MaSTR™

Enlightened Probabilistic Mixture Analysis Software

Validation of MaSTR™ Software (Mixture analysis from STRs): a fully-continuous probabilistic mixture analysis validation using PowerPlex®Fusion 2 – 5 contributor mixtures

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ABSTRACT

Probabilistic genotyping (PG) for evaluation of mixtures provides many advantages compared to historical (CPI, binary likelihood ratio) approaches. Fully continuous approaches utilize more of the information from the evidentiary profile; such as peak heights, stutter percentages, mixture ratio, probability of drop-in/out to provide weighted genotypes from the mixture profile. The weighted genotypes are used to calculate a likelihood ratio that a profile is included, or not included, in the mixture. Mixture profiles are compared to hypothetical profiles created based on these parameters and hypothetical combinations of genotypes present in the sample in order to calculate how probable it is that a given individual contributed to the mixture. Integration over a large number of interrelated parameters is required to calculate relative probabilities but doing so directly is not feasible. Marcov Chain Monte Carlo (MCMC) is a widely used sampling method to approximate such complex integrals with high accuracy.[1,3] Statistically sound, rigorous software tools are essential for forensic interpretation of complex mixtures that often have multiple contributors, low-level DNA of one or more of the contributors, shared alleles, minor contributor alleles in stutter positions to major contributors and drop-out. Thorough validation of methodologies is required for forensic laboratories. Conclusions of a preliminary evaluation of MaSTR software indicated MaSTR software provides the rigor of a fully continuous probabilistic approach[1] in a straight-forward software program. A full validation was proposed as the results of the preliminary study were concordant with the known mixture contributors. The validation study of MaSTR software provides the forensic community with a detailed report of the capabilities and reliability of this newly available tool to assist the forensic analyst in applying their expertise to evaluate mixture profiles.

The validation study was designed in accordance with the Scientific Working Group on DNA Analysis Methods (SWGDAM) and Organization of Scientific Area Committees (OSAC) guidelines. Purified, de-identified DNA from 40 donors was obtained from the Nebraska Biobank (University of Nebraska Medical Center), quantified using the Quantifiler Human DNA Quantification kit (Thermo Fisher), amplified with PowerPlex®Fusion (Promega Corporation) and analyzed on an ABI PRISM® 3130 Genetic Analyzer (Thermo Fisher). Replicates of single source samples were genotyped (GeneMarker®HID software). Files were selected to provide a range of allele calls and stutter information. Results of these replicates and their dilutions were imported into MaSTR software to calculate the variance coefficient and stutter ratios required for the protocol data set. The protocol data set provides the software with context of expected peak height variation over the range of potential RFU values and stutter during the evaluation of mixture profiles. Mixtures ranged from simple, two-contributors with major/minor components with few shared alleles to complex four and five-contributor mixtures with a range of mixture ratios and shared alleles. Mixture samples were also diluted to examine low-template effects across the range of contributors.

The study utilized the standard model within the software. This model utilizes peak heights, drop-in/out, degradation, stutter, co-ancestry (NRC I and NRC II) and allele frequencies from USA populations.[5] Tests included evaluation of mixtures with: 1) a known contributor profile and no person of interest (POI) profile(s), 2) a known contributor profile and POI profile(s), 3) no known and no POI profiles(s) to evaluate deconvolution capabilities and 4) contributor and non-contributor samples to evaluate exclusion capabilities. Additional parameters included use of the elimination database, containing some of the known contributor genotypes, to mimic detection of staff profiles in a mixture in order to evaluate MaSTR's capability to detect contamination. The number of MCMC iterations and chains required increased with complexity of the mixtures. Results were consistent with known contributors and exclusions. Methodology and tabulated results will be presented.

METHODS

DNA Quantification, Mixture Construction, Amplification, Capillary Electrophoresis

All human DNA samples used in this study were purchased as de-identified extracts from the Nebraska BioBank, and were used according to the University of Nebraska-Lincoln's Institutional Review Board's (IRB) approved policies for ethical standards and methods for human testing. Sample collection and experimentation commenced only after the IRB review process was completed.

