elsevier_FSIGEN_1339

Short ~~C~~communication

~~Single~~ A novel multiplex assay for simultaneously analysing 13 ~~Rapidly M~~rapidly mutating Y-STRs

Rashed Alghafri[a, b]

Will Goodwin[a]

Arwin Ralf[c]

Manfred Kayser[c]

Sibte Hadi[a, *]

shadi@uclan.ac.uk

[a]University of Central Lancashire, School of Forensic and Investigative Sciences, Preston, UK

[b]General Department of Forensic Sciences and Criminology, Dubai Police General Head Quarters, Dubai, United Arab Emirates

[c]Department of Forensic Molecular Biology, Erasmus MC University Medical Centre Rotterdam, Rotterdam, The Netherlands

*Corresponding author at: School of Forensic and Investigative Sciences, University of Central Lancashire, JB Firth Building, Preston, PR1 2HE, United Kingdom. Tel.: +44 1772 89 4395; fax: +44 1772 894981.

Abstract

A multiplex polymerase chain reaction (PCR) assay (RM-Yplex) was developed which is capable of simultaneously amplifying 13 recently introduced~~Rapidly M~~ rapidly mutating Y-STR markers (RM Y-STRs). This multiplex assay is expected to aid human identity testing in forensic and other applications to improve differentiating unrelated males and allow separating related males. The 13 RM Y-STR markers included in the multiplex are: DYF387S1, DYF399S1, DYF403S1ab, DYF404S1, DYS449, DYS518, DYS526ab, DYS547, DYS570, DYS576, DYS612, DYS626 and DYS627. This study reflects the proof of concept to analyse all currently known RM Y-STRs simultaneously and describes the optimization of the multiplex assay. The RM-Yplex~~assay generated~~ assay generated complete RM Y-STR profiles down to 62.5 pg of male template DNA, and from male–female DNA mixtures at all ratios tested. We herewith introduce and make available for widespread use in forensic and anthropological studies, an effective and sensitive single multiplex assay for simultaneous genotyping of 13 RM Y-STRs.

Keywords: Y-chromosomal STRs; Y-STRs; Rapidly ~~M~~mutating Y-STRs; Haplotypes

1 Introduction

The use of Y chromosome short tandem repeats (Y-STR) in various studies involving population lineage and human migration has been significant over the years as a result of its ability to differentiate between male individuals of different paternal lineages [1]. Y-STR analysis has also been considered an important tool in forensic genetics, especially in the analysis of sexual offences; paternity testing and missing person investigations [2–4]. The most advantageous aspect of Y-STR analysis in forensic genetics is that it can detect low level of male DNA in a high background of female DNA [5] as typically confronted within cases of sexual assault. Previously available single multiplex Y-STR systems using 9 (PowerPlex-Y) or 17 (AmpFℓSTR® Yfiler®) markers provide high haplotype diversities in many outbred populations [6] but are limited in differentiating male lineages in inbred and rarely studied populations [7,8]. Adding additional Y-STRs selected based on population diversity can help to enhance male lineage resolution [9]. However with these additional markers, the lineage resolution is typically enhanced to anon-maximum extent as illustrated for PowerPlex-Y23 kit [10]. Furthermore, commonly used Y-STR markers have limited value in differentiating members of the same paternal lineage. This poses a general shortcoming of current Y-STR profiling assays. To overcome both limitations, it has been suggested to use Y-STRs with higher mutation rates [11].

Recently, 13 rapidly-mutating Y-STR (RM Y-STR) markers (Table 1) have been discovered via a comprehensive Y-STR mutation study [11]. This RM Y-STR set identified based on mutation rates and not on population diversity as previous Y-STR sets, revealed strikingly increased male lineage differentiation in 51 worldwide populations (HGDP–CEPH) in comparison to the 17 Y-STR markers available in Yfiler® (Life Technologies) and was also able to differentiate many pairs of male paternal relatives [12]. The immense value of the 13 loci RM Y-STR set to differentiate related and unrelated male has been further explored in a recent worldwide study carried out by the International RM Y-STR Study Group [13]

and a most recent national study carried out by the Italian Working Group (GEFI) of the International Society for Forensic Genetics [14]. All previous studies either used the initially introduced three multiplex assays [11–14] or recently introduced two multiplex assays [15] for amplifying the 13 RM Y-STR markers. In the present study, we developed and optimised the single RM-Yplex assay for the simultaneous amplification of all 13 RM Y-STR markers.

Table 1 13 RM Y-STR markers, their repeat motifs, allele ranges and PCR product sizes as used in the single RM-Yplex developed here

