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Internal validation of *LRmix Studio* biostatistical software

Summary

The source of DNA in a stain is never known with full certainty despite the fact that the evidential profile may match a DNA profile of a given person from the population. The statistical methods, including the likelihood ratio (LR) allow estimating the evidential power of the obtained result and assessing the ratio of the odds between competing hypotheses as to the origin of a DNA profile or mixture. Therefore using analyses based on probabilistic methods seems to be logically justified and allows reducing the subjectivism of interpretation of results. Thorough knowledge and understanding of the principles of operation and limitations of the tools used for statistical interpretation of the results of biological traces analyses in forensics is the key stage that precedes the formulation of conclusions. The process of checking the efficiency of *LRmix Studio* software as well as reliability and repeatability of results involved single profiles and mixtures from two and three persons. 1971 comparisons with referential profiles were performed. The correctness of generated conditional probabilities was determined and the limits, i.e. *drop-outs* number in the evidential profile whose exceeding might bring about false LR values were identified.

Key words: DNA mixtures, likelihood ratio, LRmix Studio, validation, forensic DNA analysis

Introduction

The analysis of crime scene stains containing DNA from two or three persons is one of the most difficult challenges faced by forensic DNA experts. Such traces require specialist knowledge, superb examination practice and maintaining particular caution when formulating conclusions. It is forensic expert's duty to estimate the value of evidence achieved by scientific examination and presenting the result in a manner that is logical and clear for the judicial authority but, first of all, compliant with the standards accepted in the forensic genetics community. In the light of fast development of biostatistical methods and tools, DNA Commission of International Society for Forensic Genetics (ISFG) argues that the appropriate method for assessing the value of evidence from DNA analysis is the probabilistic method based on likelihood ratio (LR) (Gill et al., 2012), particularly in cases of analyses of mixtures of DNA from two and more persons, especially when the quantity of template DNA originating from the donors is low (Coble i in., 2016).

The analysis performed by means of probabilistic models based on estimating the likelihood ratio value involves assessment of the determination from the genetic examinations in relation to alternative hypotheses (H_p – prosecutor's hypothesis and H_d – defence attorney's hypothesis) regarding the origin of DNA in the crime scene stain, formulated based on known circumstances of the crime or put forward by the judicial authority. The likelihood ratio measures the supporting power provided by the analytical findings in order to differentiate between competing forensic hypotheses of interest. It constitutes a scientifically accepted and logically justified method of presenting conclusions from DNA analyses (Haned et al., 2015). Upon formulating the hypotheses mathematical analysis is still quite complex and it can be performed only with use of specialist software (Coble et al., 2016).

LRmix Studio is an expert system designed for statistical evaluation of DNA profiles facilitating determination of evidential value of any DNA profile, in particular high order DNA mixtures (Haned, Jong, 2016). The statistical model adopted in *LRmix Studio* takes into account allele labelling and the number of DNA mixture components. It is referred to as the *qualitative continuous model* (ENFSI, 2015). The software facilitates calculating likelihood ratios for DNA profiles and mixtures with many components and replications, and accounts for stochastic effects related to PCR, such as *drop-out* and *drop-in* (Haned, Gill, 2011). In this article we describe research aiming at internal validation of *LRmix Studio* software performed according to the recommendation of ISFG DNA Commission (Coble et al., 2016) and ENFSI

Table 1. Simulated DNA mixtures with respective DNA contents.

For example, sample no. 0.5 contains three components, A, B and C. The *Drop-out* column comprises the number of alleles from respective components of mixture, which are not present in the sample (that is, their peak heights are below the detection threshold amounting to 50 RFU), for example, sample 1.1 has a combination of referential alleles (D, E, F) with *drop-out* cases (0, 3, 2). Column „Above ST" refers to components of simulated DNA mixtures, for which all the alleles heights were above the stochastic threshold (here: above 200 RFU). The values in brackets in "Samples" column refer to the numbers of repeats.