DNA was extracted at the Nebraska BioBank from 40 donor samples using the Qiagen QIAcube and the QIAamp 96 DNA QIAcube HT kit optimized for downstream genomic assays. Addition of proK and AL buffer and incubation at 56°C resulted in lysis of the cells. The samples were added to the spin columns in the Qiacube for washing and elution of DNA. The DNA was washed via a salt wash (AW1) followed by a salt eliminating wash (AW2) then an ethanol wash. DNA eluted from the column via a low ionic elution buffer. The resulting DNA was quantified using the NanoDrop™ One microvolume UV-Vis spectrophotometer at the Nebraska BioBank.

In order to further determine the concentration of DNA in each sample after they were delivered, the extracts were quantified using an Applied Biosystems 7500 real-time PCR system and the Applied Biosystems Quantifiler™ Human DNA Quantification Kit according to the manufacturer's recommendations.

Prior to the analysis of mixed samples, a protocol data set was created for the MaSTR software to use in the assessment of stutter peaks and the calculation of a variance factor. Ten samples were chosen that demonstrated the greatest level of allelic variation available. Each sample was amplified using an initial quantity of 0.5 ng with five replicates. The samples were then diluted 1:2 and 1:4 and amplified again, each with five replicates, yielding a total of 150 analysis samples with 10 distinct genotypes and a broad range of peak heights for the MaSTR software to use.

Mixture samples were created using two, three, four, or five of the single source samples. Two different strategies were used in creating the two and three-person mixtures. After each single source sample was genotyped, an appropriate number of samples (two or three) were selected that showed minimal or maximal allele sharing amongst the available genotypes, in order to test the MaSTR software under these different conditions. Two different two-person mixture combinations were created, one in which the samples shared a total of five alleles amongst five loci (low share) and one in which the samples shared 19 alleles amongst 18 loci (high share). The same process was used for the three-person mixtures, resulting in one combination with 16 alleles shared amongst 15 loci between the three contributors (low share) and a different combination of contributors showing 31 alleles shared amongst 21 loci (high share). The four- and five- person mixtures were created by randomly selecting previously unused samples and combining them without regard to allele sharing. This gave a total of 19 different single source genotypes used to create 6 mixture genotypes, no genotype was used in more than one combination. Two-person mixtures were made in the following ratios; 1:1, 1:2, 1:3, 1:5, and 1:10. Three-person mixtures were made in the following ratios; 1:1:1, 1:1:2, 1:3:5, and 1:2:10. Four-person mixtures were made in the following ratios; 1:1:1:1, 1:2:2:5, and 1:1:3:10. Five-person mixtures were made in the following ratios; 1:1:1:1:1, 1:2:2:5:10, and 1:1:5:5:10. The mixture ratio balance for each sample was assessed by multiple methods prior to final amplifications. Initially, the peak heights of each single source sample selected for combining were compared to the others in the planned mixture. The amount of input DNA for each contributor was then adjusted so that the peak heights in the mixture should correspond to the desired ratio. Test mixture samples were then assembled and run in triplicate to assess if the amount of DNA in the respective amplifications gave acceptable peak height ratios or needed further adjustment. Once the test mixture samples demonstrated peak heights that fit expectations for the desired mixture ratio, those quantities of DNA were used to create the experimental samples. Each mixture sample was also serially diluted 1:2, 1:4, and 1:8 in order to examine the same contributor ratios with and without stochastic amplification effects. Every final mixture/dilution combination sample was run in triplicate.

All samples were amplified using the Promega PowerPlex® Fusion 5C kit following the manufacturer's protocols. The target initial quantity of DNA for amplification was 0.5 ng and was subsequently adjusted according to the requirements of the specific experimental conditions. The amplification reaction final volume was 25 µL for all samples and thermal cycling was performed in an Applied Biosystems GeneAmp PCR System 9700 using maximum ramp speed for 30 cycles. All amplification sets included a positive control (cell line 2800M DNA, included in kit) and a negative control (deionized water used for sample dilutions).

