

## PowerPlex Y23 System: A Fast, Sensitive and Reliable Y-STR Multiplex System for Forensic and Population Genetic Purpose

Toshi Jain<sup>1,2,\*</sup>, Pankaj Shrivastava<sup>1\*\*</sup>, Bansal DD<sup>1</sup>, Dash HR<sup>1</sup> and Veena Ben Trivedi<sup>1</sup>

<sup>1</sup>DNA Fingerprinting Unit, State Forensic Science Laboratory, Sagar-470001 (MP), India

<sup>2</sup>School of Studies in Microbiology, Jiwaji University, Gwalior (MP), India

\*Authors equal Contributed

### Abstract

Y-STR genotyping is an excellent tool for the identification of male DNA masked by female DNA, particularly in sexual assault cases where, more often the male and female components cannot be separated completely. Resultant, the female component could exist prominently even in the male component after separation. When the "male DNA sample mixed with female DNA" undergoes PCR by using autosomal STR multiplex amplification process, the female DNA component is also amplified, mostly masking the male DNA. Recently launched Y-STR kit, PowerPlex Y23 (PP Y23) System is a 5-dye multiplex genotyping kit that analyzes 17 Y-STR loci, commonly available in other Y-STR multiplex kits (DYS19, DYS385a/b, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, and Y-GATA-H4) along with that 6 new Y-STR loci (DYS481, DYS533, DYS549, DYS570, DYS576, and DYS643). The addition of 6 new markers has made the Y-STR analysis more discriminating. The purpose of the present study is to show the amplification of male DNA from a wide variety of forensic samples using half the manufacturer's recommended reaction volume. Also the present study validates the kit for its use in Forensic casework. The results demonstrate that the PPY23 System is a robust and sensitive multiplex system, capable of giving reliable and consistent amplification results from a wide variety of forensic casework samples. Even the presence of high concentration of commonly encountered inhibitors such as hematin (from Blood) and humic acid (from soil), male/female DNA mixtures and low amounts of DNA template (15.62 pg) could not hamper the process of amplification.

**Keywords:** Y chromosome; Y-STR; Forensic; DNA typing; Short tandem repeat (STR); PowerPlex Y 23

### Introduction

Over the years forensic DNA typing has earned the credibility of forensic scientists. Different variants of DNA fragment analysis technology is being used for different purposes in forensics [1]. Autosomes and X chromosome (only in daughters) are inherited from both the parents after recombination, while Y chromosome is inherited paternally and mitochondrial DNA is inherited maternally. The inheritance pattern of these markers makes them suitable for establishing paternal and maternal lineage [1].

In routine forensic biological and serological examination of rape cases presence of semen and/or spermatozoa is detected, which cannot link the evidence to a particular suspect. Because of this fact DNA typing in sexual assault cases has proven to be a better solution. Sexual assault cases, that routinely consists of evidentiary stains that are a mixture of body fluids from the victim and suspect. Though differential analysis technique permits the separation of the male and female components of the mixed stains [2]. However, a complete separation is not always possible [3-7]. These limitations of the separation process, makes it challenging for the forensic experts to interpret the mixed DNA profiles, with two or more contributors. In many circumstances the PCR based amplification methods fail to amplify the minor (e.g. male) component of DNA mixtures as they have to face the opposition with the alleles from the major (e.g. female) component, which tends to get amplified first. One approach towards resolving this issue is to target male-specific polymorphisms on the non recombining portion of the Y chromosome (NRY). The NRY is strictly inherited paternally and, therefore, is the best material to trace the paternal lineage with the additional advantages of small effective population size, low mutation rate, sufficient markers and population-specific haplotype distribution [8,9]. With the availability of genotyping markers for the NRY region, Y-DNA typing has become one of the most indispensable techniques in

