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"Pee Value": Storing Urine for Subsequent DNA Analysis

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ABSTRACT

Drug abuse is a prevalent problem in the Philippines. With the increased drive to apprehend individuals who partake in the use of illegal drugs, there is a need to re-examine the handling and storage procedures for urine samples, which may be tested to prove or disprove allegations of drug use. With the availability of forensic DNA technology as the most powerful tool for human identification, the inclusion of DNA testing in decision trees used by law enforcers and government laboratories during drug investigations is expected to improve the process of determining actual drug users while promptly addressing allegations of misconduct. Because DNA testing is a relatively novel procedure previously not considered in drug investigations in the Philippines, there is a need to test whether storage procedures for urine that showed positive results allow for subsequent DNA testing. Samples that test positive for drugs are routinely stored at -20°C for up to one year prior to disposal. In this study, urine DNA samples were extracted from 20 male individuals. The samples were subsequently stored at room temperature, 4°C, and -20°C for 2 months and 9 months. This was followed by DNA profiling using the PowerPlex® 21 System. Overall, DNA extracted from urine samples stored at cool temperatures (4°C and -20°C) were found to provide more consistent DNA profile results compared to samples that were stored at room temperature. We propose here a decision tree for drug testing from start to end that should serve as a decision support tool for Philippine government agencies engaged in drug investigations.

Keywords: DNA profiling, short tandem repeats, storage duration, storage temperature, urine DNA extraction

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INTRODUCTION

Use of illegal drugs or substances, such as methamphetamine hydrochloride (known locally as "shabu", "ice", or "meth") and cannabis (or "marijuana"), is a prevalent problem in the Philippines. In 2017, the Philippine Dangerous Drugs Board (DDB 2019) reported 4,045 individuals who were positive for illegal drugs, with the majority of drug users being male (91%). To curb the use of illegal drugs, the Philippine government passed Republic Act 9165 or the "Comprehensive Dangerous Drugs Act of 2002" which prescribes the testing of samples from persons applying for certain types of licenses and/or jobs, as well as suspected drug users. In addition, RA 9165 allows the routine testing of samples from students of secondary and tertiary schools, employees of private and public offices, officers and members of law enforcement agencies, and candidates for public office to be done by government forensic or accredited drug testing laboratories (Comprehensive Dangerous Drugs Act of 2002). Because of the increased severity of punishment meted out to suspected drug users, there is a need to formulate a decision tree to cover all aspects of testing of human urine samples. The decision tree should start from collection to drug testing until the conduct of additional confirmatory drug tests and DNA testing, when required.

Institutions mandated by national governments to stop the illegal use of drugs follow different operating procedures. Hence, the compatibility of procedures for handling and storing urine, including DNA profiling methods, must be tested in each jurisdiction. For example, in the US and Europe, storage temperatures of urine in different laboratories vary from 4°C to -80°C (Brinkmann et al. 1992, Yasuda et al. 2003; Castella et al. 2006; Soltyszewski et al. 2006; Zhang et al. 2012, Devesse et al. 2015). In the Philippines, urine samples that test positive for drugs are stored up to one year at -20ºC. This temperature is known to preserve metabolites that can be detected if urine samples undergo further testing in cases of contention (DOH 2004). Notably, -20ºC is also used for prolonged storage of most biological and DNA samples because DNA degradation and breakdown of biological material are reduced at this temperature.

DNA testing of urine samples to evaluate allegation of sample switching has been reported in other countries (Junge et al. 2002; Soltyszewski et al. 2006). While not all urine samples are required to be authenticated, storage of urine at an ideal condition is still being done should any contention arise (Srisiri et al. 2017). We foresee the same happening in the Philippines, particularly if the proposed Death

Penalty Bill for drug users is passed into law. Autosomal Short Tandem Repeat (aSTR) DNA profiling is commonly used to identify human sources of urine samples (Junge et al. 2002, Marques et al. 2005, Thevis et al. 2007), as well as other types of samples that may be submitted as evidence (Butler 2010). This study reports the testing of urine samples stored at different temperatures (room temperature, 4ºC, and -20ºC) after two months and nine months, in order to determine if the present storage conditions will allow the future DNA testing of urine to resolve allegations of sample switching and laboratory misconduct.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the University of the Philippines Manila Research Ethics Board (UPMREB-2012-0271).

