars.^{58,59} Benedict et al.⁵⁹ determined that creatinine

interacts with the starting reactant, picric acid, and sugar reacts with an intermediate form of picric acid.

This study sought to explore the extent of the cross-reactivity between glucose and other sugars with picric acid. Cross-reactivity refers to non-specific or unintended interactions between two substances, which in this case are the Jaffe reagents or Urित्रace[®] reagent strips and the non-urine fluids. Table 11 lists the four beverages used in this study, as well as information regarding the relevant sugar molecule contained in the beverage.

Beverage	Relevant Sugar Molecule	Composition of Sugar
Lemon-Lime Powerade[®]	High fructose Corn Syrup	Glucose and fructose monosaccharides
Nantucket Nectars[®] Lemonade	Sucrose	Glucose-fructose disaccharide
Coors[®] Light Beer	Maltose	Glucose-glucose disaccharide
Red Bull[®] Energy Drink	Glucose	Glucose monosaccharides

Table 11: False positives for urinary creatinine: beverages containing different forms of glucose.

The manufacturer's protocol for urine stains was followed for the analysis of the beverages.⁵¹ Using the Jaffe test, positive results were obtained in all edge cuttings, although the color change was weak for Lemon-lime Powerade[®]. Red Bull[®] was the only beverage to produce strong positive Jaffe results for both center and edge cuttings, whereas beer produced weak positive results for both.

Using the Uritrace[®] test, positive results were again observed for both cuttings for Red Bull[®], but in only one of two cuttings for the other three beverages. Notably, only the edge cutting of the lemonade produced a positive result using the Jaffe, whereas only the center cutting produced a positive result with the Uritrace[®] card. This discrepancy may possibly be attributed to human error; however, the extent of distribution of the sucrose in the stain may also play a role. The samples that generated positive results in this study may have been extracted from areas where more sucrose molecules settled upon drying of the liquid on the substrate.

Lemon-lime Powerade[®] produced positive results only when the edge of the stain was tested. This may indicate that the fructose and glucose travel with the liquid as it spreads out on the fabric, and is preferentially deposited near the perimeter of the stain. Red Bull[®] and beer, which contain uncomplexed glucose and maltose, respectively, do not appear to localize at any particular area in the stain. All four beverages demonstrate an affinity for picric acid, implicating possible complications for analysis of questioned urine stains if these beverages were present at a crime scene. It must be noted that although glucose and its variants are postulated to be reacting with picric acid, other components of the beverages may also be reacting with the picric acid to produce a color change.

Two previous studies reported that Sprite[®], a commonly consumed soft drink containing high fructose corn syrup, produced a positive Uritrace[®] result.^{56,57} The authors did not specify which region of the Sprite[®] stain was

sampled, thus it cannot be determined if the findings were entirely consistent with those from the high fructose corn syrup beverage (Powerade[®]) in this paper.

4.2.2 RSID™-Urine: Serum

In evaluating the results, it was apparent that the rabbit anti-human antibodies present in the reagent strip were specific enough to react with only urinary THP as opposed to serum THP, though serum THP levels were not evaluated. The samples derived from the center and edge of the serum stains generated negative results using the RSID™-Urine test. Given the results of Part I in which THP preferentially stayed towards the center of the stain, if a detectable level of THP was present in serum, a positive result should have occurred for the center cutting. However, the results suggest that though THP is present in human serum, THP is not present at a high enough concentration to elicit a positive response using the RSID™-Urine test.

4.3 Part III: Analysis of Urinary Sediment from Aged Stains with Different Storage Conditions

The inability to visualize nucleated epithelial cells in the urine sediment using the described extraction and H&E staining methods reinforces the notion that urine is a difficult matrix for the retention of nuclear material.^{16,40,41,42,45} The buccal cell sample was provided to demonstrate the workability of the procedure and because cells were observed in the slides containing the buccal cells, it can be concluded that the aged urine samples did not contain sufficient levels of

nucleated epithelial cells in the pellet for detection. The absence of nucleated epithelial cells suggests that the stains may have either contained no such cells from the start or that the presence of the cells diminished as the stain aged. Thus, subjecting a sample to any type of environmental insult can potentially accelerate cellular degradation and render the stain unsuitable for DNA analysis.^{16,40,41,42}

