Employment-Related Drug Testing of Biological Matrices

MARKERS OF SPECIMEN VALIDITY IN URINE, ORAL FLUID AND HAIR

ROBERT M. WHITE & RUTH E. WINECKER | NATIONAL LABORATORY CERTIFICATION PROGRAM (NLCP),
RTI INTERNATIONAL, DURHAM, NC, *USA* | vwquailsole@comcast.net

Since 1986, RTI has supported the Department of Health and Human Services (HHS) by managing the National Laboratory Certification Program (NLCP). RTI conducts all aspects of the program, including assisting with the development of mandatory guidelines for federally regulated workplace drug testing, the review and assessment of laboratory applications; inspections of applicant and certified laboratories; design, preparation, and distribution, scoring performance testing samples; identifying problem areas, and monitoring corrective actions. These activities are designed to identify issues before they impact drug test results. We operate all aspects of the NLCP, including the inspection program for forensic drug testing laboratories and the manufacture, distribution, scoring, and reporting of proficiency testing (PT) samples. From the beginning of the program to 2024, the NLCP has accredited 148 Laboratories across the US, two in Canada, and one Initial Instrumented Testing Facility (IITF) in Canada and has performed over 4000 inspections and shipped more than 200,000 urine, oral fluid, and hair PT samples. The NLCP publishes a newsletter Drug Testing Matters (DTM) on topics written by subject matter experts that are of interest to the drug testing community. Portions of this article were previously published in the DTM series. More information on the NLCP can be found online at https://forensicrti.org/nlcp/

This is the second of a two-part series on drug testing of urine, oral fluid, and hair. This article provides background on specimen validity testing and identification of adulterated and substituted specimens.

Background

Drug testing is used for numerous administrative and criminal justice purposes. In living subjects, the testing can be performed using urine, oral fluid, or hair. Although drug testing can be used to make certain that a patient or donor is taking a drug as prescribed (e.g., pain management), the preponderance of drug testing is carried out to make certain that none of a predetermined list of drugs/drug metabolites is present, such as in employment-related, driver license re-granting and probation testing. In cases where the donor suspects that they will be positive for a prohibited drug, there is incentive to alter their specimen so that it will be negative but will also appear as an authentic specimen that will not arouse suspicion of specimen tampering.

The current requirements for certification for employment-related urine drug testing under the United States Department of Health and Human Services Substance Abuse and Mental Health Services (U.S. DHHS SAMHSA) Mandatory Guidelines [1, 2] include specimen validity testing (SVT) in addition to drug/drug metabolite testing. Under both Urine and Oral Fluid Mandatory Guidelines, a biomarker is defined as "An endogenous substance used to validate a biological specimen." Biomarkers serve as a basis for SVT regardless of the biological sample submitted for testing. SVT is the analysis of selected endobiological parameters, including biomarkers, of a submitted donor specimen to ensure that the specimen originated from the donor and is not diluted, adulterated, or substituted.

The biological matrix (test specimen) used for testing an individual for drugs/drug metabolites may be urine, oral fluid, or hair. The most commonly used and easiest to adulterate specimen--urine--will be discussed first using an updated version of material from Drug Testing Matters, "Drug Testing in Urine, Oral Fluid, and Hair Part 2: Analysis" [3].

Urine

So long as unadulterated, undiluted urine for drug testing is used for drug/drug metabolite testing, the drug testing process proceeds smoothly from initial testing to reporting a negative result or through confirmatory testing and reporting a negative or confirmed positive. The general process has been described briefly in Part 2 of the "Drug Testing Matters" series May 2019. The process will not be repeated here.

Conversely, if a urine specimen is adulterated or substituted, the manipulation of the specimen may adversely affect initial and/or confirmatory drug tests. To detect manipulation of a urine specimen to determine whether the urine is suitable for drug/drug metabolite testing, SVT is performed on the urine specimen. Usually, SVT is performed contemporaneously with initial drug testing to identify specimens where adulteration or substitution has occurred before proceeding further with the testing process. Table 1 describes general adulteration and substitution methods presented in a previously published "Drug Testing Matters" article on the collection and testing of each matrix [3]. A brief, updated description of substitution methods is presented at the end of this section.

