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Extending STR markers in Y chromosome haplotypes

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Abstract Two multiplex reactions were developed to amplify 16 Y-STRs (DYS19, DYS385, DYS389 I and II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, GATA A7.1, GATA A7.2, GATA A10, GATA C4, GATA H4). Here we extend previous population studies done in a sample from northern Portugal for the GATA A7.1, GATA A7.2, GATA C4 and GATA H4 loci. A total of 199 different haplotypes identified by the 16 Y-STR markers were observed in a sample of 208 male individuals, of which 190 were unique and 9 were found twice. The overall haplotype diversity was 0.9996. The haplotype diversity of the Y-STR set composed of the 8 new markers is higher than the Y-STR core set included in the Y-STR haplotype reference database. Sequence structure of new alleles for GATA C4 and GATA H4 is reported. The usefulness of the inclusion of this new set of Y-STRs in forensic casework was also assessed. The increase in haplotype diversity with the addition of any new Y-STR marker to the 8 Y-STR core set is dependent not only on the gene diversity (positively) but also (negatively) on the degree of gametic association between the markers and the haplotypes previously defined. For instance, in our sample the addition of the DYS437, DYS438 and GATA A7.2 to a 13-locus set increased haplotype diversity only by 0.0001.

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Introduction

Typing of Y chromosome-specific STRs has become very useful in evolutionary studies and forensic casework, namely in deficiency paternity testing and in rape cases involving one or more semen donors. All of these studies have generated great amounts of data that must be organised in DNA databases. The most extensive survey on Y-STRs was included in the Y-STR haplotype reference database (YHRD; http://www.ystr.org).

The Y-STR core set included in the YHRD consists of a restricted set of 8 STRs that have been well characterised and analysed in a multicenter study (Kayser et al. 1997b).

More recently, new series of Y-specific STRs have been described (Ayub et al. 2000; White et al. 1999; Iida et al. 2001) and studied in several populations (Gusmão et al. 2001, 2002a; Grignani et al. 2000; Hou et al. 2001; Mohyuddin et al. 2001).

It is clear that the addition of new STR markers will slowly enlarge the number of different lineages in a population, mostly due to the effect of spontaneous slippage mutation, increasing the discrimination power. However this cannot be a never-ending process, also taking into account that the application of Y chromosome variation in forensic casework requires large population genetic studies. Therefore it is crucial to balance cost and efficiency.

The question addressed in this work is whether or not the Y-STR "minimum haplotype" established in the YHRD should be enlarged and, if so to what extent. To make this aim possible we have developed a new strategy for the amplification of 16 Y-specific STRs (DYS19, DYS385, DYS389 I and II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, GATA A7.1, GATA A7.2, GATA A10, GATA C4, GATA H4) in a small number of PCR multiplex reactions, that facilitates routine work in a forensic laboratory, and have analysed our collection of

males from the northern Portuguese population for the GATA A7.1, GATA A7.2, GATA C4, GATA H4 loci in order to extend the population studies already reported in the same sample (Gusmão et al. 2002a). Finally, the utility of the combination between the new set of Y-STR markers DYS437, DYS438, DYS439, GATA A7.1, GATA A7.2, GATA A10, GATA C4, GATA H4 and the classical ones in forensic casework is discussed.

Material and methods

DNA samples

A sample of 208 unrelated healthy male blood donors already typed for the classical Y-STR core set and for DYS437, DYS438, DYS439 and GATA A10 was selected from the northern Portuguese population.

Genomic DNA was extracted either by chelex extraction or using the salting out method according to Miller et al. (1988).

Multiplex amplification

A total of 4 multiplex PCR reactions for typing 16 Y-STRs in monochromatic platforms were constructed, that can be reduced to 2 multiplex PCR kits if typing the same STRs in polychromatic platforms. Primer sequences are given in Table 1 and PCR cycling conditions for each approach are given in Tables 2 and 3. PCR amplifications were performed in a PE GeneAmp PCR system 2400 thermocycler, with 5–50 ng of genomic DNA in a 12.5 µl reaction volume comprising 1.5 mM $MgCl_2$, 200 µM dNTPs, 1× Gold buffer (AB Applied Biosystems) and 1 U of Taq Gold polymerase (AB Applied Biosystems). For the decaplex Y set, 2 U of Taq Gold polymerase was needed, and the final concentration of $MgCl_2$ was 2.5 mM.