Locus	Repeat type	Repeat motif (variant in bold)	Allele ranges	Product sizes
DYF387S1	Tetra, complex	$(AAAG)_3(GTAG)_1(GAAG)_4N_{16}(GAAG)_9$ $(\mathbf{AAAG})_{13}$	34–42	240–272
DYF399S1	Tetra, complex	$(GAAA)_3N_{7-8}(\mathbf{GAAA})_{10-23}$	15–31	264–322
DYF403S1a	Tetra, complex	$(\mathbf{TTCT})_{10-17}N_{2-3}(\mathbf{TTCT})_{3-17}$	4–20	298–348
DYF403S1b	Tetra, complex	$(TTCT)_{12}N_2(\mathbf{TTCT})_8(\mathbf{TTCC})_9(\mathbf{TTCT})_{14}N_2(TTCT)_3$	42–55	416–468
DYF404S1	Tetra, complex	$(\mathbf{TTTC})_{10-20}N_{42}(TTTC)_3$	11–17	174–198
DYS449	Tetra, complex	$(\mathbf{TTTC})_{12-18}N_{22}(TTTC)_3N_{12}(\mathbf{TTTC})_{12-18}$	25–36	300–344
DYS518	Tetra, complex	$(AAAG)_3(GAAG)_1(\mathbf{AAAG})_{14-22}(GGAG)_1(AAAG)_4N_6(\mathbf{AAAG})_{11-19}N_{27}(AAGG)_4$	34–47	258–308
DYS526a	Tetra, complex	$(\mathbf{CCTT})_{10-17}$	11–18	132–160
DYS526b	Tetra, complex	$(CCCT)_3N_{20}(\mathbf{CTTT})_{11-17}(\mathbf{CCTT})_{6-10}N_{113}(\mathbf{CCTT})_{10-17}$	30–42	340–388
DYS547	Tetra, complex	$(\mathbf{CCTT})_{9-13}T(\mathbf{CTTC})_{4-5}N_{56}(\mathbf{TTTC})_{10-22}N_{10}(CCTT)_4(TCTC)_1(\mathbf{TTTC})_{9-16}N_{14}(TTTC)_3$	42–52	408–448
DYS570	Tetra, simple	$(\mathbf{TTTC})_{14-24}$	14–22	120–152
DYS576	Tetra, simple	$(\mathbf{AAAG})_{13-22}$	12–22	170–210
DYS612	Tri simple	$(CCT)_5(CTT)_1(TCT)_4(CCT)_1(\mathbf{TCT})_{19-31}$	31–40	185–213
DYS626	Tetra, complex	$(\mathbf{GAAA})_{14-23}N_{24}(GAAA)_3N_6(GAAA)_5$ ~~(AAA)1~~ $(AAA)_1(\mathbf{GAAA})_{2-3}(GAAG)_1(GAAA)_3$	24–35	222–266
DYS627	Tetra, complex	$(AGAA)_3N_{16}(AGAG)_3(\mathbf{AAAG})_{12-24}N_{81}(AAGG)_3$	15–24	308–344

2 Materials and methods

2.1 Sample collection and extraction

Eight DNA samples were used to develop and optimise the multiplex assay consisting of two male controls 9948 (Promega) ~~&~~and TaqMan (Life Technologies), 4 unrelated male DNA samples (M1–M4), 1 female control (9947A), and 1 female DNA sample (F1). Samples M1, M2, M3, M4 and F1 were consented population samples that were collected earlier. These were extracted using organic extraction method [16] and quantified using Plexor® HY (Promega) as recommended by the manufacturer.

2.2 Multiplex development and primer design

Basic Alignment Search Tool (BLAST) was used to obtain the sequence of all 13 RM Y-STR loci from GeneBank® (~~www.ncbi.nlm.nih.gov~~www.ncbi.nlm.nih.gov). Previously published allelic ranges (Table 1) were considered to leave sufficient space between the loci for detecting any additional alleles [11]. New primers were designed for four~~loci, DYF387S1, DYS570, DYS576 and DYS612~~ loci, DYF387S1, DYS570, DYS576 and DYS612 using Primer 3 software~~ (~~http://bioinfo.ut.ee/primer3-0.4.0/) for adjusting their positions within the design of this 5-dye RM-Yplex assay (Fig. 1) [17,18].

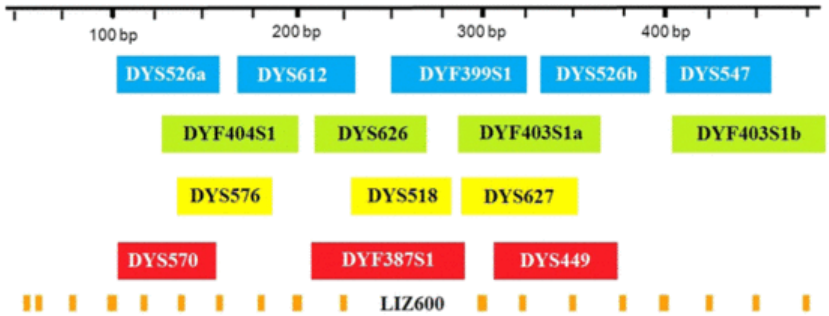

Fig. 1 Diagram illustrating fluorescent dye label colours used and PCR product size ranges achieved for the 13 RM Y-STR markers incorporated in the single RM-Yplex assay.

Forward primers for each locus were labelled with an appropriate fluorescent dye to generate a labelled PCR product for detection using ABI 3500 Genetic Analyser (Applied Biosystems) (Table 2). All primers were quality-control tested by Eurofins MWG Operon using HPLC method [19]. GeneScan™ 600 LIZ® (Life Technologies) was used as an internal standard. G5 matrix (Life Technologies) compatible dye panel was selected for the assay.

Table 2 Primer sequences and concentrations developed and optimised for the RM-Yplex assay. Different flouropores are also indicated.

Locus	Primer sequence	Concentration µM	Reference
DYF387S1	Forward: ~~ATTO565-~~ATTO565- ACAGAGCTAGATTCCATTTTACCC	0.05	Present ~~S~~study
	Reverse: GCCACAGTGTGAGAAGTGTGA	0.05	
DYF399S1	Forward: ~~6-FAM-~~6-FAM- GGGTTTTCACCAGTTTGCAT	0.06	[11]
	Reverse: CCATGTTTTGGGACATTCCT	0.06	
DYF403S1a/b	Forward: ~~YakimaYellow-~~YakimaYellow- CAAAATTCATGTGGATAATGAG	0.40	[11]
	Reverse: ACAGAGCAGGATTCCATCTA	0.40	
DYF404S1	Forward: ~~YakimaYellow-~~YakimaYellow- GGCTTAAGAAATTTCAACGCATA	0.11	[11]
	Reverse: CCATGATGGAACAATTGCAG	0.11	
DYS449	Forward: ~~ATTO565-~~ATTO565- TGGAGTCTCTCAAGCCTGTTC	0.06	[11]
	Reverse: CCATTGCACTCTAGGTTGGAC	0.06	
DYS518	Forward: ~~ATTO550-~~ATTO550- GGCAACACAAGTGAAACTGC	0.12	[11]
	Reverse: TCAGCTCTTACCATGGGTGAT	0.12	
DYS526a/b	Forward: ~~6-FAM-~~6-FAM- TCTGGTGAACTGATCCAAACC Reverse: GGGTTACTTCGCCAGAAGGT	0.40 0.40	[11]
DYS547	Forward: ~~6-FAM-~~6-FAM- TCCATGTTACTGCAAAATACAC	0.40	[11]
	Reverse: TGACAGAGCATAAACGTGTC	0.40	
DYS570	Forward: ~~ATTO565-~~ATTO565- CTGGCTGTGTCCTCCAAGTT	0.04	Present ~~S~~study
	Reverse: GGCAACCTAAGCTGAAATGC	0.04	
DYS576	Forward: ~~ATTO550-~~ATTO550- GTTGGGCTGAGGAGTTCAATC	0.03	Present ~~S~~study
	Reverse: GGCAGTCTCATTTCCTGGAG	0.03	

