TECHNICAL NOTE

Roland A. H. van Oorschot,[1] *Ph.D.; Stephen J. Gutowski,*[1] *Ph.D.; Sarah L. Robinson,*[1] *B.S.; Julie A. Hedley;*[1] *and Ian R. Andrew,*[1] B.S.

HUMTH01 Validation Studies: Effect of Substrate, Environment, and Mixtures

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ABSTRACT: New DNA typing methods need to be thoroughly validated prior to use in forensic investigations. This includes determining the effects different sample conditions have on the typeability of those samples. Biological samples routinely encountered in forensic case work were exposed to a series of different substrates, environmental conditions, and mixtures and typed for the STR HUMTH01 using PCR. None of the conditions resulted in a false typing or preferential allele amplification. It is demonstrated that the application of HUMTH01 typing methods in forensic case work can be reliable, robust, and efficient.

KEYWORDS: forensic science, HUMTH01, DNA, PCR, validation, substrates, environments, mixtures

HUMTH01 (Human Tyrosine Hydroxylase, intron 1) is a highly polymorphic short (4bp- AATG) tandem repeat (STR) locus on chromosome 11p 15.5–p15. It can be typed for by silver staining PCR amplified product run on a horizontal polyacrylamide gel. Stringent validation studies of new techniques are required prior to introduction into routine forensic casework. HUMTH01 validation studies have been reported in the following areas: chromosomal location, and allele sequences (Polymeropoulos et al. 1991, Edwards et al. 1991, 1992, Puers et al. 1993); mode of inheritance using family studies (Edwards et al. 1992, Wiegand et al. 1993; reproducibility, consistency and sensitivity (Gill et al. 1994, Wiegand et al. 1993a, van Oorschot et al. 1994); species specificity (van Oorschot et al. 1994) and population studies (Edwards et al. 1992, Puers et al. 1993, Wiegand et al. 1993a, Lareu et al. 1994, Nellemann et al. 1994, van Oorschot et al. 1994). To our knowledge, however, other necessary validation studies—like determining the effect of substrate, environment and mixtures specifically on the typing of HUMTH01—have not been published.

[1]Forensic Officer, Premier Caseworker, and Forensic Officers, respectively, Victoria Forensic Science Centre, Macleod, Victoria, Australia.

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This paper reports on our investigations of the effect of different substrates, environmental conditions, and mixtures on the typeability and application of HUMTH01 to forensic case work investigations.

Material and Methods

Samples, General

The samples (blood, semen, vaginal swabs, buccal swabs, saliva, hair) were from laboratory personnel of known HUMTH01 type. Hair samples comprised three hair roots. Blood samples, unless specified otherwise, are dried blood stains on cloth (blood coming from sequestrene collection tubes). Swabs were taken using cotton swabsticks. Where more than one buccal swab or vaginal swab was collected from the same individual at a given time, it was attempted to collect approximately equal amount of tissue on each. Where blood and semen were involved, quadruplicates were prepared. Samples were placed in plastic petri dishes. Samples were stored at −20°C after collection/receiving specified treatment. DNA was extracted from these samples within 1 month of being stored. All samples were of heterozygous type with up to five repeat units difference between the two alleles. Additional samples from forensic case work were handled as indicated below.

Substrate Studies

25 μL of fresh blood was dropped on a series of different substrates (see Table 1). Where possible the substrates were 10 × 10 mm and used completely in the DNA extraction procedure. Alternatively the dried blood was scraped or swabbed off the substrate (Table 1).

Environmental Studies

Various samples; blood (25 μL on 10 × 10 mm cloth), blood liquid (received environmental insult as liquid blood in a sequestrene tube then handled as blood), seminal stains (10 μL on 10 × 10 mm cloth), vaginal swabs, buccal swabs and hair were exposed to various environmental conditions for various lengths of time. For blood and seminal stains older than 96 days a dried stain of approximately 5 × 10 mm was used for extraction. The conditions include storage at −70°C (dark), 4°C (dark), room

TABLE 1—*Yield and HUMTH01 amplification of 25 μL of blood taken from various substrates.*