Forensic DNA typing, especially in sexual assault cases which bump into issues like unsuccessful separation of male and female cells. Complete separation of male and female cells is not feasible in aforementioned cases because of certain reasons like, age and/or quality of semen stains, azoospermic men, the presence of other body fluids from victim and suspect of different sex, and, two or more male semen donors. The ability to target polymorphisms on the NRY region and the recent availability of improved multiplex markers for NRY, has made it possible to analyse such samples that were previously difficult to interpret. To understand the demographic history of human populations, Y chromosome has been widely used in anthropology and population genetics as well [10]. Two classes of NRY markers, short tandem repeats (Y-STRs) and single nucleotide polymorphisms (Y-SNPs) are being used in the present scenario. The combination of alleles at multiple Y-STR loci, on a single Y chromosome defines a Y-STR haplotype, whereas the combination of alleles at multiple SNPs defines a NRY haplogroup [11]. The high geographic specificity of many NRY haplogroups [8,12] aids forensic scientists in narrowing down the geographic location of the perpetrator (male) with the help of circumstantial evidence left at the crime scene. The forensic utility of Y-STRs is the result of their high levels of polymorphism in human populations [13], their small size in base pairs

**\*Corresponding author:** Pankaj Shrivastava, DNA Fingerprinting Unit, State Forensic Science Laboratory, Sagar-470001 (MP), India, Tel: +91 9424371946; E-mail: [pankaj.shrivastava@rediffmail.com](mailto:pankaj.shrivastava@rediffmail.com); [ecsdjsbp@gmail.com](mailto:ecsdjsbp@gmail.com)

**Received** April 12, 2016; **Accepted** April 28, 2016; **Published** April 30, 2016

**Citation:** Jain T, Shrivastava P, Bansal DD, Dash HR, Trivedi VB (2016) PowerPlex Y23 System: A Fast, Sensitive and Reliable Y-STR Multiplex System for Forensic and Population Genetic Purpose. J Mol Biomark Diagn 7: 281. doi:[10.4172/2155-9929.1000281](https://doi.org/10.4172/2155-9929.1000281)

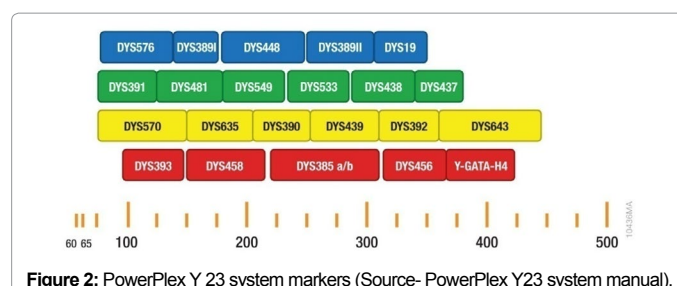
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(~100-400 bp), and the ability to type multiple Y-STRs in a single PCR reaction [14,15]. Two decades ago, since the first case investigation by Y-STR markers [16-18], the application scenario of Y-STR continuously evolved in various aspects like the number of markers. The original 9-Y-STR set, named as the minimal haplotype, expanded in number with time to increase the discrimination power of the Y-chromosome test [19] (e.g. PPY12 (PowerPlex Y12, Promega Corp., USA), Yfiler [AmpFlSTR Yfiler kit, (Applied BioSystem/Life Technologies/Thermo-AB/LT/Thermo)]). The PP Y23 system (Promega Corp., USA) is a newly developed multiplex kit that allows the simultaneous analysis of 23 Y-STR markers, incorporating six new markers in addition to the previous markers of the Yfiler kit. This new Y-STR panel was recently validated for forensic use and for concordance with the Yfiler [20-22]. Multiple utility of PP Y23 system is presented in Figure 1.

Though a few publications report validation of PP Y23 in last few years [20-22] for forensic purpose but this is the first study reporting the trial of kit on a wide variety and number of forensic samples with interesting findings along with the validation study and proving the utility of PP Y23 system in forensic DNA typing casework from India, a country with diverse population. All the validation experiments were performed using half reaction volume of the recommended protocol of the manufacturer and complete Y23 STR profile was detected without any drop-ins or drop-outs. This was confirmed further by full volume PCR of tested samples as well.