Urine samples

Twenty males and five females between 18 and 40 years provided urine samples for the study. Female urine samples were included as positive controls for DNA extraction. Female urine is known to contain more cells than male urine (Prinz et al. 1993) because of anatomical differences between their urogenital systems. Hence, the inclusion of female samples which are expected to yield sufficient DNA for DNA profiling served as positive control in case the extraction and genotyping of male urine samples provide negative results. Volunteers who were not able to produce at least 160 mL of urine, those with kidney disease or urological conditions, as well as females that were menstruating, were excluded. Blood from menstruation contains DNA from white blood cells which may inflate DNA yield readings. Each donor provided at least 160 mL of urine, which was thoroughly mixed by inverting the tubes several times prior to aliquoting into 16 tubes with 10 mL of urine each. Four urine aliquots were stored in each of the following conditions: room temperature (RT), refrigerated temperature (RefT; 4°C), and freezing temperature (FT; -20°C). Storage temperatures are approximated. Samples are stored in one compartment per storage temperature. DNA extractions were performed on two aliquots of urine after two and nine months post-collection.

Reference samples

To generate the reference DNA profile per individual, male and female volunteers provided blood samples that were blotted on Whatman® FTA® cards (GE Life Sciences). Blood on FTA® samples were extracted following manufacturer's protocol. The samples were amplified using PowerPlex® 21 (PP21) System (Promega) and analyzed using Applied Biosystems® 3500 (AB3500) Genetic Analyzer (Thermo Fisher Scientific) and GeneMapper® ID-X v.1.2 (Thermo Fisher Scientific) software. The aSTR DNA profiles from blood served as reference for comparison with urine DNA profiles in order to assess concordance and % allele recovery.

Processing of urine samples

For each storage condition, 10 mL of urine samples were aliquoted into smaller tubes with approximately 2 mL of urine each then centrifuged at 8000 rpm for 10 minutes to collect the cells. The supernatant was discarded and the precipitate was washed thrice with 200 µL Phosphate Buffered Saline (PBS). DNA per volunteer was extracted in duplicate using QIAamp® DNA Micro Kit (Qiagen) following manufacturer's protocol and eluted using 20 µL of elution buffer. For all extraction events, a negative control without urine sample that served as reagent blank was included.

Measuring DNA quantity and evaluating DNA quality

DNA yield and presence of inhibitor were determined using Plexor® HY Quantitation kit (Promega) and the Applied Biosystem® 7500 Real-Time PCR System (Thermo Fisher Scientific) following manufacturer's instructions. The PP21 aSTR kit, AB3500 Genetic Analyzer, and GeneMapper® ID-X v.1.2 software were used to generate the aSTR DNA profiles. Whenever possible, 0.5 ng of input DNA was amplified in a 10-µL PCR reaction. The exact DNA input amounts are shown in Figures 1 and 2. To generate the DNA profile per volunteer, one DNA extract that did not show inhibition results from the real-time PCR assay was selected for amplification. A total of 120 amplifications were performed using samples from 20 volunteers stored at three temperatures and at two periods.

Figure 1. Heat map of the DNA amplification results using male urine samples after 2 months of storage. Visual representation of the autosomal alleles generated using PowerPlex® 21. PP21 markers are arranged based on the increasing molecular weights (bp) of expected range of amplicon size for a given aSTR DNA marker on the X-axis. Storage temperature, sample name, and amount of input DNA are shown on the Y-axis. Samples within a storage temperature group are listed in decreasing amount of DNA. UT05, UT06, and UT16 stored at RT, either have no DNA or have DNA below the assay's detection limit (approximately 0.001 ng). These samples were amplified to test whether the multiplex PP21 reaction is robust enough to amplify DNA below the Plexor® HY limit of detection.