Substrate[a]	Yield Estimate[c]	Amplification Score[d]
Denim blue	175 ng	++
Cotton 100%	225 ng	++
Polyester 100%	900 ng	++
Wool 100%	300 ng	++
Corduroy	600 ng	++
Terry toweling	NK	++
Lycra	250 ng	++
Leather-natural	150 ng*	—
Leather-dyed	NK	—
Carpet-100% wool	230 ng	++
Carpet-synthetic	250 ng	++
Nylon stockings	180 ng	++
Sanitary napkin	85 ng	++
Tampon	38 ng	++
Cotton wool	75 ng	++
Tissue paper	144 ng	++
Newspaper—black	200 ng	++
Brown paper	190 ng	++
Bandaid	88 ng	++
Soil-dirty sand, 0.1g	450 ng	++
Soil-dirty clay, 0.1g	180 ng	++
Soil-pot mix, 0.1g	625 ng*	++
Rubber	NK	+
Glass (scraped off)	480 ng	++
Plastic (scraped off)	700 ng	++
Foil (scraped off)	330 ng	++
Cork (scraped off)	230 ng	±
Leaf (scraped off)	525 ng	++
Grass	520 ng	++
Wood-untreated (scraped & swabbed off)	500 ng	++
Wood-treated (scraped & swabbed off)	250 ng	++
Acetic acid[b]	500 ng	++
Sodium hydroxide 1M[b]	475 ng	++
Sodium hypochlorite (5%)[b]	500 ng	++
Petrol-unleaded[b]	780 ng	++
SDS 20%[b]	125 ng	++
Motor oil (SAE 20 W 50)[b]	208 ng	++

[a]10 × 10 mm where possible

[b]10 × 10 mm cloth soaked in solution and allowed to dry (oil sample was still slightly damp) prior to adding blood.

[c]Yield estimate: NK = not known (<2 ng/8 μL), * = some low mol. wt. DNA.

[d]Amp. score: — no bands, ± weak, + strong, ++ very strong.

temperature (dark and light), 50°C (dark), as well as in a humidity box (closed plastic box with a moist underlay kept at room temperature plus light). DNA was isolated and typed from blood stains, seminal stains, buccal swabs, and hair samples exposed to 11d and 96d for each of the following conditions: 4°C, room temperature, 50°C, humidity. It was also attempted on additional blood stains, seminal stains, buccal swabs and hair samples stored at −70°C, 4°C and room temperature for various durations up to 10 years 5 months, as well as liquid blood samples stored at −70°C for 5 years 4 months, 4°C for 7 months and 50°C for 7d. An additional set of fresh samples (blood stains, seminal stains, vaginal swabs, buccal swabs and hair) was exposed to ultra violet light (G30T, 30W, UV-C) in a PCR workstation for various lengths of time: 5 min, 15 min, 30 min, 1 h, 3 h, 27 h and 5 days (vaginal swabs only for 30 min and 3 h).

Mixture Studies

Various mixture ratios of different fresh samples were prepared; DNA + DNA, blood + blood, saliva + blood, saliva + semen, vaginal swab + semen (Table 2). It was attempted to keep the total amount of sample equal. Blood + blood, saliva + blood, and saliva + semen mixes were prepared in titre tray wells and transferred to 10 × 10 mm cloth in plastic petri dishes. With the vaginal swab + semen mixes various volumes of seminal fluid were added to separate vaginal swabs. Samples were allowed to dry for 7 days at room temperature prior to storage. DNA + DNA mixes were prepared in eppendorf tubes just prior to amplification. Mixes were of two different heterozygous types where each type had at least one unique allele.

DNA Extraction and Yield Estimation

DNA was obtained by standard proteinase K/SDS digestion followed by phenol/chloroform extraction and ultrafiltration washing using centricon −30 concentrators (Kanter et al. 1986; Hochmeister et al. 1991) except for a few case work samples where extensive dialysis was used instead of ultrafiltration washing. DNA yield estimates were obtained after electrophoresis of 8 μL genomic DNA through a 0.8% agarose gel stained by ethidium bromide and comparison to known standards. Where no visible DNA was observed in the yield gel this was recorded as '-' or 'NK' (less than 2 ng/8 μL).

Amplification and Typing

Amplification (including the use of bovine serum albumin), polyacrylamide gel electrophoresis, silver staining and genotyping were performed as described by van Oorschot et al. 1994. Where the genomic DNA concentration could be determined, 5 ng was used for amplification. Where the yield was less than 2 ng/8 μL, 30 μL was used per amplification. The amplification scores given in Tables 1 and 2 are based on band intensity in the acrylamide typing gels. DNA samples that gave negative amplification scores were repeated for confirmation. Those found to be negative in the substrate studies were also repeated using both one-third, and three times, the normal amount of template.