### The PP Y23 System

The PP Y23 System allows co-amplification of the several loci recommended in the European Minimal Haplotype (DYS19, DYS385a/b, DYS389I/II, DYS390, DYS391, DYS392, and DYS393) [16,23], the two additional loci recommended by SWGDAM (DYS438 and DYS439) [24], as well as twelve additional loci (DYS437, DYS448, DYS456, DYS458, DYS481, DYS533, DYS549, DYS570, DYS576, DYS635, DYS643, and Y-GATA-H4). Six of the additional loci are highly discriminating Y-STR loci (DYS481, DYS533, DYS549, DYS570, DYS576 and DYS643) [25-27]. The PP Y23 System is configured with the majority of the highly discriminating loci with smaller amplicon size. Due to smaller amplicon size even trace amounts of DNA can be amplified and the discrimination potential of partial profiles can be maximized [20]. The PP Y23 System provides all the necessary materials to amplify the Y-STR regions of human genomic DNA. It includes a hot-start DNA polymerase, and is compatible with the 3100-Avant Genetic Analyzers and Applied Biosystems 3130, 3130xl, 3500 and 3500xl Genetic Analyzers. The PP Y23 System is a 5-dye system. The blue channel consists of the fluorescein labelled STR



**Figure 2:** PowerPlex Y 23 system markers (Source- PowerPlex Y23 system manual).

amplicons for DYS576, DYS389I, DYS448, DYS389II, and DYS19. The green channel includes DNA fragments labelled with JOE for DYS391, DYS481, DYS549, DYS533, DYS438, and DYS437. The yellow channel consists of amplification products for DYS570, DYS635, DYS390, DYS439, DYS392, and DYS643 which are labelled with TMR-ET. The amplified products for DYS393, DYS458, DYS385a/b, DYS456 and Y-GATA-H4 are in the red channel and are labelled with CXR-ET. Fragments included in the internal lane standard are detected in the orange channel and are labelled with CC5 (CC5 Internal Lane Standard 500 Y23) (Figure 2).

## Materials and Methods

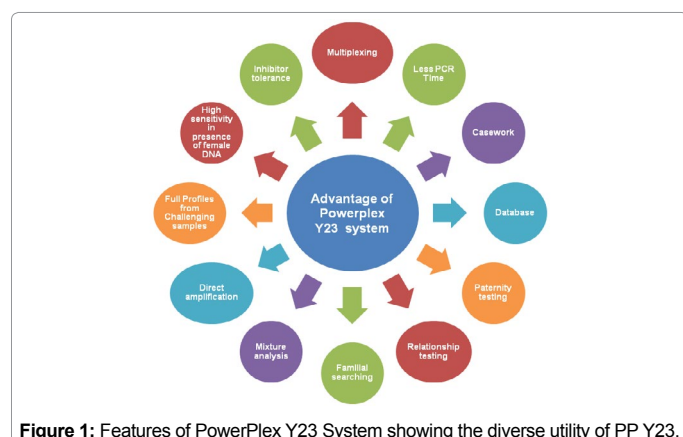
### System sensitivity

DNA samples organically extracted using automated DNA extraction system 12 GC (PSS, Matsudo, Japan) and quantified using real time PCR ABI 7500 (AB/LT/Thermo). Two male DNA samples were used for the sensitivity study. A serial dilution was performed with the following template amounts: 1000 pg, 500 pg, 250 pg, 125 pg, 62.5 pg, 31.25 pg, and 15.62 pg and 8.00 pg.

For the inhibition study, 1 ng DNA sample mixed either with humic acid (final concentrations 10 ng/μL, 100 ng/μL, 200 ng/μL and 300 ng/μL) or hematin (final concentrations 20 μM, 100 μM, 200 μM, 500 μM and 700 μM) were used. The amplification performance of male:female (M:F) mixtures was tested using M:F mixture ratios. 1000 pg of female DNA was mixed with varied quantity (1000 pg, 500 pg, 250 pg, 125 pg, 62.5 pg, 31.25 pg, 15.62 pg and 8.00 pg) of male DNA. For this, samples with a measured DNA concentration higher than 0.1 ng/μL were diluted with TE buffer to appropriate quantity. For accessing the direct Amplification compatibility, 1 and 0.5 μL of saliva samples of 50 different males was amplified without any prior processing/treatment. In all the tested samples complete Y 23 STR profile was detected. No drop-ins or drop-outs were observed in all the tested samples, this was confirmed with the Y 23 profile of same samples amplified after DNA isolation. Direct amplification reactions were also performed using one 1.2 mm punches from FTA cards (having buccal/blood cells). Punches were added directly to the reaction mixture to check the direct amplification compatibility of FTA Cards (GE Healthcare/Whatman) with PP Y23 system. Amplification was performed by using half the manufacturer's recommended reaction volume.