Detection system

For genetic typing, the ABI310 automatic sequencer (AB Applied Biosystems) along with the Genescan 2.1 analysis software were used.

Allele designations were based on comparison with the allelic ladders obtained by the mixture of previously sequenced samples for the most common alleles. Allele nomenclature was as proposed by Kayser et al. (1997a) and by Gusmão et al. (2002b).

Table 1 Sequence and dye label of the primers used in multiplex optimisation

Locus	Dye label	Forward and reverse primers (5'-3')	Refence[a]
DYS19	FAM	CTA CTG AGT TTC TGT TAT AGT GGG TTA AGG AGA GTG TCA CT A	Kayser et al. (1997a) New[b]
DYS385	HEX	AGC ATG GGT GAC AGA GCT A CCA ATT ACA TAG TCC TCC TTT C	Schneider et al. (1998)
DYS389	FAM	CCA ACT CTC ATC TGT ATT ATC TAT TCT TAT CTC CAC CCA CCA GA	Kayser et al. (1997a)
DYS390	FAM	TAT ATT TTA CAC ATT TTT GGG CC TGA CAG TAA AAT GAA CAC ATT GC	Kayser et al. (1997a)
DYS391	TET	CTA TTC ATT CAA TCA TAC ACC CA CTG GGA ATA AAA TCT CCC TGG TTG CAA G	Kayser et al. (1997a) Gusmão et al. (2000)
DYS392	FAM	AAA AGC CAA GAA GGA AAA CAA A AGA CCC AGT TGA TGC AAT GT	Ruitberg et al.[c] (pers. comm.) Kayser et al. (1997a)
DYS393	FAM	GTG GTC TTC TAC TTG TGT CAA TAC AAC TCA AGT CCA AAA AAT GAG G	Kayser et al. (1997a)
DYS437	TET	GAC TAT GGG CGT GAG TGC AT AGA CCC TGT CAT TCA CAG ATG A	Ayub et al. (2000)
DYS438	HEX	TGG GGA ATA GTT GAA CGG TAA GTG GCA GAC GCC TAT AAT CC	Ayub et al. (2000)
DYS439	TET	TCC TGA ATG GTA CTT CCT AGG TTT GCC TGG CTT GGA ATT CTT TT	Ayub et al. (2000)
GATA A7.1	TET	AGC AAG CAC AAG AAT ACC AGA G TCT ATC CTC TGC CTA TCA TTT ATT A	New White et al. (1999)
GATA A7.2	FAM	AGG CAG AGG ATA GAT GAT ATG GAT TGA TGC TGT GTC ACT ATA TTT CTG	White et al. (1999) New
GATA A10	HEX	CCT GCC ATC TCT ATT TAT CTT GCA TAT A ATA AAT GGA GAT AGT GGG TGG ATT	White et al. (1999)
GATA C4	FAM	AGT GTC TCA CTT CAA GCA CCA AGC AC GCA GCA AAA TTC ACA GTT GGA AAA ATG T	White et al. (1999)
GATA H4	TET	GTT ATG CTG AGG AGA ATT TCC AA CCT CTG ATG GTG AAG TAA TGG AAT TAG A	New White et al. (1999)

[a]References given in the order forward/reverse primers.
[b]Modified from Szibor et al.2000.
[c]Poster presentation at the 11th International Symposium on Human Identification 2000, Promega, Wisc.

Table 2 PCR cycling conditions and primer concentrations for four multiplex reactions constructed for typing 16 Y-STRs markers in monochromatic platforms