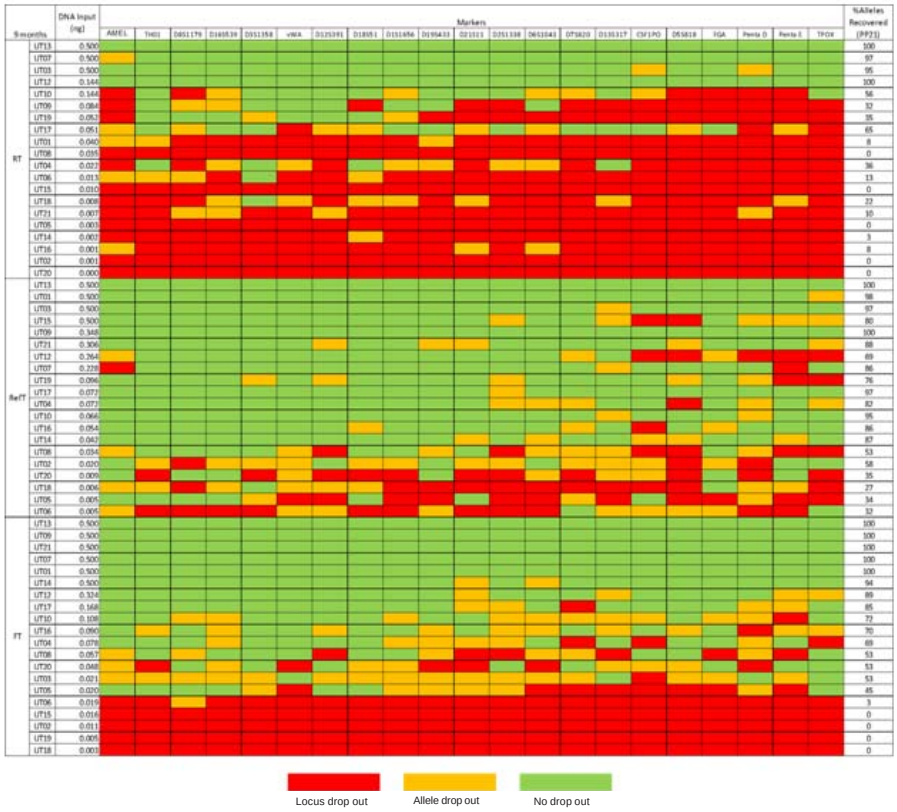

Figure 2. Heat map of the DNA amplification results using male urine samples after 9 months of storage. Visual representation of the autosomal alleles generated using PowerPlex® 21. PP21 markers are arranged based on the increasing molecular weights (bp) of the expected range of amplicon size for a given aSTR DNA marker on the X-axis. Storage temperature, sample name, and amount of input DNA are shown on the Y-axis. Samples within a storage temperature group are listed in decreasing amount of DNA. UT20 stored at RT, either has no DNA or has DNA below the Plexor® HY assay's detection limit (approximately 0.001 ng). This sample was amplified to test whether the PP21 multiplex reaction is robust enough to amplify DNA below the Plexor® HY limit of detection.

DNA quality was evaluated based on allele recovery (AR) expressed as percentage of alleles generated over the total number of expected alleles, and peak height ratio (PHR) when the DNA profile yields heterozygous alleles at that DNA marker. PHR in percent (%) was calculated by dividing the peak height of the allele with a smaller peak by the peak height of the allele with a larger peak, multiplied by 100.

Statistical data analysis

The statistical data analyses were performed using GraphPad Prism® version 6 (GraphPad Software, Inc).

RESULTS AND DISCUSSION

Yield of human DNA and presence of inhibitors

Female urine samples, known to have more epithelial cells per unit volume, which served as controls for the DNA extraction procedure, produced sufficient amplifiable DNA (data not shown). Most male urine samples (96%) did not exceed 1 ng/mL of urine (Figure 3). Statistical analysis using a two-way ANOVA showed that storage time ($p = 0.1922$) and storage temperature ($p = 0.4338$) did not independently and interactively affect DNA yield.

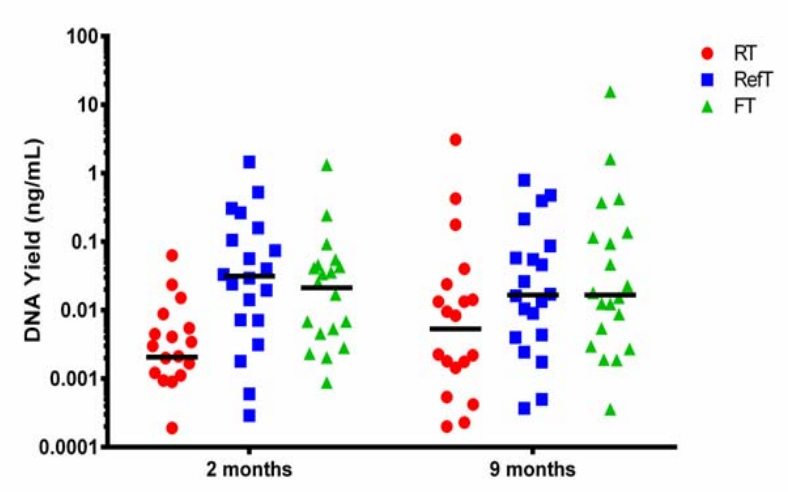

Figure 3. Average yield (ng DNA/mL urine) of human DNA extracted in duplicate using the QIAamp® DNA Micro Kit from 20 male urine samples stored at different conditions. Differences in DNA yield for samples stored for 2 and 9 months ($p = 0.1922$) and at different temperature conditions ($p = 0.4338$) were not significant. Horizontal lines indicate the median for each data set.