### Casework and population data analysis for forensic and population genetic purpose

PP Y23 system was tested with different variety of case work samples including clothing stained with different body fluids, tissues, vaginal smear slides, vaginal swabs, nail scrapings of females in case of sexual assault which might have male tissue struck in them, uterus where there was no discrimination with fetus (pregnancy of few weeks when there is no discrimination between mother and fetus) and swabs from the weapons etc. For forensic genetic analysis and comparison



**Figure 1:** Features of PowerPlex Y23 System showing the diverse utility of PP Y23.

we used DNA from unrelated males from two tribal populations of the central India (Table 1). The PP Y23 system was also tested on brother's pairs and father son pairs to observe the patrilineal similarity between related individuals.

DNA amplification

All of the samples were amplified using GeneAmp PCR System 9700 thermal cycler (AB/LT/Thermo). The following amplification set-up and cycling parameters, as described in the PP Y23 Technical Manual (PP Y23 System Technical Manual, 7/12, available at: <http://www.promega.com/resources/protocols/technical-manuals/101/powerplex-y23-system-protocol/>, with the modification of using half the reaction volume of the recommended protocol. Each amplification reaction contained 2.5 µl of PP Y23 Master Mix and 1.25 µl of PP Y23 Primer Pair Mix, 0.5 µl of DNA (200 pg) with up to 7.75 µl of MQ water. Extracted DNA amplification reactions containing, 200 pg of male DNA in a 12 µl volume, were cycled as per the guidelines from the manufacturer. A 10 and 7 µl reaction volume was also tested for amplification using PP Y23 System with 200 pg and 100 pg of male DNA. Samples used for direct amplification were processed using the same cycling parameters with the exception of the cycle number which was optimized for each substrate. Blood on FTA Cards was amplified with 26 cycles while buccal samples on FTA Cards were amplified with 28 cycles.

As the PP Y23 System employs five fluorescent dyes, spectral resolution was established using the PowerPlex 5-dye Matrix Standards (Promega Corporation) enabling evaluation of each fluorescent dye contained in the kit. A 36 cm capillary array was used with POP-4 Polymer (AB/LT/Thermo). Samples were prepared for separation and analysis by adding 1 µl of amplified sample or allelic ladder to 10 µl of Hi-Di Formamide (AB/LT/Thermo) and 0.5 µl of ILS provided with PP Y23 system. Samples were denatured for 3 min at 95°C followed by a snap cool in an ice bath. All analyses used the CC5 Internal Lane Standard 500 Y23 (ILS) and the allelic ladder mix provided with the PP Y23 System. Separation of amplified products was performed on

the Genetic Analyzer 3100 (AB/LT/Thermo). GeneMapper ID v3.2 (AB/LT/Thermo) was used to determine fragment size and allele calls with a 50RFU analytical threshold. Sizing precision is vital for accurate genotyping. Internal lane standard (ILS) is used in fragment analysis to ensure accurate sizing. To ensure precision with the ILS, allelic ladder were run across multiple capillaries on 3100 Genetic Analyzer.

Results and Discussion

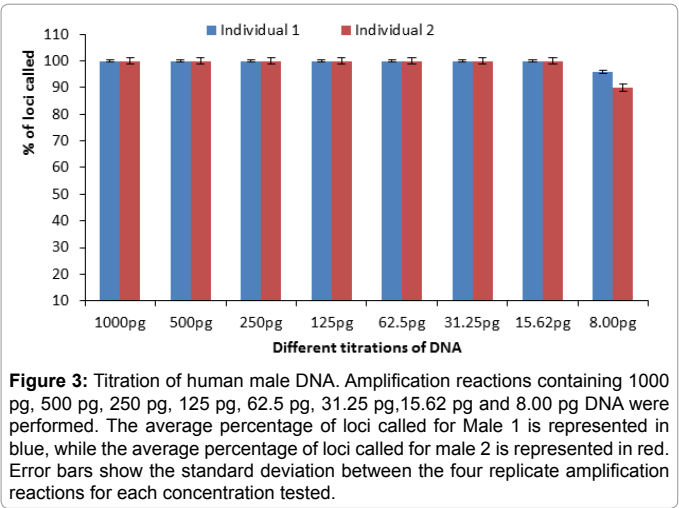
Full Y 23 STR profile was obtained from a DNA sample as low as 15.62 pg (Figure 3). The sensitivity study was performed to determine the optimal DNA concentration range that would yield quality profiles containing no pull-up, drop-out, off-scale peaks, any other artefacts or peak height imbalances of <50%. Data showed a DNA input range of 125 pg -1000 pg was optimal for half-reaction amplification protocol using the PP Y23, with best quality results at 125, 200 and 250 pg. In addition half volume reactions showed high sensitivity by exhibiting great peak heights specially when working with vaginal swabs containing high female DNA quantity.

