

# Forensic evaluation of DNA typing in equine urine samples submitted to prohibited substances control: applications in controversial confirmation analysis cases

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## SUMMARY

Presence of drugs is prohibited in post racing urine samples by horseracing authorities. In case of a positive result, it may be necessary to confirm the identity of the urine sample. This study was undertaken to verify the reproducibility of equine individual identification from urine samples in a large number of individuals. Some studies have shown that urine samples might represent a potential valuable source of DNA, but the determination of the genetic profile have been problematic due to the poor recovery of amplifiable DNA. Cellular material within urine is contained in a large volume of fluid containing a number of salts and other metabolites that can largely interfere with PCR amplification.

## MATERIALS AND METHODS

### DNA Isolation

Our study analyzed 40 equine urine B-samples corresponding to A-samples obtained from Italian racetracks that tested negatives in screening for prohibited substances in our lab. All samples were collected at least 60 days before this study and kept frozen at -20°C. Frozen urine was thawed at 4°C and mixed vigorously, and then 5 ml of each sample was taken for DNA isolation as suggested by Tobe et al. (2007), with some modifications. DNA was isolated from each sample using two different methods:

- with phenol/chloroform technique, cells are incubated 2 hours at 56°C in 200 µl of lysis buffer (1M Tris/HCl, 1M DTT, SDS 10%) containing 10 µl of Proteinase K 10mg/ml. The lysate was then purified with Phenol:Chloroform (1:1) and Chloroform:Isoamyl Alcohol (24:1). Extracted DNA was then precipitated with cold, absolute ethanol, lyophilized and resuspended in 100 µl of bidistilled water;

- with ChargeSwitch® Forensic DNA Purification Kit, DNA extraction was performed according the manufacturer's protocol (Invitrogen Corporation, CA).

The major limitation of PCR-based tests resides in inhibition of DNA amplification by substances usually found in samples.

In order to remove substances which can inhibit PCR in urine analysis in both procedures, extracted DNA was purified using Montage PCR Centrifugal Filter Devices spin columns (Millipore, Co., USA) and 50 µl of eluate containing purified DNA was obtained.

Recovery efficiency of extracted DNA was evaluated by spectrophotometric analysis at 260 nm, using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., USA). The quality of DNA was evaluated by estimating the ratio of absorbance at 260 nm and 280 nm, with an average of 1,58/1,85 value in 50% of the samples.

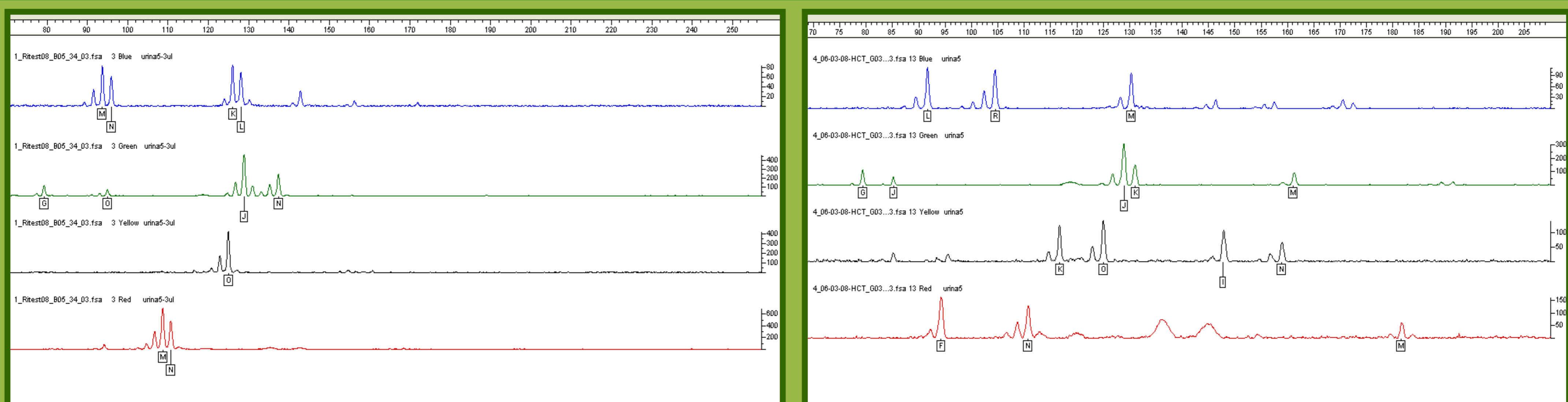
### PCR amplification and electrophoresis

STRs typing was performed alternatively with StockMarks® Equine 17-Plex Genotyping Kit (Applied Biosystem, US) and with Equine Genotypes™ Panel 1.1 Kit (Finnzymes Diagnostics, Finland). Both kits allow co-amplification of nine loci recommended by the 'Equine Genetics and Thoroughbred Parentage testing Standardization Committee of the International Society for Animal Genetics (AHT4, AHT5, HMS6, HMS7, HTG4, VHL20, ASB2, HMS3, HTG10) and eight additional loci commonly used for horse parentage testing and identification (ASB17, ASB23, CA425, HTG6, HTG7, HMS2, HMS1, LEX3). Genetic profiles were obtained by capillary electrophoresis using ABI PRISM 3100 Genetic Analyzer (Applied Biosystem, US). Experiments were performed according the ISAG guidelines.

## RESULTS

A full genetic profile (17 loci) was obtained in 37,5% of samples extracted with ChargeSwitch® Forensic DNA Purification Kit and further purification; 15, 12 and 9 loci were amplified in 12,5% of samples; no results were obtained in 25% of cases. From samples which yielded a full genetic profile, same result was obtained even when the extraction was carried out on a lower volume of urine (3 ml). With phenol/chloroform technique, DNA recovery revealed less efficiently and a good ratio 260/280 was obtained only in few purified samples. In the absence of the purification process, unspecific results and low-quality STR profiles were obtained (Fig.1).

To verify the origin of the amplified samples, genetic profiles were compared to data previously obtained from blood/hair typing of the same individual, stored in UNIRELAB equine genotypes database.



**Fig.1.** Genetic profile of a sample extracted with phenol/chloroform technique without purification (left diagram) and after purification step (right diagram).

Markers amplified: **VHL20, HTG4, AHT4, HMS7**; HTG6, AHT5, HMS6; ASB23; ASB2; HTG10, HTG7, HMS3, HMS2; **ASB17, LEX, HMS1, HMS2.**

Both profiles were compared with those stored in UNIRELAB genotypes database and in the absence of purification step unspecific results were obtained.

## CONCLUSIONS

Our results confirm that successful DNA typing in urine is not mainly depending on the amount of sample but on the presence of the PCR inhibitors. In our experience, a minimum of 3 ml of urine and a robust and reliable analytical technique are needed, since genetic typing of equine urine samples depends mostly on the purification process of extracted DNA. At this stage we were unable to identify genetic profiles of all the urine samples investigated. Better results could be obtained focusing in evaluation of PCR inhibitors in urine samples with Real-time PCR analysis. The utility of this technology extends beyond quantification and gives access to simultaneous evaluation of DNA template extraction quality. Early detection of problematic samples allows analysts to operate inhibitor mitigation strategies prior DNA analysis, thereby facilitating sample processing.

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