Primer	Final concentration (μM)	Pre-incubation	Denaturing	Annealing	Extension	Denaturing	Annealing	Extension	Final Extension
Pentaplex I[a]									
DYS19	0.12	95°C, 11 min	94°C, 30 s	58°C, 30 s	70°C, 45 s				60°C, 20 min
DYS389	0.06								
DYS390	0.18								
DYS393	0.08								
Cycles				32					
Pentaplex II									
DYS391	0.12	95°C, 11 min	94°C, 30 s	60°C, 30 s	70°C, 45 s				60°C, 45 min
DYS437	0.08								
DYS439	0.16								
GATA A7.1	0.12								
GATA H4	0.24								
Cycles				30					
Triplex I									
DYS385	0.3	95°C, 11 min	94°C, 30 s	62°C, 20 s	70°C, 30 s	94°C, 30 s	60°C, 20 s	70°C, 30 s	60°C, 20 min
DYS438	0.3								
GATA A10	0.2								
Cycles				10			22		
Triplex II									
DYS392	0.16	95°C, 11 min	94°C, 30 s	62°C, 20 s	70°C, 30 s	94°C, 30 s	60°C, 20 s	70°C, 30 s	70°C, 45 min
GATA A7.2	0.12								
GATA C4	0.16								
Cycles				10			22		

[a]This pentaplex is a modification of a previous one developed by Gusmão et al. (1999), using a new reverse primer for DYS19 (see Table 1) in order to prevent an overlap with DYS390

Sequencing

Singleplex PCR amplified fragments for GATA C4 and H4 were purified with Microspin S-300 HR columns (Pharmacia, Uppsala, Sweden). A dideoxy cycling sequencing reaction was carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (AB Applied Biosystems, Foster City, Calif.). The products were purified using a $MgCl_2$/ethanol-based protocol and run on an ABI 3100 sequencer (AB Applied Biosystems, Foster City, Calif.). The results were analysed using the 3100 Data Collection software.

Statistical analysis

Allele/haplotype frequencies were estimated by gene/haplotype counting. Observed gene and haplotype diversities were calculated according to Nei (1987). A linkage disequilibrium exact test using a Markov chain method was performed using Arlequin 2.000 software (Schneider et al. 2000).

Results and discussion

Multiplex optimisation

Since forensic laboratories use different platforms for typing STRs, such as silver staining or fluorescence detection in automated sequencers, we developed multiplex reactions where the size range of the alleles of each marker does not overlap, allowing their use in both monochromatic and polychromatic platforms. When using monochromatic platforms we recommend a protocol of 4 multiplex reactions (Pentaplex I and II, Triplex I and II; Table 2) to amplify 16 Y-STR markers, which can be reduced to 2 multiplex reactions (Decaplex Y and Hexaplex Y; Table 3) when using a polychromatic platform. The newly described Y-STR loci (DYS437, DYS438, DYS439, GATA A7.1, GATA A7.2, GATA A10, GATA C4, GATA H4) were chosen based on a few population studies already published (Gusmão et al. 2000, 2002a; Grignani et al. 2000; Hou et al. 2001; Mohyuddin et al. 2001) and on our preliminary studies, as those which were more polymorphic and therefore with higher powers of discrimination.

For the construction of these novel multiplexes, new primers were designed for some markers using the program Primer 3 (http://www2.no.embnet.org/primer3/primer3_www.cgi) (Table 1). The parameters taken into account were the size of the PCR product, the annealing temperature and primer-dimer formation.

All multiplex PCR kits amplified DNA regardless of the type of extraction used (e.g. chelex, or "salting out").

Table 3 PCR cycling conditions and primer concentrations needed for two multiplex reactions constructed for typing 16 Y-STRs markers in polychromatic platforms

Primer	Final concentration (μM)	Pre-incubation	Denaturing	Annealing	Extension	Denaturing	Annealing	Extension	Final Extension
Decaplex Y									
DYS19	0.48	95°C, 11 min	94°C, 30 s	58°C, 20 s	70°C, 30 s				70°C, 45 min
DYS389	0.32								
DYS390	0.36								
DYS391	0.20								
DYS393	0.08								
DYS437	0.06								
DYS439	0.32								
GATA A7.1	0.08								
GATA H4	0.20								
Cycles				32					
Hexaplex Y									
DYS385	0.24	95°C, 11 min	94°C, 30 s	62°C, 20 s	68°C, 1 min	94°C, 30 s	60°C, 20 s	68°C, 1 min	60°C, 60 min
DYS438	0.48								
DYS392	0.28								
GATA A7.2	0.12								
GATA A10	0.16								
GATA C4	0.12								
Cycles				10			22		

Table 4 Allele frequencies at 4 Y-STRs in a northern Portuguese population (208 individuals)