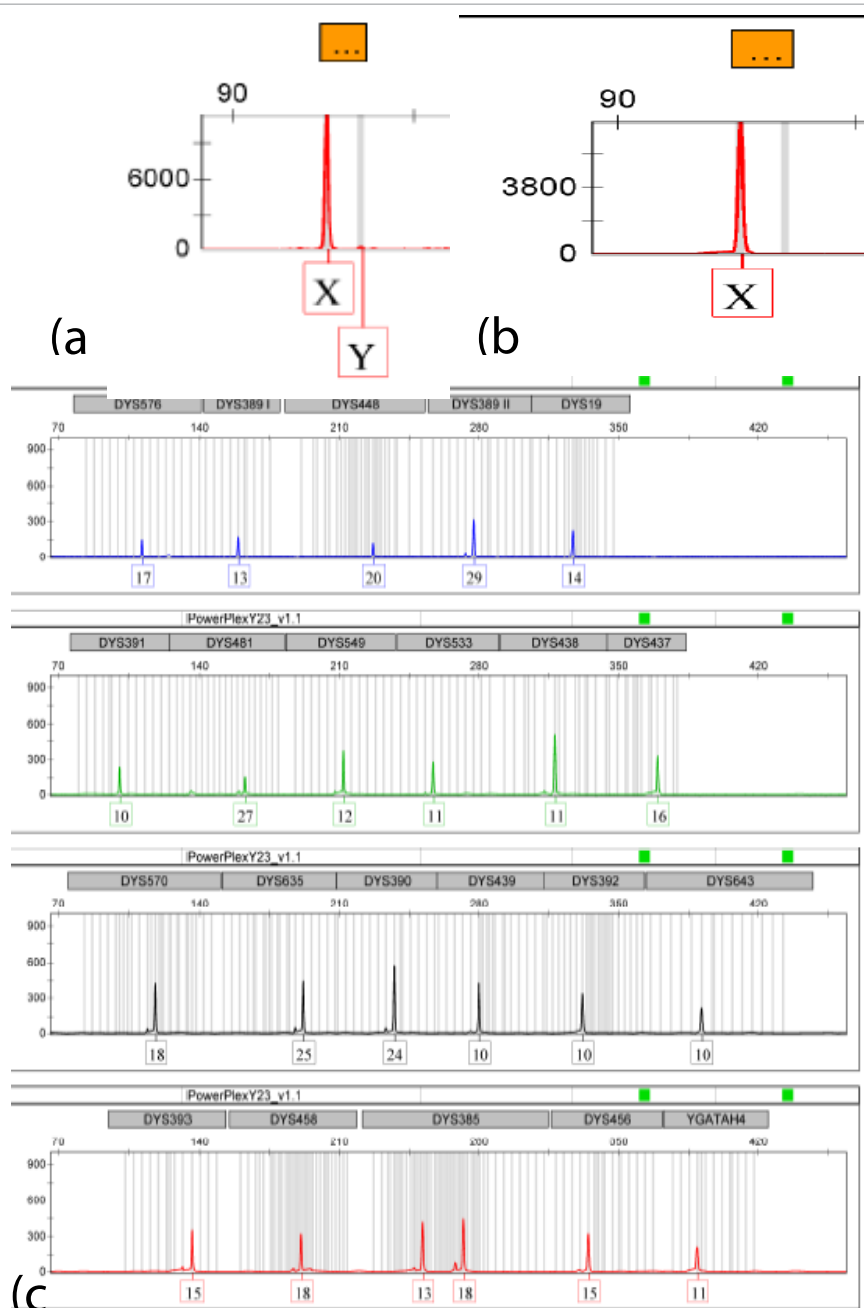
Amplifying more than 1000 pg of DNA produced peak heights above 8000 RFU that resulted in pull-up, split peaks, increased stutters and the profile was not clean however all the alleles were called. Increasing DNA quantity resulted in poor quality profiles with peaks imbalance; partial/total reaction inhibition was observed when working with too much DNA. The sensitivity was tested with extracted DNA and 1.2 mm punches from either blood or buccal samples on FTA Cards. Complete profiles were obtained for all of the tested samples with either one or two 1.2 mm punches. Full profiles were detected in 8 of the 10 replicates in case of FTA having blood sample and 9 of the 10 replicates in case of FTA having buccal cells with 1.2 mm punches added to the reaction. Reduction of reaction volume to 10 and 7 µl from 12.5 µl, showed an increase in sensitivity. All the samples tested with 200 pg DNA resulted in complete Y STR profile. The samples mixed with different concentrations of inhibitors, hematin and humic acid (up to 500 µM/µl concentration of hematin and 100 ng/µl concentration of humic acid), resulted into full Y-STR profile, showing the robustness of the kit and its improved tolerance for inhibitors. The above mentioned virtue of the kit proves its suitability for forensic casework samples. After this concentration of tested inhibitors, partial DNA profile was obtained. System reliability was evaluated with samples encountered in criminal cases. At the DNA fingerprinting facility of authors the PP Y23 kit was tested with all variety of forensic samples: sperm and epithelial fractions from underwear which is common in rape cases, underwear and sanitary pad of rape victim in menses, cases of rape with girl child i.e. with exhibits with excessive female bleeding, nails of female expecting to have male cells, coloured cotton clothing which leave colour in making them wet during DNA extraction, blood stained soil and a bloodstain from a bed sheet. In most of these samples only female autosomal profile could be detected (Figure 4a) with/without a small Y (Figure 4b) on amelogenin marker in very few samples with the Identifiler Plus (AB/LT/Thermo) multiplex autosomal STR kit which is very reliable kit for almost all the forensic samples for generating autosomal STR profile including samples having high inhibitor concentrations. All the samples produced a full YSTR profile with the PP Y23 System (Figure 4c) which otherwise if only tested with autosomal STR multiplex even after differential extraction would have resulted as a single source profile of female. The PP Y23 kit is by now tested on more than 500 casework samples successfully. The results from the samples in this study were consistent with the PP Y23 data generated from different samples (under garments, vaginal smear slide, pubic hair and/or mixed samples recovered from scene of occurrence)