Forensic Case Work

Samples were handled according to our standard laboratory procedures. Blood was kept at 4°C till plated (usually within 1 month), then kept at room temperature for seven days, followed by storage at −70°C. Stains on clothing were kept at room temperature till examined after which it was isolated and used immediately or stored at −70°C. Stains on weapons were kept at room temperature till used for typing. Vaginal swabs were stored at 4°C or −70°C.

DNA samples were only typed for HUMTH01 after they had been typed for D1S80 and/or HLA-DQA1 and no exclusions were observed.

Results

Substrate Studies

Detectable quantities of DNA were retrieved from the 25 μL of blood, from all but three of the 37 substrates tested; terry toweling, leather-dyed and rubber. DNA from leather-natural and

TABLE 2—*HUMTH01 amplification of sample mixtures.*

Samples	Amounts	Amplification Score
DNA:DNA	5 ng : 0 ng	++ : ND
	0 ng : 5 ng	ND : ++
	5 ng : 5 ng	++ : ++
	2.5 ng : 2.5 ng	++ : ++
	0.5 ng : 0.5 ng	+ : +
	4 ng : 2 ng	++ : +
	4 ng : 1 ng	++ : +
	5 ng : 0.5 ng	++ : ±
	5 ng : 0.1 ng	++ : ±
	5 ng : 0.05 ng	++ : −
	5 ng : 0.005 ng	++ : −
Blood : Blood	15 μL : 15 μL	++ : ++
	20 μL : 10 μL	++ : ++
	25 μL : 5 μL	++ : ++
	25 μL : 2.5 μL	++ : −
	25 μL : 0.5 μL	++ : ±
	25 μL : 0.25 μL	++ : −
	25 μL : 0.025 μL	++ : −
Saliva : Blood	40 μL : 10 μL	++ : ++
	40 μL : 1 μL	++ : +
	40 μL : 0.1 μL	++ : −
	2 μL : 20 μL	± : +
Saliva : Semen	40 μL : 10 μL	++ : ++
	40 μL : 1 μL	++ : ++
	40 μL : 0.1 μL	++ : +
	40 μL : 0 μL	++ : ND
	2 μL : 10 μL	++ : ++
Vaginal swab : Semen	swab : 20 μL	++ : ++
	swab : 10 μL	++ : ++
	swab : 1 μL	++ : ++
	swab : 0.1 μL	++ : ++

NOTE: Amp score: − no bands, ± weak, + strong, ++ very strong, ND not determined.

soil-pot mix were clearly degraded. The samples from terry towelling, rubber and soil-pot mix however still gave typeable amplification. Only two samples failed to give typeable amplifications: leather-natural and leather-dyed (Table 1). No false typings or preferential allele amplifications were observed in these samples.

Environmental Studies

Our studies (data not shown) showed that each of our liquid blood samples (−70°C for 5 years 4 months, 4°C for seven months, 50°C for 7d) gave very strong amplification scores. All blood stains, seminal stains, buccal swabs and hair samples gave strong to very strong amplification scores for all conditions (i.e., 4°C, room temperature, 50°C, humidity) after 11d exposure, except for a hair sample kept under humid conditions which gave a weak score. The latter sample had still given a very strong score after 4d exposure. After 96d exposure all the same samples gave markedly lower DNA yields, even to the extent that the buccal swab and hair sample kept under humid conditions were untypeable. The adverse effect on the typeability of most samples kept under humid conditions may be partly due to an increase of bacterial/fungal contamination. Of the older samples, blood stains and seminal stains stored at −70°C for 8 years and 4 years 1 month, respectively gave very strong amplification scores. While the yield and amplification scores decreased over time for items stored at 4°C and room temperature, a blood stain, a seminal stain, a buccal swab and a hair sample each stored at 4°C for 2 years 6 months, all still gave typeable results. This was also true for a 7 year old blood stain and 4 year 1 month old seminal stain kept at room temperature. There is, however, some sample to sample variation in the effect of long term storage as some other blood stains, stored at 4°C for 2 years 6 months and 4 years, gave negative results. A 10 year 5 month old hair sample was also negative.

There was a gradual decrease in DNA yield from blood stains, seminal stains, buccal swabs and hair samples when exposed to experimental UV light, resulting in undetectable yields from seminal stain after 3 h exposure, from blood after 27 h and from hair after five days. The DNA yield from buccal swabs was lowest after 5 days exposure. While very strong amplification scores were obtained from all samples after 3 h exposure, no typings were obtainable from the blood and semen samples after 5 days exposure. The buccal swab and hair sample did still give very strong amplification after 5 days exposure. These results indicate that exposure to natural UV light for an extended period could also reduce DNA yield and typeability.