DYS612	Forward: ~~6-FAM-~~6-FAM- CCCCATGCCAGTAAGAATA	0.10	Present ~~S~~study
	Reverse: GTGAGGGAAGGCAAAAGAAAA	0.10	
DYS626	Forward: ~~YakimaYellow-~~YakimaYellow- GCAAGACCCCATAGCAAAAG	0.07	[11]
	Reverse: AAGAAGAATTTTGGGACATGTTT	0.07	
DYS627	Forward: ~~ATTO550-~~ATTO550- CTAGGTGACAGCGCAGGATT	0.20	[11]
	Reverse: GGATAATGAGCAAATGGCAAG	0.20	

2.3 RM-Yplex~~A~~ assay PCR amplification conditions

The RM-Yplex assay was optimized in a reaction volume of 15 µl with 7 µl of Platinum® PCR Multiplex Master Mix (Applied Biosystems), 1.5 µl of the 13 RM Y-STRs primer mix (Table 2), 5.5 µl PCR grade water and 1 µl of DNA template.The volumes of extracts in casework can be therefore adjusted. During optimization, the male DNA template amount was kept at 1 ng/µl unless otherwise indicated. PCR was conducted on GeneAmp 9700 (Applied Biosystems) and the following conditions were established in 9600-emulation mode (i.e. ramp speeds of 1 °C/s):

- 95 °C for 10 min~~utes~~.
- 12 cycles: 94 °C for 30 s~~econds, 58 °C for 45 seconds, 72 °C for 60 second~~, 58 °C for 45 s, 72 °C for 60 s.
- 20 cycles: 94 °C for 30 s~~econds, 55 °C for 45 seconds, 72 °C for 60 second~~, 55 °C for 45 s, 72 °C for 60 s.
- 72 °C for 45 min~~utes.~~

2.4 Detection and analysis of PCR products

Amplified products were electrophoresed on ABI 3500 Genetic Analyser (Applied Biosystems) following manufacturer's protocols using the G5 matrix filter to detect the dyes used. A spectral matrix was established using matrix standard set DS-33 (Applied Biosystems). Samples were prepared using 9.6 µl Hi-Di™ Formamide (Applied Biosystems), 0.4 µl GS600 LIZ size standard and 1 µl PCR product. The prepared samples were injected into the 8-~~C~~capillary 50 cm array for 10 ~~second~~s at 3 kV. Separation was performed at 15 kV for 44 min~~utes, at~~, at a run temperature of 60 °C using the POP-6™ sieving polymer (Applied Biosystems). Following data collection, samples were analysed using GeneMapper® *ID-X* software version 1.2 at a threshold of 50 ~~RFU~~rfu. A sequenced allelic ladder was included in each injection. Allele calls of all markers including multicopy markers were made at a minimum peak height of 50 ~~RFU~~rfu. This was done using the conservative method of calling an allele without making use of relative peak height in case of multicopy markers.

2.~~4~~5 Multiplex performance studies

The developed RM-Yplex assay was tested for its reproducibility, sensitivity, mixture resolution, and family pedigree studies. All controls and samples used were genotyped a number of times to test reproducibility. In order to test the sensitivity of the developed multiplex assay, serial dilutions of male controls 9948, TaqMan and M1~~male DNAsample (31.25pg to~~ male DNA sample (31.25–3000 pg) were amplified using the multiplex assay. For the testing male/female mixtures, the amount of 9948 male DNA control was kept 1 ng and female DNA (F1) was increased from 250~~ng to 1000~~ ng to 1000 ng. In addition, samples from two different UAE Arab families were analysed using the RM-Yplex assay to demonstrate close paternal relative mutations patterns.

2.~~5~~6 Development of allelic ladder

2.~~5~~6.1 Alleles purification

For each locus, samples with different allele sizes were amplified in singleplex PCRs using optimised PCR cycling conditions as in ~~section 2.3~~Section 2.3 and 2.5 mM primer concentration for each primer set. Amplified alleles were isolated after electrophoresis from agarose gel. The band was excised using a sterile scalpel blade, and purified using a QIAquick Gel Extraction Kit (Qiagen, UK). In order to separate close allele sizes in mutli-allelic loci, alleles were separated by running amplified products on a polyacrylamide gel before purification.

2.~~5~~6.2 Alleles sequencing

Unlabelled primers were used to amplify purified alleles in singleplex PCRs using optimised PCR conditions. PCR products were purified using microCLEAN™ reagent. Sequencing reactions were carried out using BigDye® Terminator v3.1 Cycle

Sequencing Kit (Applied Biosystems). Each allele was sequenced twice using forward and reverse primers for confirming DNA sequence.

2.56.3 Allelic ladder construction

The amplified products obtained from singleplex PCRs were electrophoresed and the volumes of amplified products to be added to the ladder stock were calculated for individual alleles on the basis of allelic heights (e.g. if 1 µl volume was used for a allele showing a peak height of 500 rfu then 0.5 µl volume was added for the allele showing a peak height of 1000 rfu). The amplified products for each locus were thus mixed together in appropriate proportions to produce a locus ladder. 1 µl of this ladder was electrophoresed on 8-capillary ABI 3500 genetic analyser. The results were analysed using GeneMapper® *ID-X*. The ladder was then balanced by adjusting the volume of the amplified products for the samples exhibiting lower peak heights to generate a balanced ladder. The locus ladders were then mixed together to produce a composite ladder which was electrophoresed on 8-capillary ABI 3500 genetic analyserand analysed using GeneMapper® *ID-X*. The composite ladder was again balanced using the strategy for locus ladders. This produced a stock of balanced composite allelic ladder for further use [20].