Sl. No.	YHRD Accession no.	Haplotype count	Population
1	YA004145*	107	Chattisgarh, India (Majhi)
2	YA004144*	217	Madhya Pradesh, India (Bhil)

\*data can be accessed from [www.yhrd.org](http://www.yhrd.org)

Table 1: Population data analysis of two tribal population of central India by using PP Y23 system.





**Figure 4:** DNA typing of autosomal (Identifiler plus kit) and Y STR (PP Y23 system) in a real case, in autosomal STR amplification, with/without a small Y (a,b), female dominating single source profile was generated however the same sample on testing with PP Y23 gave a complete Y STR profile (c).

of the same victim. Except few split peaks, all the saliva samples were typed correctly when tested for direct PCR amplification with 13, 10 and 7  $\mu$ l reaction volume. Correct and complete male DNA profile was obtained from all the M:F mix, showing that the kit is quite sensitive in amplifying male DNA component. In our study the PP Y 23 system was able to generate full profiles even with the vastly masked male component in a M:F mixture sample (Figure 5).

In addition to the evaluation and validation experiments, unrelated male individuals of two different tribal populations were typed with the PP Y23 system. The data is submitted to YHRD ([www.yhrd.org](http://www.yhrd.org)) population database (Table 1).

Forensic parameters (gene diversity, haplotype diversity, discrimination capacity, and match probability) were calculated and results were then compared to the values calculated for the subsets of the 16, 12 and 9 Y-STR markers. As expected, the PP Y 23 system provided a higher power of discrimination than the other three marker sets, further proving its usefulness for forensic and population genetics purposes.