None of the mentioned environmental conditions resulted in a false typing or preferential allele amplification.

Sensitivity and Mixture Studies

Sensitivity studies (data not shown), examining serial dilutions from 0.005 ng to 50 ng DNA using our standard protocols, showed that typings were obtainable from as little as 0.05 ng DNA. An extra five cycles (total 35) of amplification produced typings from 0.01 ng. High molecular weight bands and very weak additional bands of one to two repeat units smaller than the actual alleles were frequently present when using 10 ng or more template DNA (1–5 ng when using 35 cycles). No false typings were generated when using an additional five cycles. Wiegand et al. (1993a) using a slightly different amplification protocol mention that typings can be obtained from 0.1 ng template DNA using 30 cycles and 0.05 ng from 32 cycles.

Table 2 shows that the minor component is still detectable from the following mixtures; 0.1 ng DNA: 5 ng DNA, 0.5 μL blood: 25 μL blood, 1 μL blood: 40 μL saliva, 2 μL saliva: 20 μL blood, 0.1 μL semen: 40 μL saliva, and 0.1 μL semen from normal vaginal swabs.

Forensic Case Work

Approximately 100 cases (mainly homicides and sexual assaults) have been analysed using HUMTH01 during routine case work investigations at our laboratory. Results of probative value were provided in approximately 95% of cases. DNA was extracted mainly from blood stains, seminal stains and vaginal swabs, usually within one year of collection. Positive typings were obtained from samples such as: an eleven-year-old vaginal swab, seminal stain and blood stain; a five-year-old anal swab; a four-year-old hair sample kept at room temperature; and formalin-fixed-paraffin-embedded foetal tissue. This further illustrates the utility and robustness of this system. Samples that did not amplify include a blood stain from a soiled rubber car mat, a hair sample, an old bone, and a blood stained piece of wire. All but the latter sample were also negative for all other DNA tests attempted (such as, one or more of RFLP, HLA-DQA1, D1S80).

Discussion

Unlike several AmpFLP systems that have longer core repeat units and total size, HUMTH01 does not exhibit preferential allele amplification of degraded DNA and is more likely to provide a

result. Wiegand et al. (1993a) has shown that HUMTH01 results can be obtained from severely degraded DNA extracted from stains exposed to various temperatures for varying lengths of time, and from case work stains that had given negative results using RFLP and AmpFLP typing methods. Furthermore, others have reported the ability to obtain HUMTH01 typings from urine samples (Brinkman et al. 1992); three year old stains, eight year old seminal stains, saliva from stamps and cigarette butts (Brinkman 1992); and two year old fingernail scrapings (Wiegand et al. 1993b). Van Oorschot et al. 1994 have shown that an individual's HUMTH01 type is not affected by the cell origin of the DNA.

The mixture studies illustrate that both components of various sample mixtures can be readily typed and that the minor component is still detectable when representing only 10% of the DNA within the mixture. Hammond (1992) reported similar results.

This study and others demonstrate that this HUMTH01 typing method is reliable, robust, and efficient, thus providing a useful tool in forensic case work.

While these studies have been useful in the current forensic context it is debatable that they all need to be done on scientific reasons for each new STR system to be used for forensic case work. Comparisons with similar studies on other DNA typing systems used in forensic case work, such as restriction fragment length polymorphisms (RFLPs), sequence variable PCR markers (such as, HLA-DQA) and amplified fragment length polymorphisms (such as, D1S80 and STRs) reveal that results obtained from studies into the effects of substrates, environment, mixtures, sensitivity and body sample origin (including Comey and Budowle 1991, Sajantila et al. 1992, Kloosterman et al. 1993, Laber et al. 1993, Lygo et al. 1994, Cosso et al. 1995) are predictable. It is our opinion that it is unnecessary to do extensive investigations into each of the latter type of studies for new STR to be introduced into forensic casework. Characteristics of loci, amplification optimization, population studies, species cross amplification, nonprobative casework studies and blind trials must remain essential components of a validation study. Limiting the array of necessary validation studies would reduce costs and the time involved when implementing a new STR system into casework.

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Address requests for reprints or additional information to
Roland A. H. van Oorschot, Ph.D.
Victoria Forensic Science Centre (VFSC)
Forensic Drive
Macleod
Victoria
Australia 3085