In addition to estimating DNA concentration in real-time, the Plexor® HY Quantitation Kit which contains an internal PCR control (IPC) was used to test for the presence of PCR inhibitors. Inhibitors may result in failed amplification, underestimation of DNA quantity in real-time PCR assays and/or reduced allele recovery during DNA profiling (Krenke et al. 2008, Alaeddini 2012). For example, urea in urine is known to inhibit DNA amplification (Khan et al. 1991). In this study, samples stored at RT and FT exhibit less inhibition after 9 months of storage, whereas samples stored at RefT display less inhibition after 2 months (Table 1). The decrease in the number of samples exhibiting inhibition at RT and FT after 9 months may be due to the breakdown of urea and urinary creatinine over time (Spierto et al. 1997, Panyachariwat and Steckel 2014). However, it is unclear why there appears to be less inhibition at RefT at 2 months but the reverse is true at 9 months. A closer study of the biochemical breakdown of inhibitors under different conditions and their effect on downstream DNA testing is recommended.

Table 1. Percentage of samples showing PCR inhibition at 2-month and 9-month storage

Storage Temperature	Percentage (%) of samples showing PCR inhibition	
	2 months storage*	9 months storage*
RT	20	7.5
4°C (RefT)	12.5	15
-20°C (FT)	17.5	7.5

* total of 40 male urine extracts tested

DNA quality

Urine samples stored at RefT and FT were genotyped more successfully compared to samples stored at RT (Figure 4). Based on a two-way ANOVA, storage temperature was found to be a significant source of variation in allele recovery ($p<0.0001$). At two months and nine months of storage, samples stored at RT have the highest incidences of allele drop-outs. At RT, samples with less than 0.1 ng of input DNA in 10 µL volume reaction resulted in <50% allele recovery (Figure 4). At RefT and FT, samples with 0.1-0.5 ng of input DNA have relatively higher rate of recovery at > 80% after 2 months of storage. These results are consistent with the work of Ng et al. (2018) that reported over 90% allele recovery from samples stored at 70 days (~2 months) for up to 100 days (~3 months) at 4ºC and -20ºC. After 9 months of storage, samples stored at RefT have >70% allele recovery, with a lower allele recovery (> 50%) observed for samples stored in FT.

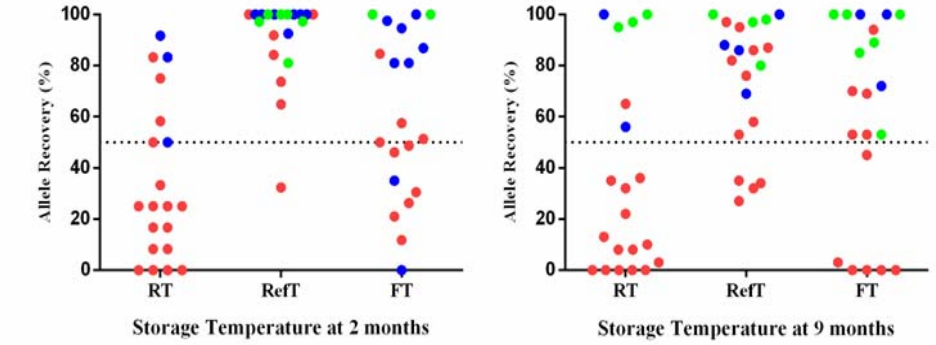

Figure 4. Allele recovery of DNA from urine at different storage conditions. The colors represent the range of the amount of DNA template used in PCR amplification (green: 0.5 ng, blue: 0.1-0.49 ng, red: <0.1 ng). DNA input of less than 0.1 ng generally results in poor allele recovery regardless of storage condition. Allele recovery greater than 50% is observed for RefT particularly at 2 months of storage even at < 0.1 ng DNA input. Storage at FT also resulted in good allele recovery at DNA input of 0.1 ng or higher. Mean allele recovery for 2 months of storage is at 33.7%, 90.75%, and 60.3% for RT, RefT, and FT, respectively. The mean allele recovery for 9 months of storage is 34%, 74%, and 59.3% for RT, RefT, and FT, respectively.