The ladder so developed was quality tested using the allelic ladder and quality control DNA samples used by the International RM Y-STR Study Group [13]2.6 .

2.7 Comparability of outcomes and nomenclature

To allow the new assay to produce data fully comparable with the International RM Y-STR Study Group Multicentre Study [13], 8 blind samples and the allelic ladders supplied by the RM Y-STR Study Group were used when analysing these samples in addition to our own allelic ladders. The International RM-YSTR group repeat nomenclature was used for allelic designation. All samples amplified robustly and the results were consistent with those of the International RM-YSTR group.

3 Results and discussion

3.1 RM-Yplex description

The RM-Yplex assay described here comprises 13 RM Y-STR markers representing 9 single copy loci and 4 multi-copy loci, which typically amplify a total of 21 male-specific alleles simultaneously [12,13]. DYS526a/b locus produces two different sizes of fragment as the forward primer has two complementary sites in the same region of Y chromosome. However, DYF387S1, DYF399S1, DYF403S1ab and DYF404S1 loci are duplicated in different regions on the Y chromosome. Additional copies may be expected as have been identified for the classical Y-STRs with increased population sample sizes (see YHRD for overview).

3.2 Development and optimisation of RM-Yplex assay

Primer sets for DYF387S1, DYS576 and DYS612 loci were modified from the initial protocol [11][11] in order to have similar annealing characteristics to the remaining pairs of primers and allowing successful combination within the same multiplex assay. The primers set for the locus DYS570 had to be redesigned from the initial protocol [11][11] to adjust product size range in order to prevent it from overlapping other loci in the same panel (Fig. 1). Multiplex PCR optimisation strategy was followed as described elsewhere [21]. A sequenced allelic ladder was developed for the RM-Yplex assay during this work (Fig. 4).

A largely balanced profile of all 13 RM Y-STR markers was successfully achieved with 32 cycles of the 2 stages PCR protocol although DYS526a showed lower peak heights in some samples (Fig. 2). PCR products were always detected above threshold (50 RFUrfu). The observed lower peak heights of DYS526a (200 250 RFU 250 rfu) were likely caused by the 3 nucleotides mismatch within the secondary primer-binding site which cannot be easily avoided due to co-amplification of DYS526b with the same primer pair. This was confirmed through sequencing (Fig. 3).

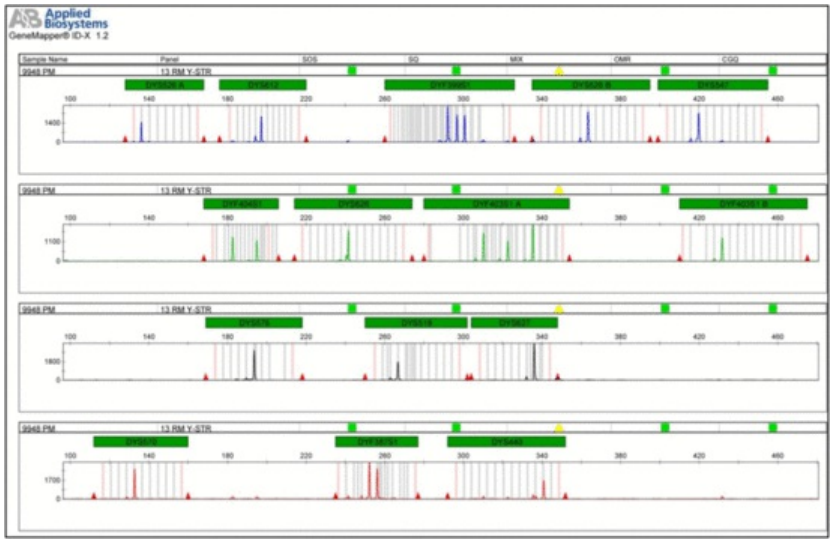

Fig. 2 Y-STR profile consisting of 13 RM Y-STR markers generated using RM-Yplex assay for male DNA control 9948 using ABI 3500 Genetic Analyser. Samples wereinjected in an 8-capillary 50 cm array for 10 s at 3 kV. Separation was performed at 15 kV for 44 min at a run temperature of 60 °C using the POP-6™ polymer.

TCTGGTGAACTGATCCAAACCTTTACTTATTTTCTTCCTTTTTTCCTCTCTCCTTCCCTCC
CTCCCTCTTTCTTCCTTTCTTTCTTCCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCT
TTCTTTCTTTCTTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCTTTCCTTCTTTCC
TTCATTTCTTCTTTCTTTCTCTTTACCT**TCTGGGAGACTGATCCAAACC**TTTACTTACTTT
CTATTTTTCTCTATTTTTCTTTCTCTATTTTTTTCTCTCCTTCCTTCCTTCCTTCCTTCCTTCC
TTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCTCTTTACCTTCTGGCGAAGTAACCC

Fig. 3 Sequencing results of DYS526ab locus illustrating the mismatch (underlined) position at the second attachment site of the forward primer producing the shorter fragment of the locus.