Samples	Components of DNA mixtures	Degradation	DNA (pg)	*Drop-out*	Above ST
0.1 (3)	(A, B)	No	(150, 30)	0, 3/ 0, 10 / 0, 6	no
0.2 (3)	(A, B)		(300, 30)	0, 4 / 0, 15/ 0, 8	A
0.3 (3)	(A, B)		(30, 150)	5, 0 / 4, 0 / 6, 0	no
0.4 (3)	(A, B)		(30, 300)	5, 0 / 7, 0 / 14, 0	B
0.5 (3)	(A, B, C)	No	(150, 6, 30)	0, 19, 13 / 2, 27, 22 / 0, 26, 11	no
0.6 (3)	(A, B, C)		(150, 30, 30)	0, 4, 8 / 0, 7, 8 / 0, 5, 12	no
0.7 (3)	(A, B, C)		(300, 6, 30)	0, 22, 11 / 0, 23,11 / 0, 23, 14	A
0.8 (3)	(A, B, C)		(300, 30, 30)	0, 7, 13 / 0, 7, 14 / 0, 8, 13	A
0.9	(M, N, O)		(500, 250, 250)	0, 0, 4	M
0.10	(S, P, R)		(500, 250, 50)	0, 0, 8	S
0.11	(A, B)	Yes	(100, 40)	6, 16	no
0.12	(C, D)		(100, 40)	5, 15	no
0.12_II	(C, D)		(100, 40)	2, 10	no
0.14	(G, H)		(100, 40)	4, 27	no
0.14_II	(G, H)		(100, 40)	1, 10	no
0.15	(I, J)		(100, 40)	9, 29	no
0.15_II	(I, J)		(100, 40)	0, 11	no
0.16	(A, B)	Yes	(250, 40)	8, 27	no
0.17	(C, D)		(250, 40)	2, 21	no
0.17_II	(C, D)		(250, 40)	0, 12	C
0.19	(G, H)		(250, 40)	0, 13	G
0.20	(I, J)		(250, 40)	0, 15	no
0.22	(C, D)	Yes	(40, 100)	16, 5	no
0.22_II	(C, D)		(40, 100)	26, 2	no
0.24	(G, H)		(40, 100)	10, 6	no
0.25	(I, J)		(40, 100)	15, 0	no
0.27	(C, D)	Yes	(40, 300)	18, 0	no
0.28	(E, F)		(40, 300)	21, 8	no
0.28_II	(E, F)		(40, 300)	21, 8	no
0.30	(I, J)		(40, 300)	17, 0	no
1.1	(D, E, F)	No	(100, 40, 40)	0, 3, 2	no
2.1	(G, H, I)		(100, 40, 40)	0, 4, 0	no
3.1	(J, K, L)		(100, 40, 40)	0, 3, 6	no
4.1	(M, N, O)		(100, 40, 40)	1, 6, 11	no
5.1	(S, P, R)		(100, 40, 40)	2, 2, 4	no
6.1	(W, T, U)		(100, 40, 40)	1, 7, 0	no
7.1	(X, Y, Z)		(100, 40, 40)	1, 4, 1	no

Tab. 1. Continue.

Samples	Components of DNA mixtures	Degradation	DNA (pg)	*Drop-out*	Above ST
1.2	(D, E, F)		(250, 40, 40)	1, 14, 9	no
2.2	(G, H, I)		(250, 40, 40)	0, 2, 5	G
3.2	(J, K, L)		(250, 40, 40)	0, 7, 6	no
4.2	(M, N, O)	No	(250, 40, 40)	0, 0, 7	M
5.2	(S, P, R)		(250, 40, 40)	0, 2, 5	S
6.2	(W, T, U)		(250, 40, 40)	0, 9, 3	no
7.2	(X, Y, Z)		(250, 40, 40)	0, 3, 5	X
1.3	(D, E, F)		(250, 250, 40)	0, 0, 7	D
2.3	(G, H, I)		(250, 250, 40)	0, 0, 7	G
3.3	(J, K, L)		(250, 250, 40)	0, 0, 11	J
4.3	(M, N,O)	No	(250, 250, 40)	0, 0, 10	no
5.3	(S, P, R)		(250, 250, 40)	0, 0, 11	S
6.3	(W, T, U)		(250, 250, 40)	2, 0, 6	no
7.3	(X, Y, Z)		(250, 250, 40)	0, 0, 9	no
1.4	(D, E, F)		(500, 40, 40)	0, 7, 4	D
2.4	(G, H, I)		(500, 40, 40)	0, 3, 10	G
3.4	(J, K, L)		(500, 40, 40)	0, 7, 9	no
4.4	(M, N, O)	No	(500, 40, 40)	0, 1, 9	M
5.4	(S, P, R)		(500, 40, 40)	0, 6, 5	S
6.4	(W, T, U)		(500, 40, 40)	0, 6, 2	W
7.4	(X, Y, Z)		(500, 40, 40)	0, 4, 7	X
1.5	(D, E, F)		(500, 250, 40)	0, 0, 7	D
2.5	(G, H, I)		(500, 250, 40)	0, 0, 8	G
3.5	(J, K, L)		(500, 250, 40)	0, 0, 4	J
4.5	(M, N, O)	No	(500, 250, 40)	0, 0, 3	M, N
5.5	(S, P, R)		(500, 250, 40)	0, 0, 4	S
6.5	(W, T, U)		(500, 250, 40)	0, 0, 2	W
7.5	(X, Y, Z)		(500, 250, 40)	0, 0, 5	X

(ENFSI, 2015). In designing the validation experiments examples presented by Øyvind Bleka were use as the base (Bleka et al., 2016).