Some of the many substitution products that have been used in place of a donor's urine to defeat urine drug testing are discussed below:

1. Water. This is probably the oldest substitution product. Water can be added to a donor's urine to dilute it, or a donor may ingest excessive amounts of water before a urine drug test. Sufficient dilution with water will produce a negative immunoassay test where the drug/drug metabolite is present in borderline concentrations but usually will not produce a valid creatinine-specific gravity combination, resulting in a dilute, invalid or, in extreme cases, a substituted result.

TABLE 1. ADULTERATION AND SUBSTITUTION. (4,5)

Attempt at Defeating Drug Test	General Class of Substance Used
Adulteration	Acids (e.g., HCl)
	Bases (e.g., NaOH)
	Oxidants (e.g., bleaching agents)
	Cross-Linking Agents (e.g., glutaraldehyde)
	Sequestering Agents (e.g., Visine®)
	Miscellaneous (e.g., soaps, detergents)
Substitution	Water
	Salt Solutions
	Household Products
	Homegrown Formulations
	Commercial Substitution Products
	Miscellaneous

Deionized and distilled water usually cannot be used as substitutes for urine because most immunoassay analyzers sense deionized and distilled water as air or "no sample." Before it can be submitted by the donor as a substitute for urine, water must be warmed to an acceptable temperature. Additionally, unlike authentic urine, water is colorless, which is often remedied by adding a small amount of yellow food coloring.

2. Salt solutions. Although salt solutions such as normal saline can resolve analytical issues like low specific gravity, they do not circumvent the problem that no creatinine will be found in the substituted specimen. Furthermore, just like water, salt solutions must be colored and warmed to body temperature before being submitted by the donor to the collector.
3. Household products. Numerous products such as sodas and sports drinks can provide the correct color to match urine, especially when diluted with water. However, substances such as diluted colored drinks may or may not contain creatinine or a substance that reacts like creatinine. Additionally, these products usually do not contain other naturally occurring urine components such as uric acid and steroids common to both sexes. Thus, common household products may or may not provide an acceptable substitute for urine, even if warmed properly prior to submission to a collector.
4. Homemade formulations. Numerous urine substitute formulations and recipes can be found on the internet. One product encountered by the author was simply undistilled vinegar, which had the proper color and contained a substance that reacted like creatinine in the Jaffé reaction, the creatinine test method. When the donor's employer added pH testing to their SVT panel, the donor added a small amount of baking soda to neutralize the pH. The donor, who was a cocaine user, was later caught when he bragged to his fellow employees about how he "beat the drug test."
5. Commercial substitution products. Several commercial manufacturers offer both "clean" authentic urine and synthetic urine products. Early versions of synthetic urine lacked components of human urine, enabling laboratory testing to detect the substitution products. However, some manufacturers have been able to remedy deficiencies by adding missing components, including uric acid, which is difficult to solubilize. Manufacturers also provide items to mask substitution products at the collection site. Examples include heating devices (e.g., handwarmers, heating pads) that warm the product to an acceptable temperature and devices to hide and dispense the product (e.g., belts with bags worn under clothing, syringes, prostheses for use during observed collections). Substitution products and their delivery systems appear to be improving with each iteration of the product since the initial publication of substitution publications [6].

One laboratory has investigated uric acid and magnesium testing to detect substituted specimens, screening all specimens for uric acid and reflexing positive specimens to additional testing for both uric acid and magnesium [6]. Using this SVT scheme on a random sample of more than 400 urine specimens, the laboratory identified 1.5% of specimens with abnormal uric acid/magnesium results, which were verified as synthetic urine.

This subsection does not provide a complete review of urine adulteration and substitution but demonstrates that numerous methods exist to suborn urine drug testing via adulteration and substitution. Such methods support supplementing or supplanting urine drug testing with other test matrices.

Based on SVT results, a laboratory may report a specimen as negative, negative-dilute, invalid, adulterated, or substituted. If a specimen is also positive for a drug, the laboratory reports all non-negative results (including positive-dilute) [1]. Examples of substances used to adulterate or substitute a urine specimen were enumerated in Part 2 of the "Drug Testing Matters" series and are briefly repeated with some updating below.