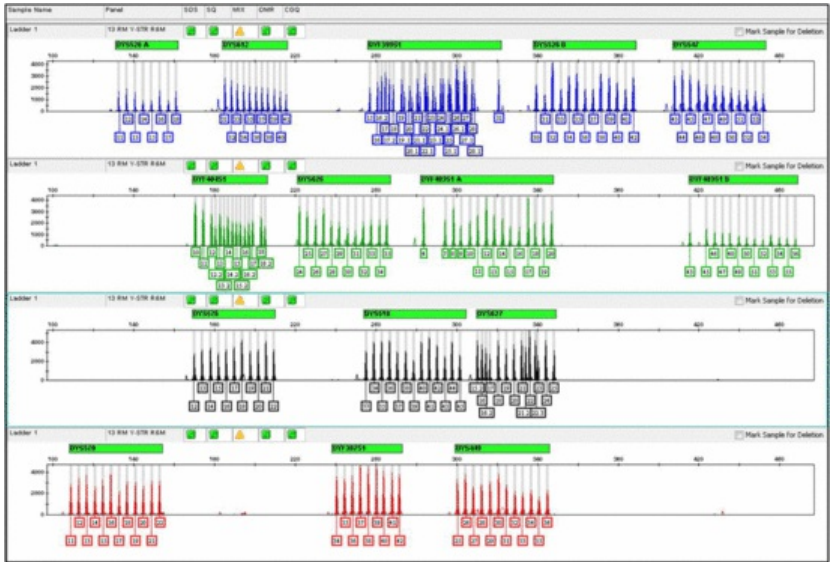

Fig. 4 Sequenced allelic ladders for 13 RM Y-STR markers targeted with the single RM-Yplex assay using ABI 3500 Genetic Analyser. Samples were injected in the 8-capillary 50 cm array for 10 s at 3000 V. Separation was performed at 15,000 V for 44 min at a run temperature of 60 °C using the POP-6™ polymer. The allele designation nomenclature was adopted from theInternational RM Y-STR Study Group's multicentre study [13].

3.3 Multiplex performance

A few pre-validation studies were conducted for demonstrating the effectiveness of the RM-Yplex assay; however, complete forensic developmental validation of the assay will be reported in a future study, while the present study is devoted to introducing the assay and making it available for widespread use. The multiplex performed well including the multicopy markers in our hands. The multiplex presented here showed quite robust allele calls for all loci in all samples including the 8 blind samples provided and the two positive controls. The previous Gglobal RM Y-STR study [13] indicated problems with accurate genotyping of two multicopy markers DYF403S1ab and DYF399S1 with some participants of this large multicentre study by using the initially described [12] three multiplex approach. Using the presented assay all variants in the multicopy markers could be easily called. With our single multiplex method, we detected some imbalance of the amplification peaks at DYS526 a/ba/b likely due to a primer mismatch at the second primer site (Figure. 3). Though this was not acute in our preliminary analysis, a third primer to match the relevant sequence could be developed to obtain fully balanced peaks at this locus. Future concordance testing as part of a complete forensic validation study will demonstrate the performance of our multiplex assay in different labs.

Furthermore, we notice that in the current design of the multiplex, the spacing between DYS518 and DYS627 appears to be small though it is adequate for other markers. Howeverfor DYS518 the large alleles 48-, for DYS518, the large alleles 48–52 were not detected in the Gglobal RM dataset [13] and alleles 46,47& 47 and 52.2 were detected at very low frequencies (0.09%, 0.06% &and 0.008%) in a dataset of over 12,000 samples. For DYS627 only the smallest allele 12 was observed at a frequency of 0.008% in the same dataset. Thus a possibility of an overlap between these two markers appears to have an extremely low probability. However, the authors are conscious of this and this might need consideration in further works.

3.3.1 Reproducibility and sensitivity testing

The male controls tested were amplified twice in triplicate and yielded consistent profiles. The female control was always included in the PCR batches. Different amounts of male DNA, ranging from 31.25 ng to 1 ng, were used as template in order to preliminarily determine the minimum amount of DNA needed to produce fully interpretable complete 13 loci RM-YSTR profiles. Three different male samples were used for the sensitivity testing. The quantities of male DNA templates used were 1 ng, 0.5 ng, 0.250 ng, 0.125 ng, 0.0625 ng and 0.03125 ng. Each quantity of male template DNA was amplified in triplicates. Full RM–Yplex Yplex profiles were obtained from 0.0625 ng to 3 ng of all three male samples tested (Fig. 5), while 0.03125 ng male template DNA yielded partial profiles. This also led to the development of haplotypes of two male controls (9948 and TaqmMan) used during the development of this multiplex assay for enabling other users for use as controls in their studies (Table 3).

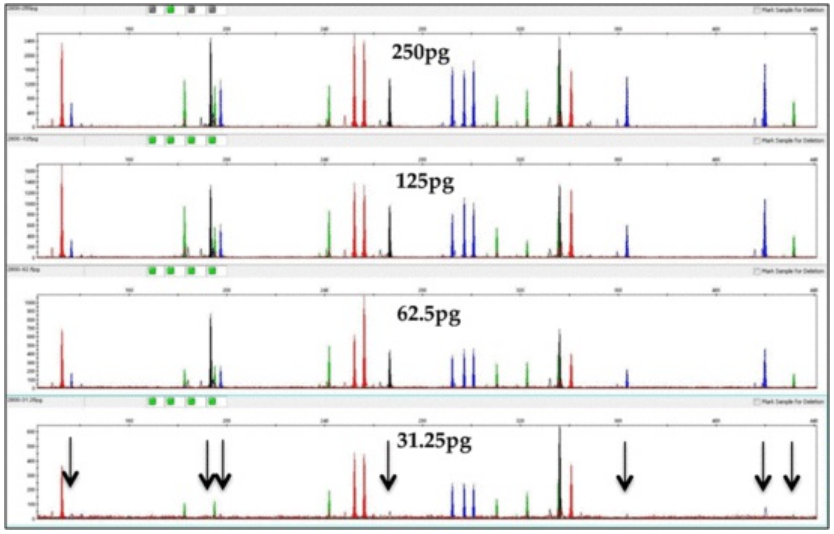

Fig. 5 Electropherograms developed using the single RM-Yplex assay targeting 13 RM Y-STR markers at different male template DNA amounts for the 9948 male control DNA. Allele drop-outs are indicated by black arrows. Run conditions were same as used for Figs. 2 and 4. Data for 3 ng and 500 pg are not shown in this figure. Note that panels are on different scales.

