Chapter 5

Assessment of the stochastic threshold, back- and forward stutter filters and low template techniques for NGM

Forensic Science International: Genetics (2012) 6:708-715

Antoinette A. Westen
Laurens J.W. Grol
Joyce Harteveld
Anuska S. Matai
Peter de Knijff
Titia Sijen

Abstract

The AmpF*l*STR® NGM™ kit shows an increased sensitivity compared to previous AmpF*l*STR® kits, and the addition of a 29th PCR cycle was found to be the major cause for this. During in-house validation, we evaluated whether the increased sensitivity requires elevation of the stochastic threshold (below which alleles are prone to drop out due to low template amplification effects). To determine the stochastic threshold, over 500 false homozygotes were examined and the threshold was set at the rfu value where 99 % of the alleles had a peak height below this value. Using 2085 Dutch reference samples, locus-specific stutter ratios were empirically determined and compared with the ones provided by Applied Biosystems. Application of sharp stutter filters is especially important for the analysis of unequal mixtures. To prevent allele calling of 99 % of the -1 repeat unit stutters, thirteen stutter ratio filters could be lowered by up to 1.79 % and for two loci the stutter ratio filters had to be elevated slightly with a maximum of 0.06 %. At all loci +1 repeat stutters were visible for the higher DNA inputs and for lower inputs at the tri-nucleotide repeat locus D22S1045 as well. The overall +1 stutter ratio filter was set to 2.50 % and for D22S1045 it was determined to be 7.27 %. To find the optimal strategy to sensitise genotyping for low template DNA samples, a comparison was made between enhancing the capillary electrophoresis settings (9 kV for 10 s) and increasing the number of PCR cycles (29 + 5 cycles).

Introduction

The AmpF*l*STR® Next Generation Multiplex (NGM™; Applied Biosystems (AB), Foster City, CA, USA) incorporates several new features when compared to SGM Plus™ (AB), which contains the core markers used in Europe until recently. To increase the discriminatory power, NGM™ combines the ten SGM Plus™ loci together with the five new European standard set short tandem repeat (STR) markers: D10S1248, D22S1045, D2S441, D1S1656 and D12S391 [1–3]. A 29th PCR cycle is added to enhance the sensitivity, and the buffer is improved to reduce the influence of inhibitory substances during PCR amplification [4]. When working under ISO 17025 guidelines, in-house validation of each new kit is mandatory. In addition to studying standard performance parameters as precision, repeatability, reproducibility, sensitivity, robustness, and suitability for mixture analysis [5] (and results not shown), we paid special attention to assessment of the stochastic thresholds, -1 and +1 repeat unit stutter filters and low template DNA techniques during our in-house validation of the NGM™, which will be described here.

When a (relatively) low single peak on a locus is analysed, it is compared against the stochastic threshold; when this peak is above the threshold, it is designated as a homozygous allele {a,a}, and when it is below the threshold, it is regarded as a potential heterozygous allele with allele drop-out of the sister allele and called {a,F} (where F stands for 'fail' and represents a 'wild card'). The stochastic threshold influences the number of false inclusions or exclusions during DNA database searches [6]; if it is set too high, homozygotes {a,a} will be called {a,F} and may result in false inclusions, and when it is set too low, heterozygotes {a,b} for which one allele has dropped out may be misdesignated as homozygotes {a,a} resulting in false exclusions [7]. The stochastic threshold is independent of DNA input; with higher inputs less allelic drop-outs occur than with lower inputs, and therefore comparison against the stochastic threshold is less often needed [6]. To assess the effect of the stochastic threshold in a NGM™ dataset, single allele peak heights were compared for heterozygous loci (after drop-out of the sister allele) and homozygous loci.

Stutter peaks are amplification artefacts that are usually ascribed to slipped strand displacement during PCR [8]. STRs, such as the tri-, tetra- and pentanucleotide repeats most widely used in forensic genotyping, predominantly form stutter products of one repeat length shorter (-1 stutter) than the parent allele. However, stutter products of one repeat unit longer (+1 stutter) or two repeat lengths shorter (-2 stutter) also occur [9]. Stutter peaks can greatly complicate the analysis of mixed stains, especially when, with unequal mixtures, the minor donor peaks are in the same peak height range as the stutter peaks of the major donor(s). The height of a stutter peak is affected by several aspects, like the number of nucleotides in the repeat, the AT-content of the repeat and the number of (uninterrupted) repeats [8,10]. As a result, different stutter

ratio thresholds are used for different STR loci in order to prevent calling of peaks on stutter position that are lower than the stutter ratio filter. We determined the -1 stutter ratio thresholds for in-house amplified samples and compared these to the AB stutter ratio thresholds. 2085 Dutch reference DNA profiles were evaluated and we found that the stutter ratio data were not normally distributed. Hence, the stutter ratio thresholds could not be calculated using the average plus 2 or 3 standard deviations from the mean without normalising the data, and we determined them empirically. In addition, we analysed whether +1 stutter ratio filters were needed for the analysis of NGM™ DNA profiles.