Materials

STR profiling

DNA profiles were generated using the PowerPlex Fusion 6C reagent kit (Promega) and the GeneAmp PCR System 9700 thermocycler (Applied Biosystems). The number of amplification cycles was 29. The products of the PCR reaction underwent capillary electrophoresis using ABI PRISM® 3130xl genetic analyzer (Applied Biosystems). The analysis of results was performed by means of GeneMapper ID-X 1.4 software (Applied Biosystems) with use of an analytical threshold at the level of 50 RFU and a Stochastic Threshold (ST) at the level of 200 RFU.

DNA profiles – pool of DNA samples

The research sample consisted of 27 single source DNA profiles, 32 two-donor mixtures and 48 three-donor mixtures, generated by combining referential samples (of known DNA profiles) of 24 non-related persons from Polish population (Table 1). Additionally, the data used for internal validation included results of DNA profiling from a series of dilutions (1–0,03 ng) of the referential sample amplified in two repetitions. The biological material consisted of blood samples collected within the implementation of NEXT (DOB-BIO7/17/2015) Project. The study was approved by the Bioethics Commission of Cracow Jagiellonian University (KBET/122/6120/11/2016). The mixtures were prepared by combining two and three components in various proportions, using material of a high content of template DNA (500 pg, 300 pg,

250 pg per component) and one or two components of low content of template DNA (100 pg, 40 pg, 30 pg, 6 pg per component). Samples with numbers from 0.1 to 0.8 were amplified in three repetitions, i.e. three separate amplifications of the same DNA extract were performed. Samples with numbers from 0.11 to 0.30 additionally underwent degradation process by seven-hour exposition to sunlight (Yoon et al., 200). DNA profiling results were exported from GeneMapper ID-X and imported to *LRmix Studio* version 2.1.3.

The phenomenon of allelic *drop-out* was observed in all the analysed DNA mixtures. The number of dropped-out alleles was determined by counting those alleles, whose corresponding alleles in the referential profiles in the simulated mixtures had the peak height below 50 RFU (homozygotes were counted twice).

Methods

Likelihood ratio *(LR) formula*

In order to estimate the power of evidential value in order to determine whether DNA of the person of interest (POI) is a component of *E* sample the following *likelihood ratio* (LR) formula of was applied:

(1) $LR = \frac{P(E|Hp)}{P(E|H_d)}$, where

H_p: DNA in the sample comes from the person of interest (POI),
H_d: DNA in the sample comes from another unknown person unrelated with POI

In the assessment of stochastic effects the *drop--out/drop-in* model implemented in *LRmix* software (Haned et al., 2015). In addition to formulating the above-mentioned hypotheses, which will be assessed by means of likelihood ratios, the said model requires determining additional input parameters:

- Frequency of occurrence of alleles in the target population used for calculating the probabilities of genotypes in profiles of unknown persons,
- F_{st} correction used for adjusting uncertainty as regards allele frequency according to the structure of the subpopulation,
- *drop-out* probability, that translates to the probability that all the alleles of a hypothetical donor within the given hypothesis have dropped out,
- *drop-in* probability, i.e. the probability that an allele/alleles whose presence is not explained by hypothetical donors within a given hypothesis will be a false allele/alleles (not originating from any of the donors) (Haned et al., 2012).

During the calculations F_{st} correction was set at the level of 0.01while the probability of *drop-in* was set at the level of 0.05. That value corresponds with expected occurrence of one allele resulting from *drop--in* phenomenon in 20 *loci*, which may overlap with the alleles originating from the actual components of the DNA mixture (Haned et al., 2012). Allele frequencies used in the calculations were elaborated by the European Network of Forensic Science Institutes (ENFSI) for the European population (Welch et al., 2012). As regards five markers, for which allele frequencies are not given in the ENFSI population database, data elaborated by the supplier of PowerPlex Fusion 6C kit (Promega) (Steffen et al., 2017).

The probability of *drop-out* in every DNA mixture was estimated by Monte Carlo simulation implemented in *LRmix Studio* software, with use of 1000 simulations performed within every hypothesis. According to the methodology described in paper by Haned et al. (2015), it was assumed that the estimated value of *drop-out* probability is the same for all the components of DNA mixture with an exception of a situation where one component of the mixture can be considered as "known" and denominated with the letter "K". In such a case zero *drop-out* probability value was assumed for that component.

Upon application of Monte Carlo simulation *LRmix Studio* software sets the limits of *drop-out* probability distribution in the range from 5 to 95 percentiles (Gill, Haned, 2013). During statistical analyses the likelihood ratio was calculated while maintaining the conservative approach, which uses the smaller quintile of LR value distribution as the measure of the evidential value. In the majority of cases the reported LR value in fact corresponded with 5% percentile of *drop-out* probability distribution achieved by Monte Carlo simulation.

Experiments design

By carrying out statistical analyses for each simulated DNA mixture (thus for each of the 80 DNA mixtures composed of 24 referential samples), the person of interest (POI) was treated in sequence as each of 24 referential samples, which gave 24 comparisons for each DNA mixture. The statistical analyses were carried out according to the following assumptions:

(2) $LR = \frac{\mathrm{POI+UN+UN}}{3\ \mathrm{UN}}$ or $LR = \frac{\mathrm{POI+UN}}{2\ \mathrm{UN}}$, where

POI – person of interest,
UN – unknown person.