4.4 Part IV: ImageJ Analysis of Uritrace[®] Results

The results of the blind verification study revealed conflicting responses by the three analysts for three of the nine samples. Two of the three analysts misreported the result of sample 6 as negative, and one of the analysts who incorrectly responded indicated that she was not confident about her response. To assess such discrepancies, ImageJ analysis was used to quantify the intensity of the colors observed in the Area 1 and Area 2 windows. The method of quantification involved measuring the integrated density of the pixels within the image. 'Integrated density' refers to the concentration of gray pixels within a selected area of an image. The software determines this value by first converting the color of the selected area of the image to grayscale and then counting the number of gray pixels which are present in that area. Theoretically, a high number of pixels in an area signifies a more intense or "dark" color. For example, a cardinal red image would have a higher pixel count than a yellow image

because it is perceived as being darker than yellow. Therefore, in grayscale, the red image would have more gray pixels than the yellow image.

The difference between the intensity of the color in Area 2 (the control area) and the intensity of the color in Area 1 (the test region) was calculated and the sign and magnitude of each difference were assessed. A negative sign in the difference column meant that the color in Area 1 was of lower integrated density, or intensity, than the color in Area 2; this should correspond with a negative assay result. A positive sign in the difference column indicated the color in Area 2 was of lower intensity (lighter) than the color in Area 1, and would be expected with a positive result. The numerical value in the difference column indicates the magnitude of the difference between the areas. A value close to zero would imply that the colors in the two areas were almost identical, and would be expected to correspond with a potentially ambiguous or difficult-to-read test result.

It is interesting to note that although a negative value should theoretically correspond to a negative test result, it was not the case in three out of five samples. Observation of the descriptive statistics in Table 9 for the negative results shows that the standard error is over three times the mean value ($-1,692.2 \pm 5,775.82$ pixels), which indicates that some results expected to appear negative do possess a positive integrated density difference. A similar discrepancy arises in the positive (urine) results, where one out of four positive samples (sample 6) yielded a negative integrated density difference. Relative to the other difference values in the set of responses, the difference value for

sample 6 has the smallest magnitude and is therefore closest to zero, indicating only a minor difference between the intensities of the color in Area 1 and the color in Area 2. Such a small difference in pixels can lead to misinterpretation of the result, as was witnessed in the study.

In addition to sample 6, there was a discrepancy with at least one of the analysts' responses for samples 4 and 7. Sample 4 was a true negative result (water; -4,897 pixels) and it was the second to smallest result in magnitude within the integrated density differences determined to have a negative value. Only one of the three analysts reported the result incorrectly, though all three analysts reported uncertainty in their responses. One might make the argument that there exists a magnitude threshold where perhaps the human eye cannot depict an intensity difference between two very similar colors; however, the misinterpretation of sample 7 contradicts that idea. Sample 7 exhibited an integrated density difference of 22,118 pixels, the second to largest value in magnitude. Such a large value should imply that the intensity difference is relatively straightforward, but two analysts incorrectly reported a negative assay result, one of whom with confidence. The disagreement exhibited here is in conflict with the notion that colors are more difficult to interpret when they are closer to zero. This demonstrates that different analysts can read the results differently and such inconsistencies can lead to inaccurate conclusions about the evidence.

When assessing the range of values for each of the two areas with respect to the expected positive and negative values (Table 10), it was observed that Area 2 had a wider range of integrated density pixel values than Area 1 in the samples where Area 1 was expected to yield a positive result (78,986 as compared to 69,226, respectively). The low and high ends of the ranges of these values were similar for both areas, demonstrating that the integrated density pixel values themselves are not reliable indicators of positive or negative results; it is the difference in intensity of these two areas that aids the analyst in interpreting the result of the test. In terms of the range of values for the expected negative (non-urine) samples, the upper and lower tail of Area 1 and Area 2 were very close in value, which was expected since neither area contained urine.

The means of the integrated density values were consistent overall with the expected results. Area 1 had a higher mean integrated density value than Area 2 when the sample (urine) was expected to generate a positive result and when the sample (distilled water) was expected to produce a negative result, the mean values of Area 1 and 2 were much more similar. The standard deviations of the expected positive and negative results were relatively close in value, indicating that the amount of variation was generally similar for Areas 1 and 2.