Please see Table 2 for updated reporting examples when a primary specimen (Bottle A) is reported as invalid, adulterated, or substituted. HHS-certified laboratories report specimens as invalid when abnormal physical characteristics or test results (i.e., drug test or SVT) indicate adulteration, but a specific adulterant cannot be identified. As an example, one laboratory notified SAMHSA of an isolated group of urine specimens which were reported as invalid due to abnormal physical characteristics (i.e., a large amount of gray sediment and an odor of decaying vegetation). SAMHSA authorized the laboratory to submit the specimens to RTI International for additional testing. Although it was obvious that specimen tampering had occurred, none of the original or additional tests could determine the adulterant(s). In another example, a separate group of specimens was reported as invalid based on a similar abnormally low pH (approximately pH 4.5). This appeared to be a failed subversion product where the manufacturer did not adjust pH back to the acceptable range after urine pH was modified with an adulterating agent. A few years later, many laboratories reported an increased number of invalid specimens with an abnormally high pH (pH ≥10), which appeared due to another failed substitution product. While an invalid report usually requires that the donor provide another specimen under direct observation, consequences for invalid reports are less severe than a report of substitution or adulteration, which constitutes a refusal to test and leads to adverse employment actions against the donor. Table 2 summarizes SVT reporting requirements for federally regulated urine specimens [1].

The federal program requires the collection of both an "A" and a "B" bottle. The B bottle is primarily reserved for instances when the two bottles have to be redesignated (e.g., Bottle A seal inadvertently broken) or the donor contests the results of the A bottle. For urine, the Mandatory Guidelines require testing

TABLE 2. REQUIRED REPORTING FOR FEDERALLY REGULATED URINE SPECIMENS

Test Result	Required Comment on Report[a]	Note
Negative for Drugs and Dilute[b]	Creatinine = (numerical value) mg/dL & SpGr = (numerical value)	Drug tests are negative, creatinine ≥ 2.0 & < 20 mg/dL and SpGr > 1.0010 & < 1.0030. Instrumented Initial Testing Facility (IITF) forwards to certified lab if creatinine ≤ 5.0 mg/dL
Positive for Drugs and Dilute[b]	(Specify drug analyte) = confirmatory test quantitative result; Creatinine = (numerical value) mg/dL & SpGr = (numerical value)	One or more positive drug analytes, creatinine ≥ 2.0 & < 20 mg/dL and SpGr > 1.0010 & < 1.0030
Adulterated	pH = (conf. test value)	pH < 4.0 or ≥ 11.0 (within the range of controls in the batch)
	Nitrite = (conf. test value) mcg/mL	≥ 500 mcg/mL nitrite
	Surfactant Present; dodecylbenzene sulfonate = (conf. test value) mcg/mL	≥ 100 mcg/mL dodecylbenzene sulfonate
	Chromium (VI) = (conf. test value) mcg/mL	adulterant ≥ limit of quantification (LOQ)
	(Specify Halogen[c]) = (conf. test value)	
	Glutaraldehyde = (conf. test value) mcg/mL	
	Pyridine = (conf. test value) mcg/mL	
	(Specify Adulterant[d]) Present = (conf. test value)	
Substituted	Creatinine = (conf. test value) mg/dL & SpGr = (conf. test value)	
	(Specify biomarker[e]) = confirmatory test value	
Invalid	Creatinine < 2 mg/dL & SpGr Acceptable	SpGr > 1.0010 & < 1.0200
	SpGr < 1.0010 & Creatinine ≥ 2 mg/dL	
	Abnormal pH = (pH value supporting the invalid result)	pH ≥ 4.0 & < 4.5 or pH ≥ 9.0 & < 11.0
	Nitrite = (conf. test value) mcg/mL	Nitrite ≥ 200 & < 500 mcg/ mL on confirmatory test
	Oxidant Activity = (≥200 mcg/mL nitrite-equivalents, ≥50 mcg/mL Cr VI-equivalents, or ≥ halogen or other oxidant LOQ)[f]	Oxidant = nitrite, chromium VI, halogen, etc.
	(Specify confirmatory drug test method) interference	Drug analyte(s) must not be included on reports for invalid results based on assay interference
	(Specify initial drug test method) Interference[f]	
	Possible (characterize as Aldehyde or Surfactant) Activity[f]	
	Abnormal Physical Characteristic – (Specify)	
	Bottle A and Bottle B – Different Physical Appearance[f]	

Table adapted from Table U-2 in the National Laboratory Certification Program (NLCP) Manual (7).