With the amplification of low-level target DNA (<0.1 ng), more stochastic effects (i.e., allelic and locus drop-out and gross peak imbalance (PHR ≤ 50%)) were observed. Allele drop-ins are non-repeated spurious alleles that are detected due to stochastic effects in amplification coupled with the sensitivity of the system used to detect alleles. Allele drop-outs occur when there is preferential amplification of shorter DNA regions, which are made more pronounced when the DNA template has undergone degradation (Caragine et al. 2009; Cowen et al. 2011,;Alaeddini 2012, Gill et al. 2015). Overall, allele drop-ins and allele drop-outs result in erroneous DNA profiles; hence, the need to evaluate results more carefully. A locus drop-out, which is a no result for the particular DNA marker, decreases the informativeness of a urine DNA profile that will be compared with a reference DNA profile (e.g., blood or saliva from the person of interest). In this study, urine samples left at room temperature for 2 months (Figure 1) and 9 months (Figure 2) exhibited considerable degradation compared to urine samples stored at RefT and FT.

Meanwhile, gross peak imbalance at heterozygous DNA markers still provides correct genotype data but is already indicative of the low DNA quality of the sample. A PHR threshold of 60% is typically used to associate and pair alleles into heterozygote genotypes (Butler 2014). We observed that DNA from urine stored at RefT for 2 months have better PHR compared to other storage conditions (Figure 5), whereas urine stored at RT exhibited extreme peak height imbalance. A summary of allele drop-outs and peak height ratios are presented in Table 2.

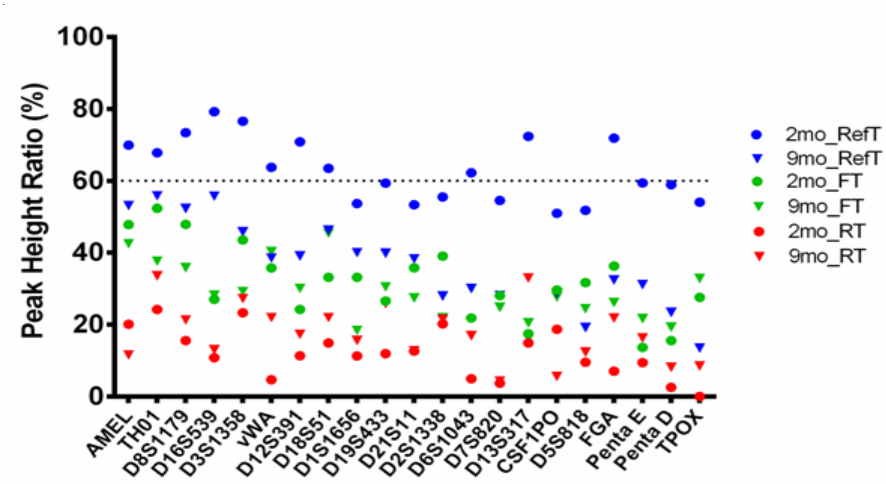

Figure 5. Average peak height ratio per locus (arranged by increasing molecular weight) for samples stored at different storage conditions. A discontinuous line marks the 60% peak height balance threshold.

Table 2. Average allele drop-outs and peak height ratio at each storage temperature and storage period.

Storage Period	Storage Temperature	Average allele drop-out (%)	Average peak height ratio (%)
2 months	RT	66.3	12.0
	RefT	9.25	62.6
	FT	39.7	32.1
9 months	RT	66.0	17.6
	RefT	26.0	36.3
	FT	40.7	29.7

Hence, we support the continued storage of urine samples at FT, as prescribed by the Philippine DOH (DOH 2004), not only to preserve metabolites that serve as targets for drug testing, but also in the preservation of DNA for subsequent genotyping, if the need arises. Drug testing centers may also opt to store urine samples at refrigerator temperatures, if freezers are unavailable or when funding is limited, provided that drug metabolites are also preserved at this temperature.