In 37 DNA mixtures one of the components could have been *a priori* conditioned as a "known person" – K. In the profiles of these components all the allele peak heights were above the stochastic threshold, i.e. 200 RFU. This provided additional several dozen comparisons for each of 37 mixtures, as expressed by the following formulas:

(3) $LR = \frac{\mathrm{K+POI}}{2\ \mathrm{UN}}, LR = \frac{\mathrm{K+POI}}{\mathrm{K+\ UN}},$

$LR = \frac{\mathrm{K+POI+UN}}{3\ \mathrm{UN}}, LR = \frac{\mathrm{K+POI+UN}}{\mathrm{K+2\ UN}}$, where

POI – person of interest,
UN – unknown person,
K – known person

Eventually, 208 comparisons were analysed, in which POI is a real component of DNA mixture and 1712 comparisons, in which POI is not a real component of DNA mixture.

Results and discussion

Analysis of single DNA profiles

The assessment of single donor DNA profiles by *LRmix Studio* software was performed according to formula (1). LR values reported by *LRMIX Studio* software for 27 single DNA profiles, for which genotypes in each *locus* were unambiguous (with no *drop-out*) were obtained in the range from 1.58×10^{28} to 1.32×10^{32} (that is log10 (LR) value were reported in the range from 28.54 to 32.14). Multiple analysis of the same DNA profile on a validated software gave repeatable results.

Analyses of DNA mixtures

Comparisons were performed of LR values for DNA mixtures and single DNA profiles, in which the person of interest (POI) is the real component of both the analysed samples. As expected, likelihood ratios for all the analysed DNA mixtures did not in any case exceed the likelihood ratio obtained for a single DNA profile of the person of interest. Moreover, a multiple analysis of the same DNA mixtures with the software subject to validation gave repeatable results.

Testing of model performance

In order to verify performance of the model implemented in *LRmix Studio* the researchers used results of profiling a series of dilutions of the referential DNA sample (Table 2). It was demonstrated that LR values reported by the *LRmix Studio* software estimated according to formula (1) for individual DNA profiles decreased accordingly with dropping amount of template DNA in the sample (1–0.03 ng), i.e. from the maximum value for the full profile (log10 (LR) = 29.21) towards the value of LR = 0, resulting from the occurrence of *the drop-out* phenomenon (Taylor, 2014). After taking into account the *drop-out* probability estimated using Monte Carlo calculations, all LR results obtained for incomplete DNA profiles were positive, supporting the H_p hypothesis. At the same time, as expected, correspondingly lower LR values for incomplete profiles were obtained as the *drop-out* phenomenon intensified, and the estimated *drop-out* probability values increased proportionally to the increasing number of alleles dropping out in sequential sample dilutions.

Analyses of samples in several repetitions

The analytical process involved using the results of profiling DNA mixtures from three people, each amplified three times. Statistical analyses were performed separately for individual DNA profiles obtained from independent amplifications of the same DNA extract, followed by simultaneous analyses of three repeated amplifications of the same sample. In the case of a low amount of DNA template in a sample, stochastic effects in subsequent PCR repeats of the same sample cause a large variation in peak height, heterozygote balance and in the number of *drop-out* phenomena (Gill et al., 2000; Benschop et al., 2011). With respect to DNA profiles with occurring *drop-out*, the simultaneous statistical analysis of three repetitions of amplification in *LRmix Studio*, taking into account the same POI component, in most cases guaranteed

Table 2. Result of LR value estimation for single DNA profiles generated by series of dilutions of one DNA sample in two repetitions: a, b.

Template DNA quantity (ng)	Number of *drop-outs*	Log10(LR)	Value of probability P(D)
1a	0	29.21	0
1b	0	29.21	0
0.5a	1	27.33	0.01
0.5b	0	29.21	0
0.25a	3	24.31	0.02
0.25b	5	24.71	0.03
0.125a	11	19.64	0.18
0.125b	7	20.66	0.09
0.06a	19	11.23	0.36
0.06b	22	10.81	0.44
0.03a	34	5.16	0.68
0.03b	26	9.46	0.50

Table 3. Results of biostatistical analysis from three samples amplified in three repetitions: a, b, c.

Sample no.	Real component POI	Number of *drop-outs*	Log10(LR)	Common Log10(LR) for three replications
0.8a	B	0, **7**, 13	5.73	8.46
0.8b		0, **7**, 14	6.56	
0.8c		0, **8**, 13	4.86	
0.6a	C	0, 4, **8**	0.005	3.43
0.6b		0, 7, **8**	2.03	
0.6c		0, 5, **12**	–1.62	
0.4a	A	**7**, 0	6.73	4.63
0.4b		**6**, 0	6.05	
0.4c		**14**, 0	1.72	

a higher LR value than in the case of analysing each amplification repetition separately (Table 3). The higher was the homogeneity of individual results estimated for three sequential repetitions, the more of repeated tests results for the joint analysis exceeded the LR values for single test results. However, when the results of repeated tests were divergent, the LR result for their joint analysis might have been smaller than the LR values obtained for individual test results, which can be seen on the example of samples marked with number 0.4.