Separation and detection of the PCR products was performed using an Applied Biosystems 3130 Genetic Analyzer with Foundation Data Collection software v3.0, following all manufacture recommended protocols. All injections were performed at 3 kV for 5 seconds. Separation occurred with Applied Biosystems Hi-DiTM formamide in Applied Biosystems POP4 Polymer. Analysis of all the injection products was performed using SoftGenetics® GeneMarker® HID v2.9.0 software. All of the two-person mixture samples were analyzed with an analytical threshold of 50 rfu, as were the three-person mixtures except for those that were diluted 1:8, which used an analytical threshold of 30 rfu. All of the four- and five person mixtures also used an analytical threshold of 30 rfu. The differing analytical thresholds reflected the prevalence of low level peaks in each condition and the desire to include allele dropout in some of the combinations in order to further challenge the MaSTR software at each mixture ratio.

Models

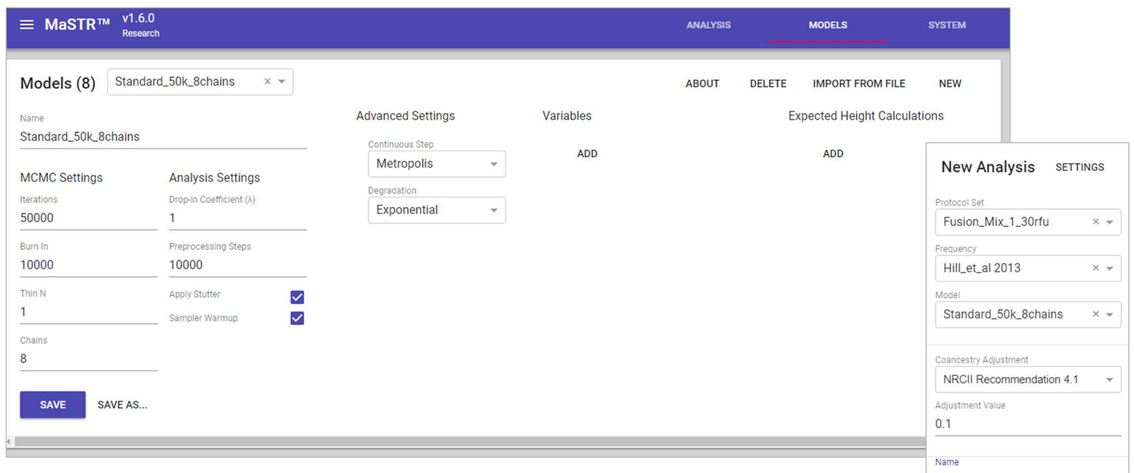

Figure 1: An example of a standard model

Models were consistent with the exception of varying the number of MCMC iterations. Models with increasing numbers of iterations were used to provide information on minimum and optimal number of MCMC iterations for 2, 3, 4 and 5 contributor mixtures.

Figure 2: The jobs submitted included the mixture sample text file and text files of single source genotypes to be evaluated, without specifying any known single source genotypes. These settings were selected in order to mimic the most difficult cases where a known contributor swab is not available. The analyses included simple 2-person mixtures with very limited allele sharing, as well as complex 3 – 5-person mixtures. Two-person mixtures with up to 70% allele sharing were used to mimic related individuals in a mixture. The NRC II 4.1 approach for co-ancestry was selected and coefficient was 0.1 Fifteen mixture analyses included non-contributor single source samples to evaluate exclusion capabilities.

Accuracy of inclusion and exclusion

Seventy-seven analyses of 2 – 5-person mixtures resulted in accurate inclusion and positive likelihood ratio for all tested samples. Fifteen of the analyses included non-contributor samples. All of the non-contributor samples were excluded, having negative likelihood ratio results.