[a] Remarks on custody and control form or CCF & on elec. report for primary specimens; Remarks on CCF/Split Specimen Report & on elec. report for split specimens. Labs and IITFs may add explanatory comments in addition to these required comments.

[b] A dilute finding is reported as a comment only in conjunction with a negative or positive result. Laboratories must report the creatinine and specific gravity values supporting a dilute finding (i.e., negative-dilute and positive-dilute) when creatinine ≥ 2.0 & < 20 mg/dL and SpGr > 1.0010 & < 1.0030

[c] Specify the halogen detected

[d] Specify the adulterant detected

[e] Specify the biomarker and critical value

[f] Lab shall contact the Medical Review Officer (MRO) to discuss the Invalid Result in accordance with the U.S. HHS SAMHSA Guidelines (82 Fed. Reg. 7920) section 11.19.g.

Note: See National Laboratory Certification Program (NLCP) Manual (7) for further guidance: IITF Checklist Question E8I and Laboratory Question E9I, CCF Decision Trees.

urine in the A Bottle for creatinine, pH, and oxidants. When SVT retesting of a B bottle is requested through the medical review officer (MRO), only those substances for which the initial testing is confirmed positive can be performed.

A considerable amount of time and effort has been invested in urine SVT to eliminate adulteration or substitution as a source of a false negative testing result. It is well worth reviewing whether substitution and adulteration methods commonly used in urine drug testing are worth employing when a positive result is anticipated and adulteration or substitution must be used in an attempt to obtain a negative drug test result. From a market survey, 3 adulterants and 32 synthetic urine preparations were identified [6]. Samples underwent preliminary testing including appearance and the Adultacheck 10 dipstick and Synthetic UrineCheck dipstick. When present, an adulterant was usually detected by routine SVT. Conversely, in this study, synthetic urines were difficult to identify using traditional SVT and the synthetic urine dipstick. In direct contrast to the Vikingsson study, an earlier study by Kim et al. [8] demonstrated good performance from the synthetic urine dipsticks. It was further noted in the Vikingsson paper that novel immunoassay reagents targeting urinary tract proteins as second-generation urine SVT are becoming available.

Using a technique known as grounded theory and online posts from both public and dark websites, urine was determined to be the most commonly discussed testing matrix [9]. The conclusion for substituted urines is that further study at the point of collection and/or in the laboratory is warranted.

Oral Fluid

Recently updated U.S. DHHS SAMHSA Mandatory Guidelines on oral fluid employment-related drug testing allow SVT but do not require it [2]. Because all oral fluid collections should be witnessed in their entirety, the topic of SVT is considerably less important for oral fluid than it is for urine. Adulterating or substituting a collected oral fluid sample is difficult. Only the following methods, which have limited possibility of success, could be identified [10]:

1. A donor coercing or compensating a collector to allow the donor to dilute a specimen or add chemicals deleterious to the testing process.
2. A donor coercing or compensating a collector to allow the donor to substitute the donor's specimen with a drug-free specimen that appears to be oral fluid.
3. A donor placing into their oral cavity a substance or substances that will interfere with the laboratory analysis.
4. A donor placing into their oral cavity a substance or substances that will dilute the specimen so that the drug/drug metabolites will be below the cutoff for a positive.

TABLE 3. GRADING SYSTEM FOR POTENTIAL ORAL FLUID SVT MARKERS

Parameter	Potential Marker					
	Amylase	AST	LD	IgA	Total Protein	Albumin
Analytical Range[a]	−2, requires 1:500 predilution	0, low end but viable	+2, fits well with neat oral fluid and collectors	+2	+2	+1, low end but viable
Transportability[b]	+1, stable enzyme, may fluctuate with mucin precipitation	+1, stable enzyme, but still a protein	+1, stable enzyme, but still a protein	+1, immunoglobulin, stable but still a protein	+1, stable but may fluctuate with mucin precipitation	+1, stable but still a protein
Cost per Test[c]	−1	−2	0	−1	+2	+1
Routine Measurement[d]	−2, easily integrated but requires predilution (offline handling)	+1, should be easily integrated, decreasing 340 signal rather than increasing	+2, should be easily integrated, increasing 340 signal if proper kit selected	−2 if ELISA, +1 if nephelometric	+2, should be easily integrated	+2, should be easily integrated
Discriminatory Power[e]	+2, more than ample analyte present	0, questionable; needs further study	+1, ample analyte appears to be present in neat and diluted oral fluid.	+2, more than ample analyte appears to be present from pre- study	+1, maybe a +2, but needs further evaluation	+1, appears to be at its low end of sensitivity from study, needs further evaluation
Influence of biological variables (needs further study)[f]	0	0	+1, housekeeping enzyme, ubiquitous	0, may fluctuate with inflammation	+1, appears reasonably stable, but may fluctuate	0, one paper says fluctuates with dentation
Overall Rating	−2	0	+7	+2 (+5)	+9 (+10)	+6