False negative results (LR < 1|H_p is TRUE)

Testing of the inclination to false negative exclusions comprises a test of system sensitivity and refers to assessment of software ability to accurate estimation of LR supporting the assumptions as to presence of DNA originating from a real person in the DNA profiling results (Scientific Working Group on DNA Analysis Methods, 2016; Moretti et al., 2017). Figure 1 shows LR results for comparisons, in which POI was the real component of DNA mixtures. Calculations of LR value were performed by the conservative method. For 20 DNA mixtures from three persons (out of 144 analysed mixtures) LR values were below 1 while DNA of the person of interest (POI) is a real component of a given mixture (H_p is real) and therefore for the above cases false negative results were obtained. In Figure 1 those results are marked with an orange frame. For two-donor DNA mixtures 10 such cases occurred (in 64). All the cases of false exclusions (LR < 1|H_p is „TRUE") were presented in Table 4. False negative LR results were characterised as a limitation of the software, however, they consider

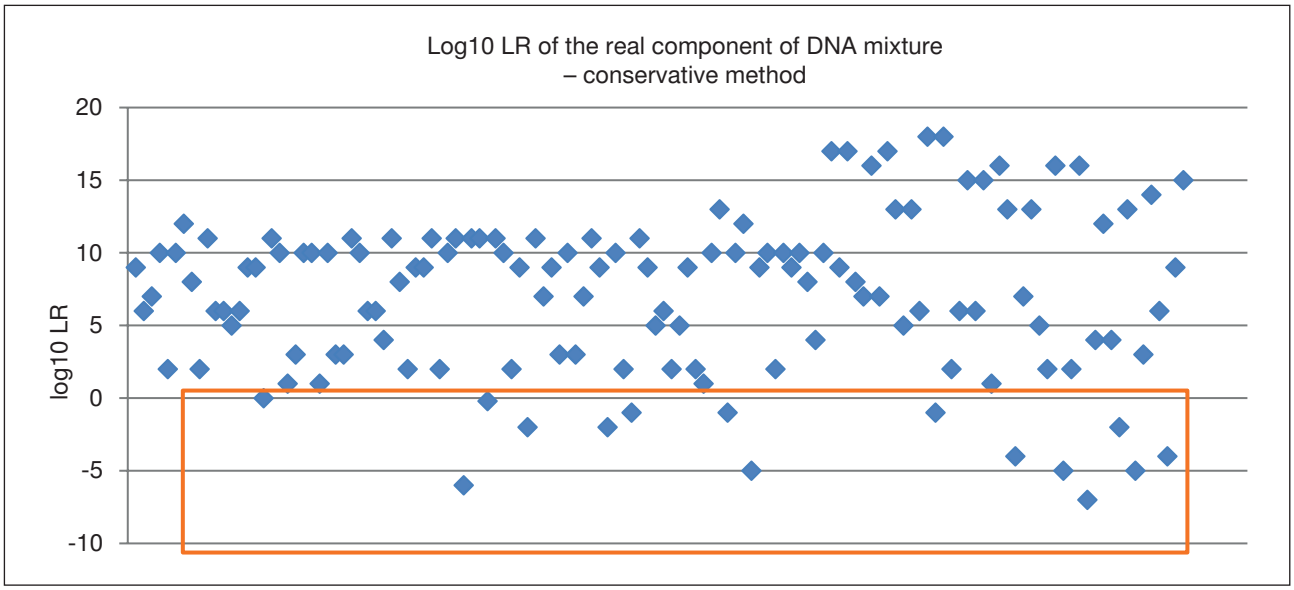

Fig. 1. Results of comparisons, in which a sample from a person of interest is a real component of DNA mixture (or H_p is true). The calculations of LR value were carried out by the conservative method. The false negative cases are marked with the orange frame.

Table 4. All the cases of DNA mixtures, for which results of statistical analysis upon taking into account the real component gave LR < 1.