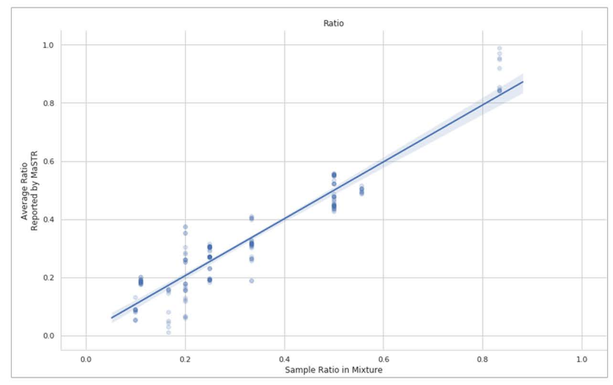

Figure 4: A Ratio Plot showing the correlation between the ratio of a contributor to a mixture and the average ratio value reported by MaSTR when that contributor's profile was tested.

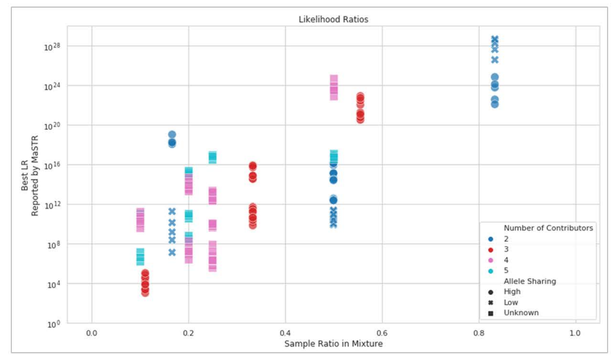

Figure 5: A Likelihood ratio plot showing the highest LR reported for contributor profiles that were tested as unknowns, showing that contributors known ratio in the mixture, the total number of contributors, and the amount of allele sharing amongst the contributors in the sample.

Example Likelihood ratio result screens in MaSTR software from a 1:1 mixture

1653_053018_AlleleReport.txt (Alternate) — Using 'All data' frequencies

Marker	Genotype	LR (Unknown 1)	LR (Unknown 2)
D3S1358	(15, 15)	0.0936	5.3974
D1S1656	(15.3, 18.3)	13.4670	15.3728
D2S441	(10, 10)	0.2033	14.9913
D10S1248	(15, 16)	0.1771	11.2936
D13S317	(10, 13)	3.6261	5.1115
Penta_E	(7, 12)	9.7514	1.5701
D16S539	(9, 14)	0.1610	0.7201
D18S51	(14, 15)	0.2761	3.9716
D2S1338	(17, 21)	15.1212	0.3201
CSF1PO	(11, 12)	0.6949	3.1183
Penta_D	(12, 13)	0.5898	1.4613
TH01	(8, 9)	12.5938	0.2109
vWA	(14, 20)	223.0850	6.2354
D21S11	(28, 32.2)	0.4947	28.2698
D7S820	(11, 11)	0.1681	12.4481
D5S818	(10, 13)	0.6305	36.0374
TPOX	(8, 8)	0.0548	0.4014
D8S1179	(13, 13)	0.7505	1.0999
D12S391	(20, 25)	95.6517	3.7627
D19S433	(13, 14.2)	0.7747	0.6477
FGA	(21, 21)	2.9948	0.7697
D22S1045	(16, 16)	9.1509	9.1509
Overall		**5.4339e+3**	**5.9451e+10**

1678_053018_AlleleReport.txt (Reference) — Using 'All data' frequencies

Marker	Genotype	LR (Unknown 1)	LR (Unknown 2)
D3S1358	(17, 18)	14.2490	0.2471
D1S1656	(14, 15)	0.9734	0.8527
D2S441	(11, 14)	5.5822	0.0757
D10S1248	(13, 14)	2.9764	0.0467
D13S317	(12, 13)	0.9883	0.7011
Penta_E	(11, 17)	7.0119	43.5500
D16S539	(11, 12)	0.0340	0.0076
D18S51	(18, 21)	101.1982	7.0341
D2S1338	(24, 25)	0.3735	17.6426
CSF1PO	(10, 12)	3.6759	0.8191
Penta_D	(8, 14)	15.4617	6.2400
TH01	(7, 7)	0.0830	4.9549
vWA	(18, 19)	0.4872	17.4311
D21S11	(27, 32.2)	120.5038	2.1087
D7S820	(8, 9)	22.7806	0.3076
D5S818	(8, 13)	141.4910	2.4756
TPOX	(9, 11)	1.4416	0.1967
D8S1179	(15, 15)	3.1702	2.1632
D12S391	(18, 18)	0.1837	4.6710
D19S433	(11, 15)	2.6680	3.1914
FGA	(22, 23)	0.4280	1.6653
D22S1045	(16, 16)	9.1509	9.1509
Overall		**1.6068e+10**	**1.4686e+3**