Table 3 RM-Yplex profiles for two commercial human male control DNAs.[a]

Locus	9948 male control	TaqMan male control
DYF387S1	35:38	36:39
DYF399S1	21:22:25.1	19:23
DYF403S1a	10:15:16	11.2:12.2:15
DYF403S1b	49	54
DYF404S1	12:14	15:18
DYS449	30	29
DYS518	38	42
DYS526a	14	13
DYS526b	36	34
DYS547	48	48
DYS570	18	19
DYS576	16	15
DYS612	37	36
DYS626	28	29
DYS627	22	21

[a] Allele designation is according to the repeat nomenclature introduced by the International RM Y-STR Study Group [13].

3.3.2 Mixture study

Preliminary male–female mixture analyses were carried out. Different amounts of female sample F1 template DNA were added to 1 ng of 9948 male control ~~DNA to produce male-~~ DNA to produce male–female DNA mixtures at different ratios i.e. 1:250, 1:500, 1:750 and 1:1000. Each of the mixtures was amplified in triplicate for consistency. Full RM-Yplex profiles were obtained at all ratios of male/female mixtures tested. A slight reduction in RM Y-STR peak height was observed with increased female DNA in the mixture ~~(Figure~~ (Fig. 6).

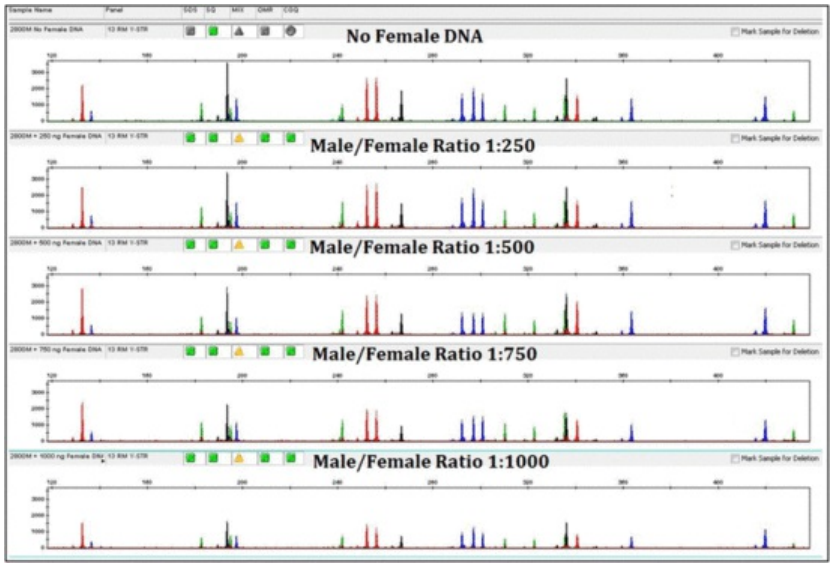

Fig. 6 Electropherograms from injecting RM-Yplex assay amplifications targeting 13 RM Y-STRs of mixturesconsisting of 1 ng male template DNA mixed with different amounts of female template DNA. Run conditions were same as used for Figs. 2 and 4.

3.3.3 UAE family pedigree samples

Samples of males from two three generations UAE Arab families comprising of 11 and 17 meiotic transfers respectively were analysed using the RM-Yplex assay developed. Both families demonstrated a pattern of mutation at different RM Y-STR loci (Fig. 7a and b). A family pedigree (Fig. 7a) comprising of 18 male individuals were connected by 17 meiotic transfers where 10 male individuals showed at least one RM Y-STR mutation. 11 mutations were observed at 5 RM Y-STR loci in this pedigree. Five mutations out of 11 were observed at DYF403S1b. Three mutations were observed at DYS626 and a single mutation each was observed at DYF387S1, DYS570 ~~& DYS547 loci.~~and DYS547 loci. In the other pedigree 4 individuals showed a single mutation at DYS 404S1, 570, 547, 399S1 and 2 showed a mutation at DYS403S1b locus (Fig. 7b). In both the pedigrees all mutations were single step changes and 13 out of a total of 16 mutations led to an increase in the allele length due to repeat gain.

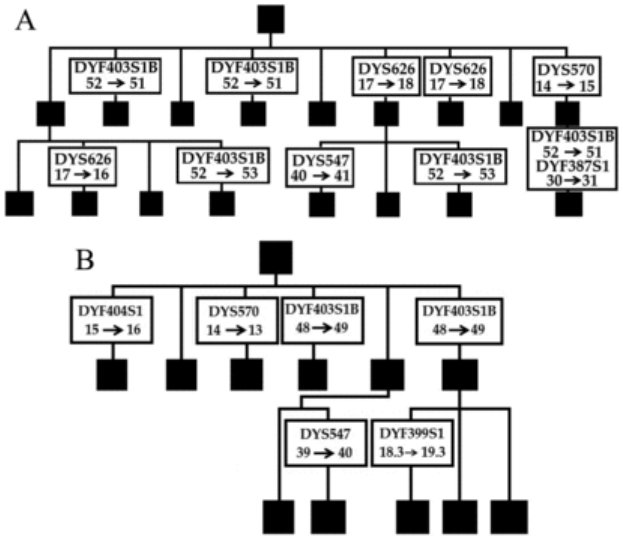

Fig. 7 (a) RM Y-STR mutation pattern and abilities to differentiate closely related males in an UAE Arab family analysed with the RM-Yplex assay targeting 13 RM Y-STR markers. Only RM Y-STRs with observed mutations are shown with the mutated alleles. (b) RM Y-STR mutation pattern and abilities to differentiate closely related males in an UAE Arab family analysed with the RM-Yplex assay targeting 13 RM Y-STR markers. Only RM Y-STRs with observed mutations are shown with the mutated alleles.