POI is a person of interest. DNA – DNA quantity for each component of the mixture (marked in bold fonts for POI). *Drop-out* – number of observed allele *drop-outs* counted for POI. LR – value of likelihood ratio estimated by means of *LRmix Studio.*

Sample no.	POI	DNA (pg)	Number of *drop-outs*	LR
6.1	T	100:**40**:40	1, 7, 0	0.4
2.3	I	250:250:**40**	0, 0, 7	4e-06
3.3	L	250:250:**40**	0, 0, 11	1e-04
1.2	E	250:**40**:40	1, 14, 9	0.23
5.3	R	250:250:**40**	0, 0, 11	3e-05
2.4	I	500:40:**40**	0, 3, 10	0.0012
3.4	L	500:40:**40**	0, 7, 9	0.03
1.5	F	500:250:**40**	0, 0, 7	0.001
2.2	I	250:40:**40**	0, 2, 5	0.9
2.5	I	500:250:**40**	0, 0, 8	4e-05
0.5	B	150:**6**:30	0, 19, 13	3e-06
0.7a	B	300:**6**:30	20, 22, 11	1e-04
0.8a	C	300:30:**30**	0, 6, 13	0.005
0.2b	B	300:**30**	0, 15	0.02
0.5b	B	150:**6**:30	2, 27, 22	2e-04
0.5c	B	150:**6**:30	0, 26, 11	8e-06
0.6a	C	150:30:**30**	0, 4, 8	0.5
0.6c	C	150:30:**30**	0, 5, 12	0.02
0.7b	B	300:**6**:30	0, 23, 11	3e-05
0.8b	C	300:30:**30**	0, 7, 14	2e-4
0.7b	B	300:**6**:30	0, 23, 11	9e-05
0.14	H	100:**40**	4, 27	2e-05
0.15	J	100:**40**	9, 29	0.0162
0.16	B	250:**40**	8, 27	1e-04
0.19	H	250:**40**	0, 13	0.5
0.25	I	**40**:100	15, 0	0.0088
0.27	C	**40**:300	18, 0	0.3
0.30	I	**40**:300	17, 0	0.1102
0.17	D	250:**40**	2, 21	0.02

one particular category of DNA mixtures – every time, when POI contribution in the mixture was equal to or lower than 40 pg.

False positive indications (LR > 1/H_p is FALSE)

Figure 2 shows LR results for comparisons, in which POI is not a real component of a DNA mixture. In 1712 comparisons there were no false positive indications, i.e. results with positive LR values (LR values a little above 1) in the situation when DNA of a person of interest was not a real component of a given mixture (H_d is real). The LR calculations were carried out by means of the conservative method. In Figure 2 it can be observed that none of the results exceeded the borderline LR = 1 value marked with the red line.

LR value as function of allele drop-out

The phenomenon of *drop-out* has an immense influence on interpretation of DNA profiles from crime scene stains by creating a risk of false inclusions or exclusions. Probabilistic systems, such as *LRmix Studio* designed particularly for samples with low DNA contents may allow the Laboratory to extend the range of DNA mixture interpretation by exactly such cases. It is still not possible to carry out probabilistic analyses of all high order DNA mixtures. It the evidential profile drops below a certain level or too large number of alleles has dropped out, its interpretation may still be impossible (Coble et al., 2016). During the analyses aiming at validation it is particularly important to determine the number of *drop-outs,* at which a DNA mixture does not qualify any longer for interpretation by means of probabilistic models. Figures 3 and 4 present the way of relating LR values estimated by the conservative method with use of *LRmix Studio* software with the number of *drop-outs* (for POI profile) in cases, where POI is the real component of DNA mixture (H_p is true) and the indications of the software remained negative. When H_p is true the limit for observed LR > 1 values obtained with *LRmix Studio* amounts to 4 occurrences of *drop-out* for DNA mixtures from up to three persons and up to 12 *drop-outs* for DNA mixtures from two persons. The set limits constitute the lowest number of *drop-outs* observed in DNA profiling results, above which false negative LR < 1 values were reported. The result of statistical analysis of DNA profiles with a larger number of *drop-outs* may be unreliable.

Conclusions

The process of checking performance of the qualitative model, as well as reliability and repeatability of results reported by *LRmix Studio* involved analyses of single DNA profiles and mixtures from two and three donors with known DNA profiles. The aim of the study was to determine the likelihood ratio for a broad range of comparisons of low template DNA traces, where quality causes the largest interpretation difficulties. Due to that samples were prepared by combining two or three components in various proportions and materials of high content of DNA template (500 pg, 300 pg, 250 pg) were used, and in addition to that always one of two components of low DNA template level (100 pg, 40 pg, 30 pg, 6 pg). Moreover, some samples underwent a process of degradation so that the DNA mixtures best reflected real traces recovered at crime scenes. In this way, partial profiles were obtained with increased *drop-out* and *locus drop-out* (when entire locus is not amplified) phenomena, which are widely observed in analyses of LT-DNA types of traces (Gill, Buckleton, 2010; Buckleton et al., 2016). The validation procedure involved 250 comparisons for real components of DNA mixtures and 1712 comparisons for materials that were not real components of DNA mixtures. To sum up, the results of the analyses demonstrated that the *LRmix Studio* software performed according to the expectations. Probabilities of genotypes determined by the system were adequate to the expectations and the statistical results were repeatable and justified.