During trial of PP Y23 on a wide variety of forensic samples during last one year, another very interesting observation was the finding of only one or two locus mismatch in Y23 STR profile of two unrelated individuals (Table 2). This further encompasses the possibility of false

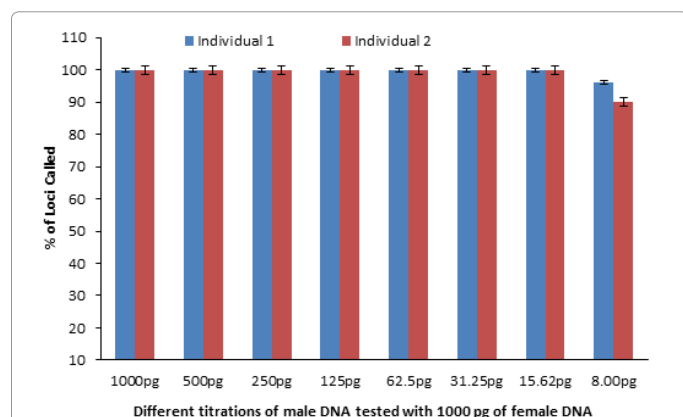


inclusion as well in sexual assault case with the use of lower marker multiplex for forensic purpose.

Male DNA profiles of brothers were also consistent for all the tested samples. The analysis of studied brother pairs indicates that the PP Y 23 system can distinguish different brother pairs (Table 3).

High genetic diversity values above 0.5 for most of the loci (21) and more than 0.4 (on 2 loci) were found when the kit was used with various population samples. The multi copy locus DYS385 reached a GD value of 0.934 in the tested tribal population (Figure 6).

Summarizing the data it is concluded that, The PP Y 23 system is able to cope with very small amounts of male DNA, as well as with



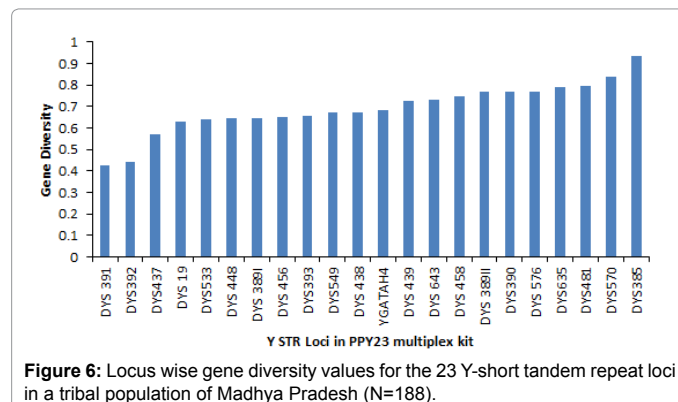
**Figure 5:** Amplification of 1000 pg, 500 pg, 250 pg, 125 pg, 62.5 pg, and 31.25 pg, 15.62 pg and 8.00 pg of male DNA was performed in the presence of 1000 pg of female DNA. The average percentage of loci called for male 1 is represented in blue, while the average percentage of loci called for male 2 is represented in red. Error bars show the standard deviation between the four replicate amplification reactions for each male with the specified DNA concentrations.

Genetic Markers	Y STR profile from the source of rape victim	Suspect I	Suspect II
DYS576	18	19	19
DYS389I	14	13	13
DYS448	20	20	20
DYS389 II	31	31	31
DYS19	16	15	15
DYS391	10	11	11
DYS481	23	23	23
DYS549	12	12	12
DYS533	12	12	12
DYS438	11	11	11
DYS437	14	14	14
DYS570	16	18	18
DYS635	23	23	23
DYS390	23	24	24
DYS439	11	10	10
DYS392	11	11	11
DYS643	10	10	10
DYS393	13	13	13
DYS458	16	17	17
DYS385	11,14	11,14	11,15
DYS456	15	15	15
YGATA4	12	12	12

**Table 2:** PP Y23 STR analysis in a real case of sexual assault, which was a case of exclusion of suspects but both the unrelated suspects (from the same cast), showed only one locus difference in Y STR profile.