Locus	Allele		Frequency (±s.d)	Gene diversity (±s.d)
	Units	bp[a]		
GATA A7.1	9	117	0.043 (±0.014)	0.5676 (±0.0162)
	10	121	0.433 (±0.034)	
	11	125	0.495 (±0.035)	
	12	129	0.029 (±0.012)	
GATA A7.2	10	152	0.034 (±0.013)	0.5706 (±0.0315)
	11	156	0.202 (±0.028)	
	12	160	0.611 (±0.034)	
	13	164	0.130 (±0.023)	
	14	168	0.024 (±0.011)	
GATA C4	19	246	0.014 (±0.008)	0.6567 (±0.0295)
	20	250	0.038 (±0.013)	
	21	254	0.192 (±0.027)	
	22	258	0.058 (±0.016)	
	23	262	0.538 (±0.035)	
	24	266	0.115 (±0.022)	
	25	270	0.034 (±0.013)	
	26	274	0.010 (±0.007)	
GATA H4	26	276	0.014 (±0.008)	0.5825 (±0.0218)
	27	280	0.341 (±0.033)	
	28	284	0.543 (±0.035)	
	29	288	0.091 (±0.020)	
	30	292	0.010 (±0.007)	

[a]Size range obtained when using the primers included in this study (see Table 1).

However, Decaplex Y showed more efficacy when using the "salting out" extraction, and a larger amount of sample was needed to amplify DNA samples extracted with chelex than with Decaplex Y.

Single locus analysis

Allele frequencies and gene diversity values for the GATA A7.1/A7.2/C4/H4 loci obtained for the northern Portuguese population are shown in Table 4. Gene diversity values for these markers are equivalent to those found by Gusmão et al. (2002a) for others Y-STRs in the same sample.

For GATA C4, 3 new alleles were found (2 alleles 19 and 1 allele 26) and for GATA H4 1 new allele was found (allele 30) (Table 5). All sequence structures of the new alleles were in accordance with the structure observed by González-Neira et al. (2001), except for one of the alleles 19 of GATA C4. For this locus the allele structure could be represented as $(TCTA)_2[(TCTA)_2(TGTA)_2]_{2-3}(TCTA)_n$ (Gusmão et al. 2002b). However, this allele 19 lacks 1 (TGTA) of the second (TGTA) block (see Table 5, allele 19, second sequence).

Haplotype analysis

By combining the allelic state of the 16 markers studied in a sample of 208 unrelated males, it was possible to define 199 different haplotypes (access to the data: www.ipatimup.

Table 5 Sequence structure of GATA C4 and H4 new alleles

GATA-C4

Consensus structure

P1–tgctgctgaatgggagcagaaatgcccaatggaatgctctcttggcttctcactttgcatagaatc**(tcta)$_4$(tgta)$_2$(tcta)$_2$(tgta)$_2$(tcta)$_2$(tgta)$_{0,2}$(tcta)$_{8–12}$**tcacattttctttatc-cattcattgattgatggatatttgggctggttcc–P2

Allele (bp) Sequence

19 (246) P1–66bp**(tcta)$_4$(tgta)$_2$(tcta)$_2$(tgta)$_2$(tcta)$_9$**50bp–P2

19 (246) P1–66bp**(tcta)$_4$(tgta)$_2$(tcta)$_2$(tgta)$_1$(tcta)$_{10}$**50bp–P2

26 (274) P1–66bp**(tcta)$_4$(tgta)$_2$(tcta)$_2$(tgta)$_2$(tcta)$_2$(tgta)$_2$(tcta)$_{12}$**50bp–P2

GATA-H4

Consensus structure

P1–tgatacacattgatactttcagcacatcacttgtatcctaggaatcatcattaaaatgttatgctgaggagaatttccaaattta**(agat)$_4$ctat(agat)$_2$ (aggt)$_3$(agat)$_{8–12}$**agaatggatag-attagatggatga**(atag)$_4$(atac)$_1$(atag)$_2$**gtgatttatcctgttaagttgtttaacaagtgggctatgta aaattttactaatattta aacataagtagtttgtagattttcttatttatt–P2