4 Conclusions

In this study, we have demonstrated that the 13 RM Y-STR markers, typically amplifying >20 male-specific alleles can be simultaneously analysed with a single multiplex assay that we called RM-Yplex. The RM-Yplex assay is robust and sensitive down to 0.06 ng template DNA, allows full RM Y-STR profile detection out of all male–female mixtures tested, and has shown reliable and consistent results with commercial male controls and~~reference~~ reference samples. With the use of the conservative allele calling method without taking into consideration the relative peak heights in case of multicopy markers; the RM-Yplex assay produced results consistent with the allelic nomenclature previously introduced by the International RM Y-STR Study Group. Haplotypes of two male controls 9948 and Taq~~m~~Man will allow for calibration and quality control when using RM Y-STRs. It was interesting that using the multiplex assay presented, the assignment alleles including all multi-copy markers even to the lowest DNA inputs was possible. The sensitivity, blind ~~&~~and control sample amplification results presented in this manuscript showed that all alleles in the multicopy markers could be discretely called. At below stochastic thresholds of input DNA (32.5 pg) there were dropouts which were expected. It might be worthwhile to include samples showing variant alleles and those amplified using the other multiplexes in the validation of the multiplex. Due to the effective, robust, and sensitive way of analysing RM Y-STRs with our tool, we expect the RM-Yplex to become widely used for differentiating related and unrelated males in forensic and other applications.

Acknowledgments

We would like to humbly thank the ~~private o~~Private Office of the Crown Prince of Dubai for enabling the Dubai Government grant support for this project. AR and MK were supported by the Erasmus MC University Medical Centre Rotterdam, The Netherlands.

References

[1]

M.A. Jobling and C. Tyler-Smith, Fathers and sons: the Y chromosome and human evolution, *Trends Genet.* **11** (11), 1995, 449–456.

[2]

L. Roewer, Y chromosome STR typing in crime casework, *Forensic Sci. Med. Pathol.* **2**, 2009, 77–84.

[3]

G.C. Calacal, F.C. Delfin, M.M. Tan, L. Roewer, D.L. Magtanong, M.C. Lara, R. Fortun and M.C. De Ungria, Identification of exhumed remains of fire tragedy victims using conventional methods and autosomal/Y chromosomal short tandem repeat DNA profiling, *Am. J. Forensic Med. Pathol.* **26** (3), 2005, 285–291.

[4]

L.A. Zhivotovsky, P.A. Underhill, C. Cinnio-lu, M. Kayser, B. Morar, T. Kivisild, R. Scozzari, F. Cruciani, G. Destro-Bisol, G. Spedini, G.K. Chambers, R.J. Herrera, K.K. Yong, D. Gresham, I. Tournev, M.W. Feldman and L. Kalaydjieva, The effective mutation rate at Y chromosome short tandem repeats, with application to human population-divergence time, *Am. J. Hum. Genet.* **74** (1), 2004, 50–61.

[5]

J.M. Butler, R. Schoske, P.M. Vallone, M.C. Kline, A.J. Redd and M.F. Hammer, A novel multiplex for simultaneous amplification of 20 Y chromosome STR markers, *Forensic Sci. Int.* **129** (1), 2002, 10–24.

[6]

W. Shi, Q. Ayub, M. Vermeulen, S. Shao R. g. Zuniga, K. van der Gaag, P. de Knijff, M. Kayser, Y. Xue and C. Tyler-Smith, A worldwide survey of human male demographic history based on Y-SNP and Y-STR data from the HGDP-CEPH populations, *Mol. Biol. Evol.* **27** (2), 2010, 385–393.

[7]

W. Wei, Q. Ayub, Y. Xue and C. Tyler-Smith, A comparison of Y-chromosomal lineage dating using either resequencing or Y-SNP plus Y-STR genotyping, *Forensic Sci. Int.: Genet.* **7** (6), 2013, 568–572.

[8]

N. Leat, L. Ehrenreich, M. Benjeddou, K. Cloete and S. Davison, Properties of novel and widely studied Y-STR loci in three South African populations, *Forensic Sci. Int.* **168** (23), 2007, 154–161.

[9]

M. Vermeulen, A. Wollstein, K. van der Gaag, O. Lao, Y. Xue, Q. Wang, L. Roewer, H. Knoblauch, C. Tyler-Smith, P. de Knijff and M. Kayser, Improving global and regional resolution of male lineage differentiation by simple single-copy Y-

chromosomal short tandem repeat polymorphisms, *Forensic Sci. Int.: Genet.* **3** (4), 2009, 205–213.

[10]

C. Davis, J. Ge, C. Sprecher, A. Chidambaram, J. Thompson, M. Ewing, P. Fulmer, D. Rabbach, D. Storts and B. Budowle, Prototype PowerPlex® Y23 System: a concordance study, *Forensic Sci. Int.: Genet.* **7** (1), 2013, 204–208.

[11]

K.N. Ballantyne, M. Goedbloed, R. Fang, O. Schaap, O. Lao, A. Wollstein, Y. Choi, K. van Duijn, M. Vermeulen, S. Brauer, R. Decorte, M. Poetsch, N. von Wurmb-Schwark, P. de Knijff, D. Labuda, H. Vuzina, H. Knoblauch, R. Lessig, L. Roewer, R. Ploski, T. Dobosz, L. Henke, J. Henke, M.R. Furtado and M. Kayser, Mutability of Y-chromosomal microsatellites: rates, characteristics, molecular bases, and forensic implications, *Am. J. Hum. Genet.* **87** (3), 2010, 341–353.

[12]

K.N. Ballantyne, V. Keerl, A. Wollstein, Y. Choi, S.B. Zuniga, A. Ralf, M. Vermeulen, P. de Knijff and M. Kayser, A new future of forensic Y-chromosome analysis: Rapidly mutating Y-STRs for differentiating male relatives and paternal lineages, *Forensic Sci. Int.: Genet.* **6** (2), 2012, 208–218.