Identifying the limitations of the probabilistic software by internal validation is crucial for determining the ranges of DNA profiles that can undergo a statistical analysis in the Laboratory. Due to that every false result was characterized as software limitation (with an assumption of LR = 1 as the borderline value). Out of 1712 tests, 86% of DNA mixtures gave LR results supporting the inclusion hypothesis and 100 falsely selected POI obtained LR results supporting the exclusion hypothesis. Thus, the validated method meets the condition of striving to minimise the number

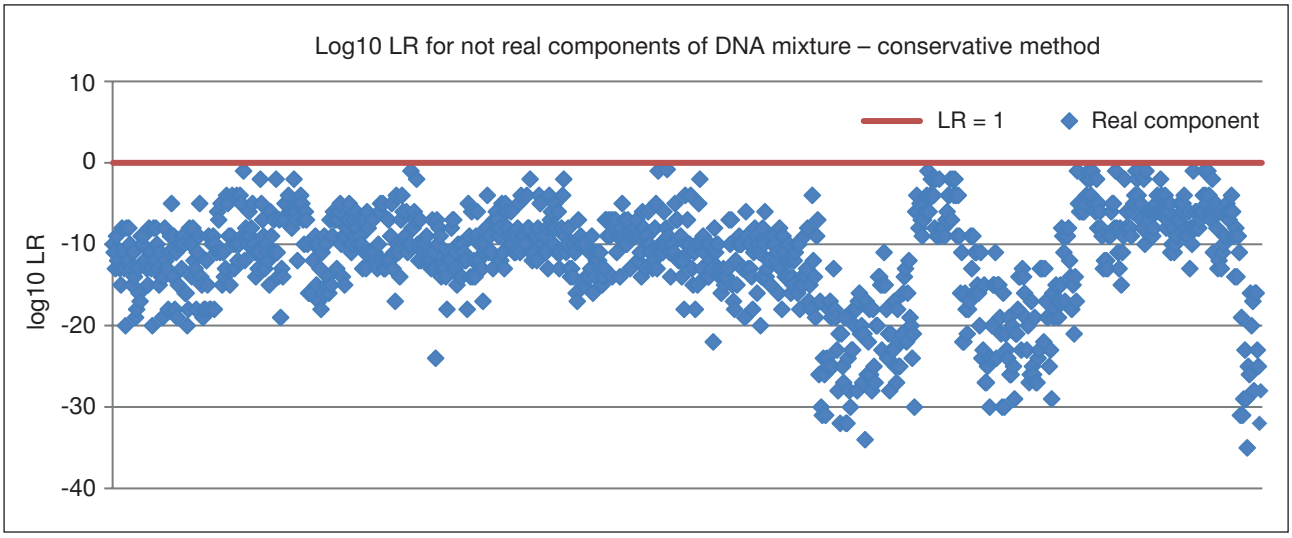

Fig. 2. Results of comparisons, in which POI is not a real component of the DNA mixture (H_p is false). Calculations of LR value were performed by means of the conservative method. LR = 1 value is marked with the red line.