Allele (bp) Sequence

30 (376) P1–85bp**(agat)$_4$ctat(agat)$_2$(aggt)$_3$(agat)$_{13}$**24bp**(atag)$_4$(atac)$_1$(atag)$_2$**92bp–P2

In bold repeat structure as recommended by Gusmão et al. (2002b)

Table 6 Number of haplotypes and haplotype diversity values found for three combinations of Y-STR markers

	8 Y-STR set core	New Y-STR markers	Total (16 Y-STRs)
Nr. of individuals	208	208	208
Nr. of haplotypes	155	166	199
Haplotype diversity (%)	99.25	99.61	99.96

pt/STR), with a haplotype diversity of 0.9996 (Table 6). This means that almost all haplotypes were unique and only 9 haplotypes were represented twice.

Comparing the number of haplotypes produced and respective haplotype diversity between the classical 8 Y-STR set core with the Y-STR set considered here (Table 6), we can see that the new Y-STR core has a higher power of discrimination and is therefore more informative than the classical set. Thus these new markers may provide useful information in addition to that already available.

Forensic assessment

One of the discussions regarding the typing of STR markers in forensic casework is the number of STRs that are needed to be typed in order to achieve the maximum exclusion probability in practical terms. This issue is even more significant when Y-STRs are in question, due to the non-recombining nature of most of this chromosome. It is accepted that the addition of an informative Y-STR to the classical set will increase the power of discrimination up to a point where, even if the marker is very polymorphic, its alleles will not be able to discriminate more haplotypes and this extra information becomes redundant. Therefore one of the strategies to tackle the problem was to rank all loci according to their diversity value and combine them to produce haplotypes. Table 7 shows the number of haplotypes produced and the respective haplotype diversity achieved when adding each marker to the top Y-STR set. All markers, except for DYS437, contributed to an increment on the number of different haplotypes. Interestingly, DYS437, although having a higher gene diversity than GATA A7.2 and A7.1, was not able to discriminate more haplotypes. This reinforces the idea that the increase in

Table 7 Number of haplotypes and haplotype diversity obtained when adding each Y-STR marker (in order of gene diversity) to the increasing top haplotype

Y-STR marker	Gene diversity (%)	Nr. of haplotypes	Haplotype diversity (%)
8 Y-STR core set	–	155	99.25[a]
DYS439	68.39[a]	173	99.72[a]
GATA C4	65.67	180	99.81
GATA A10	63.36[a]	189	99.90
DYS438	60.50[a]	190	99.91
GATA H4	58.25	194	99.93
DYS437	57.68[a]	194	99.93
GATA A7.2	57.07	197	99.95
GATA A7.1	56.76	199	99.96

[a]Values obtained in previous study (Gusmão et al. 2002a).

Table 8 Haplotype diversity obtained when adding each new Y-STR marker to the classical set core (*Nr. of linked loci* number of Y-STRs included in Y-STR haplotype reference database to which each new Y-STRs is associated)

Y-STR marker	Nr. of linked loci	Haplotype diversity (%)
8 Y-STR core set	–	99.25
8 Y-STR + GATA A7.1	1	99.59
8 Y-STR + DYS439	3	99.72[a]
8 Y-STR + GATA A10	6	99.70[a]
8 Y-STR + GATA H4	4	99.56
8 Y-STR + GATA C4	6	99.63
8 Y-STR + GATA A7.2	7	99.37
8 Y-STR + DYS438	7	99.38[a]
8 Y-STR + DYS437	7	99.52[a]

[a]Values obtained in previous study (Gusmão et al. 2002a).