Genetic Markers	Family 1 Blood relative males		Family 2 Blood relative males	
	I	II	I	II
DYS576	18	18	19	19
DYS389I	14	14	13	13
DYS448	19	19	19	19
DYS389 II	30	30	30	30
DYS19	15	15	16	16
DYS391	10	10	11	11
DYS481	22	22	23	23
DYS549	12	12	12	12
DYS533	13	13	11	11
DYS438	9	9	11	11
DYS437	14	14	14	14
DYS570	19	19	18	18
DYS635	21	21	23	23
DYS390	21	21	24	24
DYS439	12	12	10	10
DYS392	11	11	11	11
DYS643	9	9	10	10
DYS393	12	12	14	14
DYS458	18	18	16	16
DYS385	15,17	15,17	11,14	11,14
DYS456	16	16	16	16
YGATA4	11	11	12	12

**Table 3:** Y STR profile of two families using PP Y23 systems.



**Figure 6:** Locus wise gene diversity values for the 23 Y-short tandem repeat loci in a tribal population of Madhya Pradesh (N=188).

the common inhibitors which are often found in forensic samples. Full Y-chromosomal STR profiles could be generated even from mixtures with very low male component masked by high amount of female material, which is the common issue confronted in sexual assault cases. Failing to amplify and report the masked or trace male elements in sexual assault cases may lead to erroneous judgements. Seeing the current status of Y-STR DNA database (Tables 4 and 5), and the high potential of Y 23 markers, number of population studies is also expected to increase.

## Conclusion

The PP Y23 system is able to detect even very low quantity of male DNA masked by excessive amounts of female DNA. Full profile from challenging casework samples can be generated even with high concentrations of inhibitors prevailing in the samples. It is time saving with rapid-cycling protocols. It has the potential to simplify the workflows as one kit will suffice for both casework and database samples. It works well even with the direct amplification protocol of

Haplotype Format	Number of Markers	Number of haplotypes	Number of population samples	Number of national databases
Minimal	9	160,693	1015	129
PowerPlex Y	12	121,402	763	119
Y filler	17	109,137	680	112
PowerPlex Y23	23	26,869	190	58
Y filler plus	26	8,184	35	15

\*Source: [https://yhrd.org/pages/resources/stats#haplotype\\_counts](https://yhrd.org/pages/resources/stats#haplotype_counts) (last accessed on 13.03.16)

**Table 4:** Publically-available Y-STR database - YHRD.

Name	Description	Loci
Minimal	YHRD Core Loci	DYS19, DYS389I, DY389II, DYS390, DYS391, DYS392, DYS393, DYS385
PowerPlexY	Promega PowerPlex® Y	DYS391, DYS389I, DYS439, DYS389II, DYS438, DYS437, DYS19, DYS392, DYS393, DYS390, DYS385
Yfiler	AB/LT/Thermo AmpFLSTR® Yfiler®	DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385, DYS393, DYS391, DYS439, DYS635, DYS392, YGATAH4, DYS437, DYS438, DYS448
PowerPlexY23	Promega PowerPlex® Y23	DYS576, DYS389I, DYS448, DYS389II, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643, DYS393, DYS458, DYS385, DYS456, YGATAH4
Yfiler Plus	Applied Biosystems AmpFLSTR® Yfiler® Plus	DYS576, DYS389I, DYS635, DYS389II, DYS627, DYS460, DYS458, DYS19, YGATAH4, DYS448, DYS391, DYS456, DYS390, DYS438, DYS392, DYS518, DYS570, DYS437, DYS385, DYS449, DYS393, DYS439, DYS481, DYF38751, DYS533

Source- [https://yhrd.org/pages/resources/locus\\_information](https://yhrd.org/pages/resources/locus_information) (last accessed on 13.03.16)

**Table 5:** Description of loci in YHRD database.

saliva samples without any prior treatment and that too in half reaction volume of the recommended protocol.

## Acknowledgement

Authors are thankful to the Director, State Forensic Science Laboratory, Sagar (Madhya Pradesh), India for providing laboratory facility and encouragement. Authors are also thankful to Promega, (India) for providing PP Y23 genotyping kit used in the study.

## Authors contributions

PS designed the study, did genotyping of samples and contributed significantly in data analysis and drafted the manuscript. TJ helped in isolation/amplification of samples, data management, statistical analysis and drafting the manuscript. DDB, HRD and VBT did isolation/amplification/genotyping of forensic cases and helped in drafting the manuscript. All authors read and approved the final manuscript.

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