Within a locus the average -1 repeat stutter ratio is not the same for all alleles. Longer alleles tend to have higher stutter rates than shorter ones, and this is largely dependent on the length of the longest homogenous repeat stretch [8,11]. When analysing unequal mixtures and deciding on whether a peak is a stutter artefact or a minor donor allele, it might be important to know whether the stutter ratio for a specific allele is expected to be above or below the locus-specific stutter ratio threshold. Therefore, we explored the possibility to use allele-specific stutter ratio thresholds, by assessing the relation between stutter ratio and allele-specific repeat length.

The capillary electrophoresis (CE) injection settings used to analyse STR fragments differ between forensic laboratories, influenced by differences in analysts' opinions and differences between individual machines [12]. Therefore, we tested the effect of CE injection time on the stochastic threshold, percentage detected alleles, average peak height and the peak height ratio for low template (LT) DNA samples. These samples are prone to stochastic amplification effects that result in allele or locus drop-out, allele drop-in, or increased stutter peaks, especially when methods are applied that sensitise LT DNA typing. For these sensitising methods, several strategies have been proposed such as increasing the number of PCR cycles or changing the CE injection settings [13,14]. The use of replicate PCR amplifications in combination with a consensus method is advised to deal with uncertainties of LT DNA typing [6]. To determine what LT DNA technique performs best in combination with NGM™, we increased either the number of PCR cycles or the CE injection voltage.

Materials and methods

DNA samples

The reference DNA samples used for this validation study were kindly provided by the Forensic Laboratory for DNA Research (Leiden University Medical Centre, the Netherlands). They were extracted from blood samples of 2085 randomly sampled Dutch male blood donors coming from different regions in the Netherlands, who gave

their informed consent. These samples were genotyped using NGM™ and the allele frequencies will be described elsewhere (de Knijff and Sijen, in preparation). Pristine DNA007 (positive control DNA in NGM™ kit) and DNA9947a (positive control DNA in Profiler™ kit, AB) were used for determination of the stochastic threshold and LT DNA analyses.

PCR amplification and detection

The DNA was amplified in a multiplex PCR using the AmpF*l*STR® NGM™ kit (AB; early version without primer adjustments for amelogenin, D2S441 and D22S1045 [15]). Amplifications were performed with 29 PCR cycles, according to the protocol of the manufacturer. PCR products were detected by CE with an ABI Prism 3130xl Genetic Analyzer (AB) following the manufacturer's instructions. 1 µL sample or allelic ladder was analysed in combination with 8.7 µL Hi-Di™ Formamide and 0.3 µL GeneScan™ 500 LIZ™ Internal Size Standard (AB). CE injection settings were 3 kV for 15 s (3 kV/15 s), unless stated otherwise.

STR profile analysis

STR profiles were analysed using GeneMapper® ID-X software v. 1.1.1 (AB) with a peak detection threshold of 50 rfu (other settings were used for determination of the stutter ratios, see Stutter ratios section). The -1 stutter distance for D22S1045 was changed from "-4.75 to -3.25" to "-3.75 to -2.25", since it has a tri- and not a tetra-nucleotide repeat unit. The -1 repeat unit stutter filters provided by AB were used in combination with a general +1 stutter filter of 2.50 % and a 7.36 % +1 stutter filter for locus D22S1045, which shows high stutters due to its tri-nucleotide repeat structure. This initial percentage of 7.36 % was based on a subset (the first 528) of the 2085 reference samples. The 2.50 % +1 stutter filter follows from the reasoning that unequal mixtures with a minor to major ratio of 1:20 or higher, are regarded as too complex to interpret; when the major donor is homozygous and the minor donor is heterozygous, a 1:20 mixture results in a minor (heterozygous) donor peak that is 2.50 % (1:40) of the major (homozygous) donor peak.

Description of experiments

Stochastic threshold

For determination of the stochastic threshold, single alleles from heterozygous loci (representing drop-out of the sister allele) and homozygous loci were compared. Data for this comparison were derived from approximately 150 low template DNA samples, including inputs between 60 and 6 pg of pristine DNA007 and DNA9947a. In total,

511 single alleles at heterozygous loci and 138 homozygous peaks were analysed. The stochastic threshold was chosen at a relative fluorescence unit (rfu) value for which 99 % of the single alleles on heterozygous loci were below it.