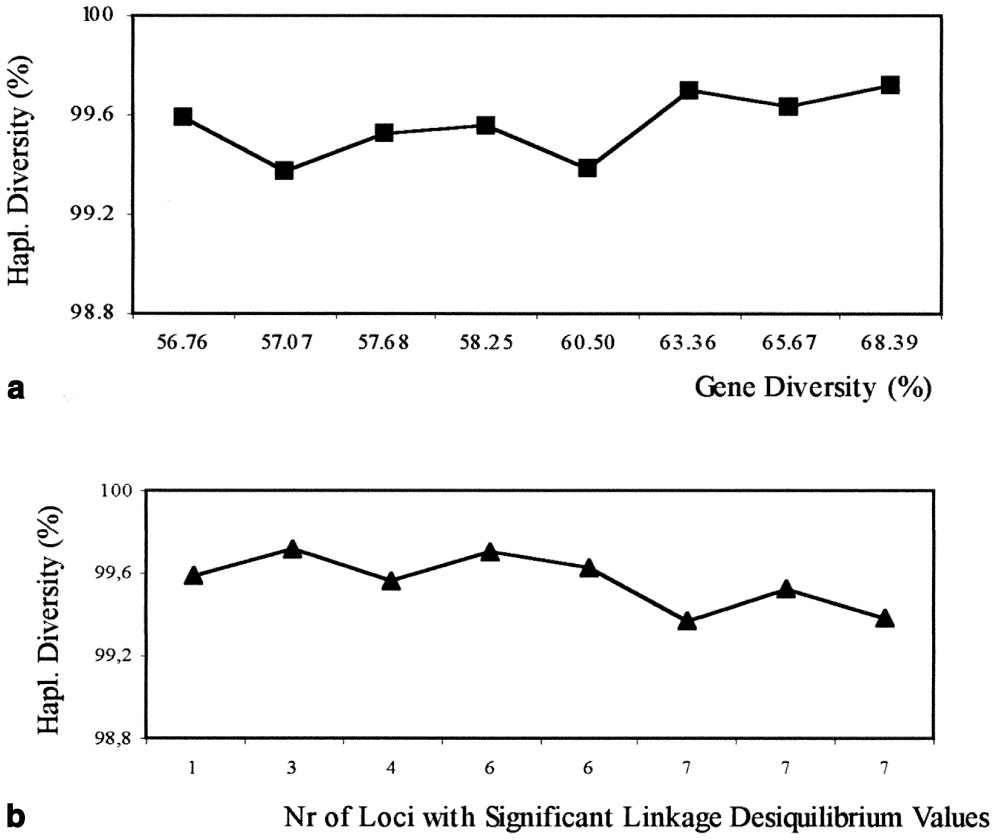

Fig. 1 Impact of **a** gene diversity per locus or **b** the number of Y-STRs included in Y-STR reference database to which new Y-STRs show significant linkage disequilibrium values on Y-STR haplotype diversity

haplotype diversity is not directly correlated to gene diversity (Gusmão et al. 2002a), but instead is mediated by the balance of a group of parameters that include gene diversity.

The next step was to perform a linkage disequilibrium exact test using a Markov chain method, to study the associations between non-alleles at all pairs of loci. The number of loci in the classical Y-STR core set to which each new Y-STR marker is associated, was also determined and its contribution to haplotype diversity was compared with the one given by gene diversity (Table 8 and Fig. 1). Comparing the two graphs in Fig. 1, it is possible to conclude that haplotype diversity has a general tendency to increase with gene diversity and a tendency to decrease with the degree of linkage disequilibrium. However, this relationship is not linear and, although some markers have high gene diversity or show small degree of linkage disequilibrium values, it may not necessarily lead to the result that they are very informative. This is the case, for instance of DYS438 which has a gene diversity of 60.50% but only increases the Y-STR classical set haplotype diversity by 0.13%. On the other hand, GATA A7.1 has the lowest individual gene diversity among the new Y-STR core but increases the haplotype diversity by 0.45%. This is probably best explained by the number of loci to which those markers show significant linkage disequilibrium values (Table 8). In fact, when excluding DYS437, DYS438, and GATA A7.2 from the whole 16-STR set we obtained the same haplotype diversity (0.9995, defining 197 different haplotypes) as when only GATA A7.1 was excluded (Table 7); the addition of the DYS437, DYS438 and GATA A7.2 loci to the 13-locus set increased the haplotype diversity only by 0.0001.

In conclusion, the decision on the inclusion of new markers to the established STR core set is a very delicate task. Indeed, even disregarding technical and economical problems, the parameters relevant to an optimal choice are not straightforward and require an extensive empirical approach. In particular, since the discrimination power is dependent on the allelic associations across loci, the best choice for a given population can result in a very poor one in a different demographic context. For this reason a thorough evaluation must be performed across the major human population groups before making a decision on expanding the 8 STR core set of the present databases.

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