4.5 Considerations for Each Method

The practical use of each method needs to be assessed in detail. As previously mentioned, an advantage to using the Jaffe test is that the reagents

can be applied directly to the urine stain. Direct application avoids diluting the stain with water or extraction buffers to a potentially undetectable level. In addition to the lack of buffer, the Jaffe test result is obtained much quicker than the other two discussed in this study. The Jaffe test requires the addition of the reagents, then a 15-minute period after which the result is read. RSID™-Urine and Uritrace® both require up to 2 hours for just the extraction period alone.

One disadvantage to using the Jaffe test is the highly reactive nature of picric acid. Aqueous picric acid produced with a volume of 30% or less of water is extremely sensitive to heat, flame, friction, and shock initiation upon drying. The dried product, picrate salts, can pose as a severe explosion hazard when initiated.⁵⁶ Sodium hydroxide has mutagenic effects and can be hazardous to skin and eyes when exposure is prolonged.⁵⁷ Another drawback of the Jaffe test is its tendency to cross-react with substances that do not contain urine, such as sugary beverages.

Uritrace® is easily portable and does not require the use of toxic reagents. Uritrace® has also been shown to be more sensitive than the Jaffe test. The NIRCL validation study showed that Uritrace® could detect creatinine in a maximum dilution of urine of 1:100 as compared to the Jaffe test, which produced a positive up to a maximum dilution of urine of 1:50.⁵¹ The sensitivity of the Uritrace® test also exceeds that reported for RSID™-Urine (1:21),⁶⁴ however, this author's experience with the Uritrace® makes the reproducibility of that threshold hard to assess.

A major disadvantage to Uritrace[®] is demonstrated in Part IV of this study, where the subjectivity of the results can lead to inaccurate conclusions regarding a potential urine stain on a piece of evidence. Subjectivity arises due to the inherent nature of the card itself – it relies on the analysts' ability to distinguish between two highly similar colors in order to determine a positive or negative result. ImageJ analysis showed that the ranges of intensity differences which constituted a positive or negative result overlapped and that in some cases, a negative result resulted in a more intense test area (Area 1) than the negative control area (Area 2). Proper interpretation of the result is an improvement that needs to be further addressed by the manufacturer.

Uritrace[®] also exhibits cross-reactivities with dog and cat urine, as well as glucose-containing liquids.⁵²

RSID[™]-Urine is similar to Uritrace in terms of portability and the absence of toxic reagents. Unlike the Uritrace[®] test, it relies on the appearance of a colored band at a distinct test region, separate from the internal control as opposed to the difference in intensity of two bands. Thus, the RSID[™]-Urine result can be easily interpreted. The manufacturer's validation study reports that even in a mixture of body fluids (e.g. blood and urine), RSID[™]-Urine has the capability to produce a positive result for urine.

The RSID[™]-Urine test, however, is not entirely specific, as it has been shown to cross-react with dog, horse, rat, and gorilla urine. Dogs, horses, and rats are all common pets and thus can be problematic for the interpretation of

RSID™-Urine results. Another disadvantage is that the stain cannot be tested directly, but must first be extracted into solution, potentially diluting the stain beyond the limit of detection.⁶⁴

4.5.1. Cost of Each Method

Method	Approximate cost of reagents/kit	Cost per test
Jaffe Test	Saturated, aqueous picric acid: \$48.25/500mL; sodium hydroxide pellets: \$45.73/100g	\$0.012
Uritrace®	\$99/25 tests/kit	\$3.96
RSID™-Urine	\$150/10 tests/kit	\$15

Table 12: Cost per test for Jaffe, Uritrace®, and RSID™-Urine methods.

Table 12 depicts the overall cost of each method using prices quoted by product distributors. The costs corresponding to the Jaffe test are based on the assumptions that only 1-2 drops of saturated, aqueous picric acid and 1 drop of a dilute sodium hydroxide solution are necessary to run one test. An analysis of the price of each method reveals that the Jaffe test would be significantly cheaper to carry out than the other two methods. Specifically, the Jaffe test is about 330 and 1,250 times cheaper than the Uritrace® and RSID™-Urine assays, respectively.^{63,65}

4.6 Suggestions for Further Research

One area for possible future research is the comparison of the limits of detection of the Jaffe and Uritrace® methods with urine and non-urine samples.

This study showed that glucose and other glucose-containing sugars can elicit false positive results using the two methods for creatinine detection but did not evaluate sensitivity. Analysts may discover that creatinine has a lower threshold for producing a color change when bound to picric acid relative to the threshold for glucose at a specific concentration. In this circumstance, diluting a false positive out of its limit of detectable color change may aid in creating a more specific test for creatinine.