Stutter ratios

Stutter ratios were obtained using the 2085 reference DNA profiles, which had a PCR input of 250–500 pg DNA. During analysis in GeneMapper® ID-X no stutter filters were applied and a detection threshold of 25 rfu (Supplementary Table S1) was used. Stutter ratios were calculated based on peak height: (stutter peak / parent allele) × 100 %. To determine the -1 stutter ratio thresholds for all loci and the +1 stutter ratio threshold for D22S1045, we used only the stutter percentage of parent alleles that were above the stochastic threshold of 400 rfu (Supplementary Table S1). Stutter peaks that were in-between two alleles on +1/-1 stutter position were regarded as -1 stutters of the longer allele. The -1 stutter ratio data were grouped into categories that comprised one percent (e.g. 2.00 – 2.99 %) and plotted against the number of observations per category. The distribution of the data was compared to a normal distribution using a Kolmogorov–Smirnov (K–S), a Lilliefors and a Shapiro–Wilk W test in Statistica v. 7.1 (StatSoft, Inc., Tulsa, OK, USA). Subsequently, all -1 stutter ratios were sorted per locus (using Excel (Microsoft Corporation, Redmond, WA, USA)), and after exclusion of the 1 % highest stutter ratios, the maximum value was chosen as the empirical locus-specific stutter ratio threshold.

When determining the stutter ratio thresholds, we noted that they are largely dependent on the number of data points that are evaluated; with more data points, more values will be in the 1 % highest stutter ratios, which facilitates a better fine-tuning of the stutter ratio threshold. GeneMapper® ID-X uses stutter ratio filter values with two decimals; although it could be more appropriate to use only one decimal for the stutter ratio thresholds, GeneMapper® ID-X would interpret a stutter ratio filter of for instance 13.3 as 13.30, and therefore we decided to present all our results with two decimals (similar to the AB stutter filters).

Low template DNA analysis

Different laboratories may choose different CE injection settings (e.g. lower settings for reference samples than for trace samples, or to minimise baseline artefacts, or higher settings to increase sensitivity). For use in routine casework, a comparison was made between 3 kV/15 s, 3 kV/10 s and 3 kV/5 s. To examine the effect of these different CE settings on LT DNA profiles, 20, 25 and 30 pg DNA007 were each amplified in 30 replicates.

A dilution series of 63, 31, 16 and 8 pg DNA007 was amplified in 6 replicates to compare our standard PCR and detection technique (29 PCR cycles and CE at

3 kV/15 s) with two LT techniques. For the first LT technique (29 + 5 cycles), after the standard 29 PCR cycles 10 µL PCR product was transferred to a new PCR tube, and after addition of 0.5 µL AmpliTaq™ Gold Polymerase (AB) 5 additional PCR cycles were performed. This method was followed by standard CE, and STR profile analysis was performed with stutter ratio thresholds that were multiplied by 1.5 (see the Results and discussion section on Low template DNA analysis). The second LT technique is based on a standard PCR followed by CE with a raised injection voltage of 9 kV/10 s. For samples that were injected at 9 kV, a Performa™ DTR gel filtration step (Edge Bio, Gaithersburg, MD, USA) preceded the CE to prevent dye blobs in the DNA profiles as described in Westen et al. [14]. For these samples, 2 µL DTR-filtered sample or 1:20 diluted allelic ladder was combined with 7.0 µL Hi-Di™ formamide and 1.0 µL 1:100 diluted GeneScan™ 500 LIZ™ Internal Size Standard (AB).

Results and discussion

Stochastic threshold

In order to determine the stochastic threshold for NGM™ STR profiles, 511 single alleles from heterozygous loci were compared with 138 homozygous peaks occurring in the same low template data set. Fig. 1A shows the empirical cumulative distribution of the homozygous and heterozygous single allele peak heights. The solid horizontal line at 0.99 crosses the data points between 387 and 435 rfu. Hence, we have set the stochastic threshold at 400 rfu (vertical line). The stochastic threshold intersects the homozygous data points at 0.75 (dotted horizontal line). Thus, for a stochastic threshold at 400 rfu, 75 % of the single peaks at homozygous loci are marked as {a,F} and 25 % are correctly designated as {a,a}, while for the single alleles at heterozygous loci 99 % are correctly marked as {a,F} and 1 % is misdesignated as {a,a}. For the latter category, the peak heights ranged between 435 and 613 rfu (compared to a maximum peak height of 994 rfu for homozygous loci). A consequence of determining the stochastic threshold as such is that allele drop-outs may still occur in profiles that have peak heights above the stochastic threshold, albeit uncommon.