Figure 6: After an analysis is completed in MaSTR software, the user clicks on the job name to open the result screen. As an example, we present the above example from a two-person 1:1 mixture without a known contributor specified. Select the name of the file of interest from the drop-down box. Sample 1653 (left) has a strong likelihood corresponding to the deconvoluted unknown 2. Sample 1678 has a strong LR corresponding to the deconvoluted unknown 1. Likelihood ratios are calculated from the weighted genotype results.

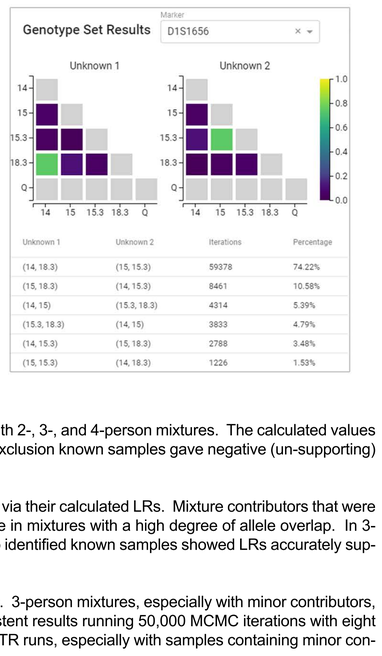

Figure 7: Weighted genotype results are viewed by selecting the Genotype Set Results from Results tool bar. Each locus is displayed by selecting from the drop-down list. A heat map indicates the most likely genotype combinations of the tested samples on a scale of 0 – 1; least likely (dark purple) to most likely (yellow). The weighted genotype combinations are displayed in a table and are used in calculating likelihood ratios.

Summary

Experimental data have shown that the MaSTR software gives accurate Likelihood Ratio results when used with 2-, 3-, and 4-person mixtures. The calculated values for each contributor LR fit well with the true identities and mixture ratios of the synthesized samples. All true-exclusion known samples gave negative (un-supporting) LRs for the appropriate unknown "position" in MaSTR.

The "Major" and "Minor" contributors (mixture ratios of at least 3:1) were clearly and accurately distinguishable via their calculated LRs. Mixture contributors that were at or close to a 1:1 ratio were accurately distinguishable in 2-person mixtures with no identified known sample in mixtures with a high degree of allele overlap. In 3- and 4-person mixtures with a high degree of allele overlap, contributors present in near equal amounts with no identified known samples showed LRs accurately supporting both samples being part of the mixture, but were sometimes difficult to tell apart.

Preliminary work shows that using 10,000 MCMC iterations with eight chains works well for 2-person mixtures. 3-person mixtures, especially with minor contributors, worked well using 20,000 MCMC iterations with eight chains and 4+ person mixtures showed the most consistent results running 50,000 MCMC iterations with eight chains. Increasing the number of MCMC iterations gave more consistent LR values across the replicate MaSTR runs, especially with samples containing minor contributors, allele dropout, shared alleles, and close ratio contributor data.

MaSTR software runs using less than 100K total MCMC iterations were resolved within 15 minutes or less, while longer runs (400K) were completed within 45 minutes using a desktop computer (Intel® Core™ i7-7700 cpu 3.60 GHz 4 core 8 logical processors) with 8 GB RAM operating with Windows 10.

Further interrogation of the data set is planned to evaluate likelihood ratio results and analysis time when known contributor(s) are included in the analysis procedure, as well as peak height variation in replicate samples.

Acknowledgements

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References

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