DNA from urine samples stored at 9 months, regardless of temperature, exhibited highly imbalanced PHR (< 40%). In forensic investigations, DNA profiles showing variable PHR are more difficult to interpret. Our data shows that the storage of urine samples at RefT for 2 months is the best condition for preserving the amount and quality of DNA for genotyping. Hence, there is need for law enforcement and

forensic drug testing laboratories to evaluate if a shortened storage period for urine samples is warranted. Overall, the conduct of all drug tests and DNA testing, when needed to evaluate allegations of sample switching or laboratory misconduct, should be performed as soon as possible. This study demonstrates the applicability of DNA testing for the identification of the source of urine samples in drug use cases when necessary. The result of this evaluation was included in the proposed decision tree for the testing of urine in drug investigations (Figure 6).

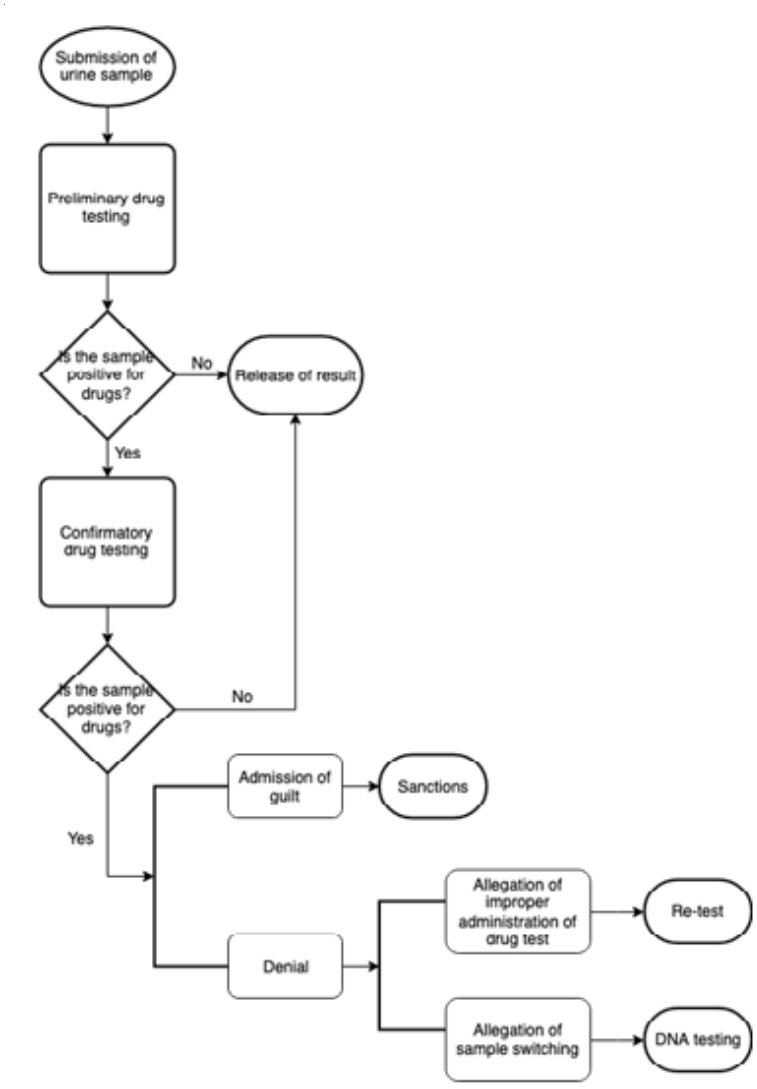

Figure 6. Proposed decision tree for urine testing in drug investigations.

CONCLUSION

Low amounts of DNA exhibiting characteristics of inhibition and degradation were extracted from urine samples stored at varying conditions. Useful DNA profiles were generated for most of the samples, regardless of storage condition. However, limited DNA input (<0.1 ng) was observed to affect allele recovery. Male urine contains a small amount of DNA; thus, the quality of generated DNA profile is affected by stochastic effects, inhibition, and degradation brought about by prolonged storage. While storage duration and temperature were not found to be significant sources of variation in DNA yield, storage temperature evaluated using % allele recovery and peak height balance, was a significant source of variation in DNA quality. DNA extracted from male urine samples stored at cooler temperatures amplified better especially at two months of storage. Thus, we recommend the storage of at least 10-mL aliquots of urine samples submitted for local drug testing at 4°C or lower and the generation of DNA profiles within this prescribed 2-month period, in order to generate good quality DNA profiles.

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