[13]

K.N. Ballantyne, A. Ralf, R. Aboukhalid, N.M. Achakzai, M.J. Anjos, Q. Ayub, J. Balažic, J. Ballantyne, D.J. Ballard, B. Berger, C. Bobillo, M. Bouabdellah, H. Burri, T. Capal, S. Caratti, J. Cárdenas, F. Cartault, E.F. Carvalho, M. Carvalho, B. Cheng, M.D. Coble, D. Comas, D. Corach, M.E. D'Amato, S. Davison, P. de Knijff, M.C.A. De Ungria, R. Decorte, T. Dobosz, B.M. Dupuy, S. Elmrghni, M. Gliwiński, S.C. Gomes, L. Grol, C. Haas, E. Hanson, J. Henke, L. Henke, F. Herrera-Rodríguez, C.R. Hill, G. Holmlund, K. Honda, U.-D. Immel, S. Inokuchi, M.A. Jobling, M. Kaddura, J.S. Kim, S.H. Kim, W. Kim, T.E. King, E. Klausriegler, D. Kling, L. Kovačević, L. Kovatsi, P. Krajewski, S. Kravchenko, M.H.D. Larmuseau, E.Y. Lee, R. Lessig, L.A. Livshits, D. Marjanović, M. Minarik, N. Mizuno, H. Moreira, N. Morling, M. Mukherjee, P. Munier, J. Nagaraju, F. Neuhuber, S. Nie, P. Nilasitsataporn, T. Nishi, H.H. Oh, J. Olofsson, V. Onofri, J.U. Palo, H. Pamjav, W. Parson, M. Petlach, C. Phillips, R. Ploski, S.P.R. Prasad, D. Primorac, G.A. Purnomo, J. Purps, H. Rangel-Villalobos, K. Rębała, B. Rerkamnuaychoke, D.R. Gonzalez, C. Robino, L. Roewer, A. Rosa, A. Sajantila, A. Sala, J.M. Salvador, P. Sanz, C. Schmitt, A.K. Sharma, D.A. Silva, K.-J. Shin, et al., Toward male individualization with rapidly mutating Y-chromosomal short tandem repeats, *Hum. Mutat.* **35**, 2014, 1021–1032.

[14]

C. Robino, A. Ralf, S. Pasino, M.R. De Marchi, K.N. Ballantyne, A. Barbaro, C. Bini, E. Carnevali, L. Casarino, C. Di Gaetano, M. Fabbri, G. Ferri, E. Giardina, A. Gonzalez, G. Matullo, A.L. Nutini, V. Onofri, A. Piccinini, M. Piglionica, E. Ponzano, C. Previderè, N. Resta, F. Scarnicci, G. Seidita, S. Sorçaburu-Cigliero, S. Turrina, A. Verzeletti and M. Kayser, Development of an Italian RM Y-STR haplotype database: results of the 2013 GEFI collaborative exercise, *Forensic Sci. Int.: Genet.* 2013.

[15]

A.A. Westen, T. Kraaijenbrink, L. Clarisse, L.J.W. Grol, P. Willemse, S.B. Zuniga, E.A. Robles de Medina, R. Schouten, K.J. van der Gaag, N.E.C. Weiler, A.J. Kal, M. Kayser, T. Sijen and P. de Knijff, Analysis of 36 Y-STR marker units including a concordance study among 2085 Dutch males, *Forensic Sci. Int.: Genet.* **14**, 2015, 174–181.

[16]

J.G. Shewale and R.H. Liu, Forensic DNA Analysis: Current Practices and Emerging Technologies, 2013, Taylor & Francis.

[17]

A. Untergrasser, I. Cutcutache, T. Koressaar, J. Ye, B.C. Faircloth, M. Remm and S.G. Rozen, Primer3 – new capabilities and interfaces, *Nucleic Acids Res.* **15**, 2012, 115.

[18]

T. Koressaar and M. Remm, Enhancements and modifications of primer design program Primer3, *Bioinformatics* **23** (10), 2007, 1289–1291.

[19]

R. Schubbert and S. Rittler, Multiplex microsatellite analysis with 5 dye-labelled marker sets using FAM, Yakima Yellow, ATTO550, ATTO 565 and ATTO633 on an ABI 3130XL genetics analyzer, Applied Genetics, 2010, Eurofins Medigenomix; Anzinger Str. 7a, 85560 Ebersberg.

[20]

R.A.L. Griffths, M.D. Barber, P.E. Johnson, S.M. Gillbard, M.D. Haywood, C.D. Smith, J. Arnold, T. Burke, A.J. Urquhart and P. Gill, New reference allelic ladders to improve allelic designation in a multiplex STR system, *Int. J. Legal*

Med. **111** (5), 1998, 267–272.

[21]

R. Schoske, P. Vallone, C. Ruitberg and J. Butler, Multiplex PCR design strategy used for the simultaneous amplification of 10-Y chromosome short tandem repeat (STR) loci, *Anal. Bioanal. Chem.* **375** (3), 2003, 333–343.

Highlights

- We present RM-Yplex amplifying 13 ~~Rapidly M~~rapidly mutating Y-STR markers.
- A sequenced allelic ladder developed for calling alleles of all loci is described.
- 8 PT samples with known RM haplotypes showed consistent results using RM-Yplex.
- Robust allele calls for all markers including multicopy markers up to 62.5 pg DNA.
- Two ~~M~~male controls 9948 and Taq~~m~~Man haplotypes are presented for improving calibration.

Queries and Answers

Query: Please confirm that given names and surnames have been identified correctly.

Answer: Yes

Query: Please check the address for the corresponding author, and correct if necessary.

Answer: Yes

Query: Your article is registered as a regular item and is being processed for inclusion in a regular issue of the journal. If this is NOT correct and your article belongs to a Special Issue/Collection please contact a.healey@elsevier.com immediately prior to returning your corrections.

Answer: It is a regular item.

Query: The unit "RFU" has been changed to "rfu" for consistency here and in subsequent occurrences. Please check, and correct if necessary.

Answer: That is fine with me

Query: One or more sponsor names and the sponsor country identifier may have been edited to a standard format that enables better searching and identification of your article. Please check and correct if necessary.

Answer: Yes