Further research can aim to determine whether a difference exists in the microscopic detection of nucleated epithelial cells between fresh urine and urine stains from the same urine void. Researchers can determine if the progressive decline in useful cellular material correlates with that of the liquid urine samples studied by Vu et al.⁴⁷

In addition, a long-term study could be initialized to track the progress of a set of sample urine stains to determine a relative maximum age of a urine stain at which a positive result could still be detected using any of the three methods discussed in this paper. Goodall et al.⁶⁶ used a radioimmunoassay technique to assess the effects of freezing on salt-free solutions of THP and discovered that changes occurred within the protein structure of THP, subsequently increasing its ability to bind to rabbit anti-human THP antibodies. Theoretically, THP detection should be improved in aged liquid urine as compared to fresh liquid urine, given constant and controllable storage conditions. Further studies should investigate

this concept to determine if a tool like RSID™-Urine could be the method of choice for the forensic analysis of aged urine stains.

Another interesting avenue to explore would be the persistence of creatinine and THP in different substrates. Researchers can observe if a difference exists between detection of each of the target molecules in substrates with dissimilar properties, such as porous and nonporous materials. Researchers can also use a variety of substrates to assess the limitations of the Jaffe, Uritrace®, and RSID™-Urine methods with regards to extraction and detection of their respective target molecules. For example, both research questions would assist an analyst in determining whether a highly absorbent material, such as carpet, is likely to yield a false negative result due to the distribution of THP.

5. Conclusion

A presumptive test for any biological fluid should be sensitive, specific, and simple to use. This study compared three biological screening tests to find the most accurate and efficient method of detection of the presence of urine.

In Part I, the Jaffe test results demonstrated a possible correlation between the visible color intensity of a liquid urine sample and the resulting color reaction. Weak positive results tended to be associated with lighter-colored urine voids. There appeared to be no noticeable pattern in the results when attempting to determine if creatinine localized in the center or edges of a stain. Manipulation of buffer volumes using the Uritrace® test showed that samples extracted with

smaller volumes usually generated a positive result, regardless if the sample was taken from the center or edge of the stain. However, in some instances decreasing the volume of extraction buffer yielded a negative result, thus no conclusive statements could be made regarding buffer volume. Conversely, RSID™-Urine results showed a definite trend. Tamm-Horsfall protein appears to have a preference for migrating to the center of the stain, thus providing investigators with a target area of the stain likely to yield the most probative results when using this assay.

In Part II, the Jaffe and Uritrace® tests exhibited cross-reactivities with beverages containing glucose and glucose derivatives. RSID™-Urine did not cross-react with serum, which reinforces its purported specificity to urinary THP.

In Part III, no nucleated cellular material was detected in the aged urine stains, demonstrating that, either cells were never present at detectable levels in the stains or that the persistence of cellular material in the stains diminished as the stains and frozen urine voids aged. Urine stains are not typically considered a rich source of nuclear material, therefore introducing other detrimental variables, such as age and unfavorable storage conditions, likely further degrade or decrease the little DNA that existed in the fresh stain.

Part IV emphasized the fact that analyst subjectivity can contribute to discrepant interpretations in situations where testing results are not easy to visually differentiate. The Uritrace® test relies on the analyst's ability to distinguish between two regions which exhibit similar color intensities. Assessing

the color intensities using ImageJ software did not resolve the issue of interpretation because the ImageJ values did not always coincide with the actual samples.

Taking into account the advantages and disadvantages to each test, the Jaffe test appears to be the optimum choice for urine detection in a forensic setting when cost is a consideration. The Jaffe test is a simple and inexpensive test to perform that does not require lengthy extraction periods or dilution of the sample stain. When compared to the Urित्रace[®] test, which also detects creatinine, the Jaffe test is easier to interpret. Although RSID[™]-Urine exhibits fewer known cross-reactivities, the kit itself is costly and has a shorter shelf life than the Jaffe test reagents. However, RSID[™]-Urine would be an excellent choice for laboratories that do not experience a high volume of casework where urine identification is prevalent because it is the more accurate and is easy to operate. Urित्रace[®] also has a shorter shelf life than the Jaffe reagents. Overall, from a practical and budget-conscious perspective, the Jaffe test would be the best choice of the three tests for urine screening in most forensic laboratories.

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