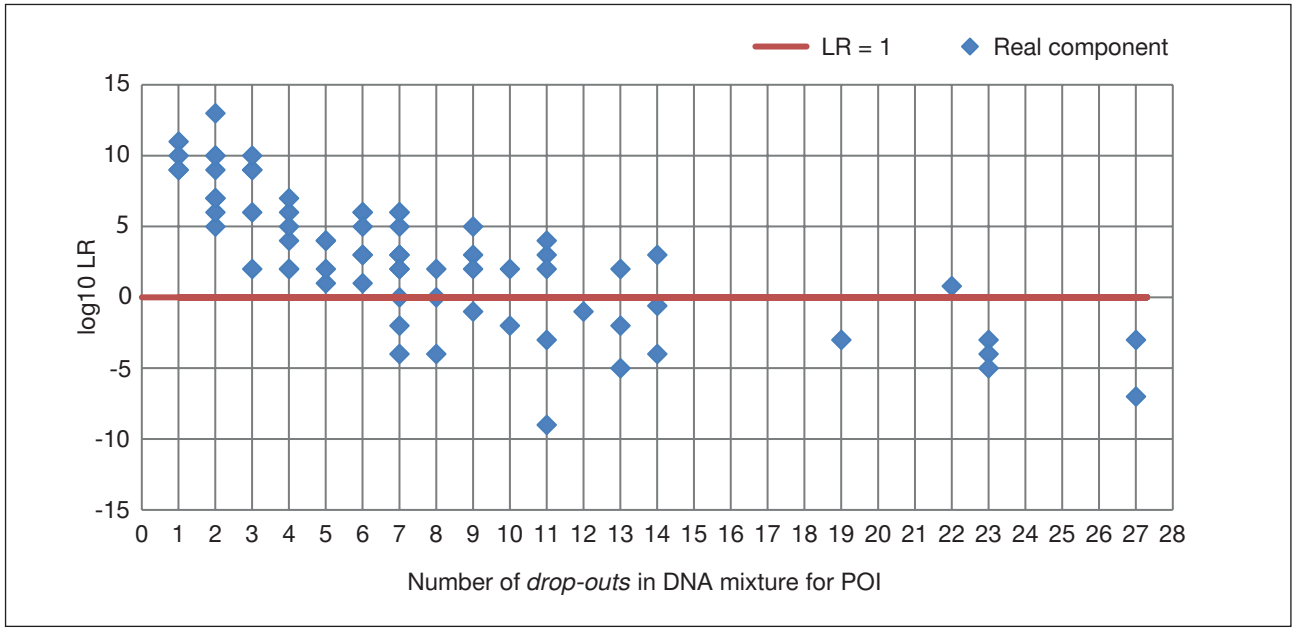

Fig. 3. The manner of relating LR value with the number of *drop-outs* for POI, in cases when POI is a real component of DNA mixture from three donors.

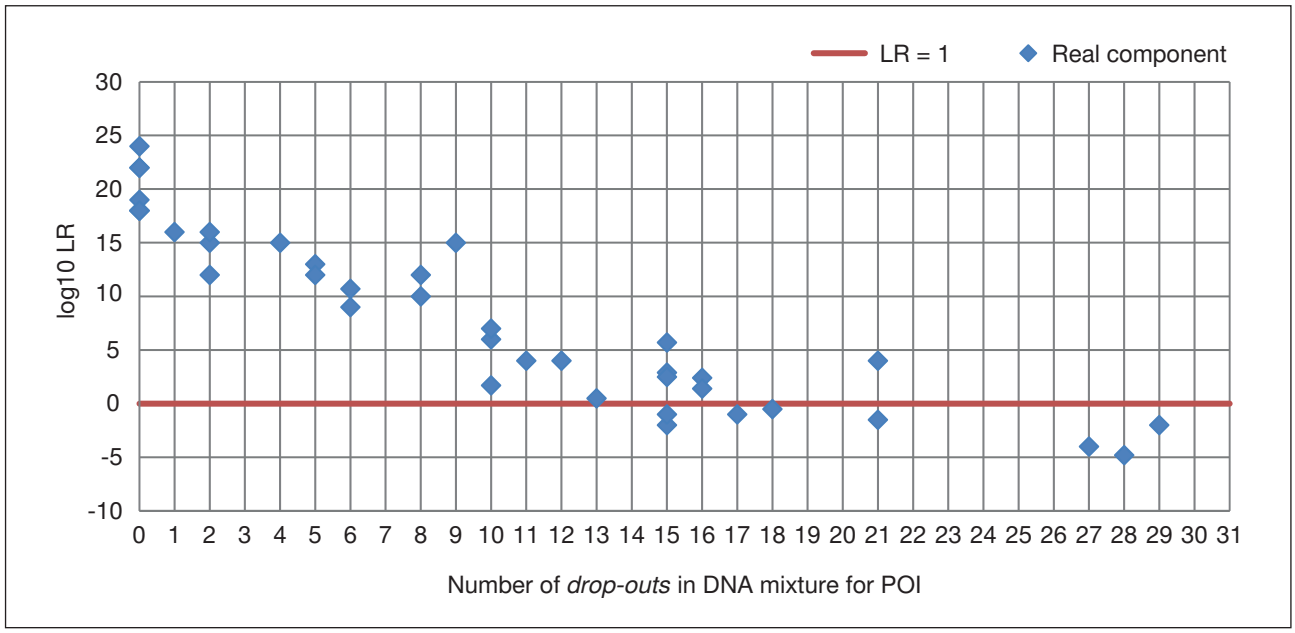

Fig. 4. The manner of relating LR value with the number of *drop-outs* for POI, in cases when POI is a real component of DNA mixture from two donors.

of false positive matches. At the same time, it was confirmed that applying the conservative method in statistical calculations (Bleka et al., 2016), as well as analysis of DNA mixtures analysis in STR multiplexes containing at least 23 short tandem repeats limits the occurrence of false positive results both in case of two--component and thee-component mixtures. Minimising false positive indications is achieved at the cost of a slight increase in false negative results (Bleka et al., 2016). However, in every case false POI exclusions referred to DNA mixtures that were complex in terms of quality and made of material from two and three donors, in which the real POI component had a low concentration of DNA template in the PCR reaction.

The conducted validation tests have proven that *LRmix Studio* can be successfully implemented in a forensic laboratory as a reliable tool for interpretation of DNA profiling results by means of probabilistic models based on estimation of likelihood ratio value. The statistical evaluation may refer both to complete DNA profiles as results of LT-DNA traces analyses. The application of this method allows forensic DNA

experts to bypass the need of taking binary decisions in the event of occurring stochastic effect and alleles below the detection limit. Thus the validated software decreases the subjectivism in assessing analytical results and contributes to standardisation of formulate conclusions.

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Translation Ewa Nogacka

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