[a] Basis is a good match between the routine analytical range and the amount or activity of the analyte in both neat and 1:3 (Oral-Eze and Intercept) or 1:4 (Quantisal) dilutions.

[b] Based on serum, plasma, urine, or serum and body fluid amylase: 14 days room temperature; urine amylase, 14 days refrigerated. Serum aspartate aminotransferase, 7 days or 48 hours −4°C; Serum lactate dehydrogenase, 14 days room temperature. IgA 72 hours room temperature. Total protein 14 days room temperature. Albumin,7 days room temperature. Any SVT analyte chosen for further study also should be reviewed for transportability at room temperature using oral fluid and in the buffer/preservative selected by the laboratory.

[c] Cost per test was calculated using a discounted price to client divided by 3 since a rough clinical formula is expendables × 3 = total cost, including capital, expendables, labor, and overhead. The actual price for a given lab will vary with reagent manufacturer and volume discounts. The best cost per test (albumin at $1.38/test) was a +2. The most expensive test (IgA at $7.29) was a −2. Other test values were calculated as a point on that scale and rounded to the nearest whole number.

[d] Based on whether the marker offline (analysis separate from oral fluid drugs on an immunoassay analyzer) or online (analysis concurrently with oral fluid drugs on an immunoassay analyzer) and any other steps such as predilution.

[e] Ample separation between the lower limit of the oral fluid reference range and substituted.

[f] From pre-study and clinical experience only. The effect of biological variables such as diurnal variation, state of hydration, psychosomatic variation, and disease states needs to be proven in an extended study.

If given the opportunity, the donor can modify the oral fluid collection kit; numerous ways of suborning the drug test such as substituting water for oral fluid exist. However, numerous oral fluid markers to catch such tricks also exist. When SVT is required for employment-related oral fluid drug testing, there exists a plethora of biomarkers suitable to determine the acceptability of a submitted oral fluid specimen and useful in the production of large batches of oral fluid initial drug testing as currently is performed for urine submitted specimens.

As discussed in Part 1 of this series, IgG and albumin are found in measurable amounts in oral fluid. In the current Oral Fluid Pilot Proficiency Testing Program, at least one laboratory used IgG but no longer does and one laboratory previously used albumin as an SVT marker for one collection device and transitioned to total protein in 2023 for another device [11].

In a pre-study (approved by both the Wake Forest University and RTI International Institutional Review Boards) conducted to identify potential oral fluid SVT markers, Friesen et al. [12] collected neat oral fluid in salinized Biophor (RapidEASE®) collectors and three different pad-type collectors (first-generation Intercept®, Quantisal™, and Oral-Eze®). A contemporaneous urine specimen was collected only to determine if donors were potentially over- or underhydrated. No drug testing was performed on the urine or the oral fluid samples [12, 13]. Essentially, samples were collected and transported to the testing laboratory on the same day. Testing for routine biomarkers (microalbumin, amylase, alkaline phosphatase or ALP, aspartate transaminase or AST, creatine kinase or CK, inorganic phosphate, lactate dehydrogenase or LD, lipase, potassium, total protein, urea, and uric acid) was performed using a high-throughput analyzer (Beckman DXC 800). Immunoglobulin analysis (IgG, IgA, and IgM) and urine creatinine was performed using ELISA on a Zepto Metric. Any required specific gravity measurements were made using the Siemens Clinitek. An aliquot of each collected oral fluid was diluted 1:3 with 0.9% saline and held in reserve in case of technical problems with any collected oral fluid. Inorganic phosphate, total protein, and potassium were ruled out as potential markers because of their presence in either the Oral-Eze buffer/preservative or the Quantisal® buffer/preservative. Although uric acid was eliminated as a candidate marker because it was below detection in two of the five neat oral fluid samples, it was reconsidered as a marker if a lower cutoff could be realized. Although amylase did not appear to demonstrate stability in one neat sample upon refrigeration, amylase appeared to be present in high activity in both neat oral fluid and oral fluid collected in a "pad device," as anticipated. IgA was also present abundantly and appeared to demonstrate stability. Table 3 details a grading system developed by Friesen et al. [12] and Wong and White [13] to evaluate potential biomarkers. It is well worth noting that even though amylase received a low rating because of the need to dilute the sample to bring the activity of most samples into a measurable range, most clinical assays for drugs/drug metabolites require a dilution that may be sufficient to use amylase as an SVT marker. Furthermore, the only interest would be in low amylase activities, not elevated activities. Uric acid probably should be considered further if a lower cutoff can be attained methodologically. Please see the use of uric acid and Magnesium for urine SVT, discussed previously.

If tampering with an oral fluid specimen can occur, two types of specimen containers must be considered—direct expectoration and a "pad-type" collector. For a direct expectoration collector, oral fluid may simply be substituted with water or a saline solution. Unless an error is detected by the instrument (e.g., deionized or distilled water was used and detected as air by the immunoassay analyzer) or an oral fluid SVT marker is included in the initial testing, a colorless fluid such as water or saline will produce a negative result. For a pad-type collector, the pad can simply be immersed in water or a saline solution yielding a negative result unless an SVT marker is included in the laboratory's initial test.

If properly applied, the use of an SVT marker in oral fluid drug testing can be quite useful in the small number of instances when an individual is successful in suborning an oral fluid drug test. Not surprisingly, the number of oral fluid tests discussed with respect to attempts to suborn the test is less than urine or hair due to the low number of oral fluid tests performed relative to urine and hair and the difficulty involved in tampering with the collection process [9].

Hair

Like oral fluid, hair for drug testing is extremely difficult to adulterate if collected properly. However, there are methods such as weaves where one person's hair is woven into another's. Synthetic hair may also be combined with real hair. In both of these cases, an observant collector can notice either and cut the hair for testing from a different area or the weave can be noted during the hair weighing process at the laboratory [14]. In the web monitoring study examining adulteration and substitution methods, hair drug test subversion strategies fell into three categories: substitution as discussed above, hair treatments such as bleaching, and hair removal [9]. By far, the most common method discussed in the study was hair treatments such as bleaching or perming.

Perhaps of greatest concern is the porosity of the hair cut for testing [15]. Increased hair porosity affects contaminants (such as external drug) entering the hair and compounds present from actual ingestion being lost from the hair. Studies examining the effect of cosmetic treatments on drug concentrations in hair are limited but, in general, show that these processes result in lower drug concentrations [16, 17].

Although the actual laboratory protocol and chemistry is complicated, several methods have been identified to determine hair porosity. Methods for determining the porosity of hair submitted for drug testing include staining with methylene blue, observing the dissolution of hair in a non-proteolytic system containing dithiothreitol (DTT), observing the rate of dissolution in a solution containing DTT and proteinase K, and measuring protein leakage from hair samples when exposed for 2 hours in a non-proteolytic system containing DTT. A hair integrity testing system (scale of 0–8, with 8 being untampered hair) and a wash system to determine categories such as "Negative/Contaminated" and "Negative" has been developed [15]. Recently, novel methods for detecting potential biomarkers for cosmetically altered hair samples using untargeted hair metabolomics have been proposed [18].

Again, due to the difficulty involved in tampering with the collection process and the lower number of hair tests performed relative to urine tests, the number of discussions involving the possibility of suborning the hair test is less than that for urine [9].

Conclusions

Numerous SVT methods currently exist for urine that is to be tested for drugs/drug metabolites. If synthetic urine can be smuggled into a collection site and successfully substituted for a donor's real urine, they are becoming more and more difficult to detect without specialized testing as synthetic urine manufacturers become more and more astute in producing a useful product. Improvements in the collection site process and in laboratory testing for synthetic urine may be necessary.

Oral fluid and hair for employment-related drug testing are collected as observed specimens. Thus, SVT is less likely to be required for either oral fluid or hair. However, if SVT is required for a hair or oral fluid specimen, effective SVT methods for both types of specimens exist and can be employed in a drug testing laboratory.

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