For the analysis of SGM Plus™ DNA profiles we used a stochastic threshold of 175 rfu. These profiles were generated after 28 amplification cycles and a CE injection at 3 kV/15 s. For NGM™, the same CE settings, but an additional 29th PCR cycle was performed. We infer that the higher stochastic threshold for NGM™ relates to this extra PCR cycle.

After the in-house validation of NGM™ (that included determination of the stutter ratio thresholds and assessment of low template DNA techniques, as described in more detail below), NGM™ has been implemented in routine casework. However, using the

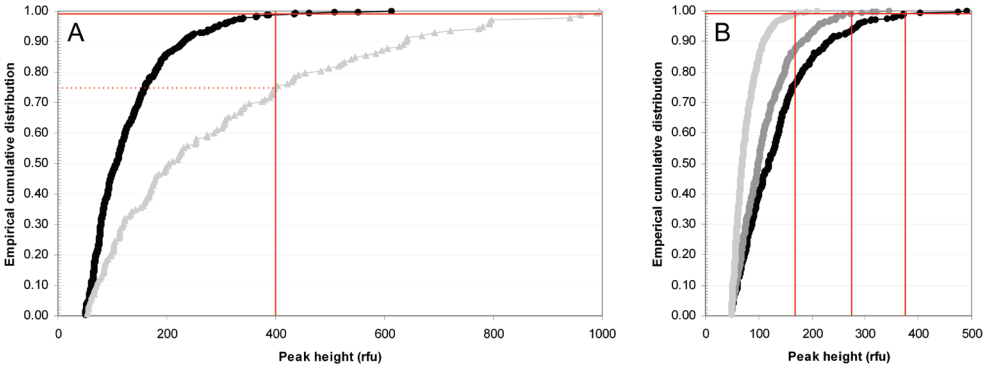

Fig. 1 (A) Empirical cumulative distribution of homozygous and heterozygous single allele peak heights for standard CE settings (3 kV/15 s). Homozygous peaks are plotted as grey triangles (n = 138), and heterozygous alleles (with drop-out of the sister allele) are represented by black dots (n = 511). The solid horizontal line at 0.99 intersects the single heterozygous alleles around 400 rfu (99 % of the alleles has a lower peak height), which is set as stochastic threshold (vertical line). The stochastic threshold crosses the homozygous data at 0.75 (75 % of the alleles has a lower peak height; dotted horizontal line). (B) Empirical cumulative distribution of heterozygous single allele peak heights for CE settings at 3 kV/15 s (black dots, n = 331), 3 kV/10 s (dark grey dots, n = 468) and 3 kV/5 s (light grey dots, n = 583). The solid horizontal line at 0.99 intersects the single heterozygous alleles around 372, 273 and 168 rfu (99 % of the alleles has a lower peak height for that CE setting), as indicated by the vertical lines, for 3 kV/15 s, 3 kV/10 s and 3 kV/5 s, respectively.

standard CE injection settings (3 kV/15 s) in routine casework for some time, profiles showing pull-up peaks (especially for homozygous peaks) were encountered repeatedly. To lower the number of pull-up peaks, we tested reduced injection times of 3 kV/10 s and 3 kV/5 s. These settings lowered the average peak height for DNA profiles with a PCR input of 500 pg DNA007 (n = 2) from 3552 rfu, to 2298 rfu and 1188 rfu for 3 kV/15 s, 3 kV/10 s and 3 kV/5 s, respectively. To assess how these CE injection times affect the stochastic threshold, the percentage detected alleles, the average peak height and the peak height ratio, a set of 90 LT DNA samples was examined (Table 1). When lowering the injection settings from 3 kV/15 s to 3 kV/5 s, the percentage detected alleles drops from 84 to 49 %, while the number of single alleles on heterozygous loci increases (Table 1). The average peak height goes down from 170 to 80 rfu, resulting in a decrease of the empirically determined stochastic thresholds (Table 1). Fig. 1B shows the empirical cumulative distribution of the heterozygous single allele peak heights for 3 kV/15 s, 3 kV/10 s and 3 kV/5 s. The stochastic thresholds for these three settings have been determined empirically in the same way as described above, and decrease from around 400 to 175 rfu (rounded values, Table 1 and Fig. 1B). The peak

Table 1
DNA profile characteristics for different CE settings.

Setting	# single heterozygous alleles[a]	Empirically determined stochastic threshold[b]		Average percentage detected alleles	Average peak height	Peak height ratio (*n*)
		Precise	Round-off			
3 kV/15 s	331	372 rfu	400 rfu	84%	170 rfu	0.62 (1048)
3 kV/10 s	468	273 rfu	300 rfu	74%	125 rfu	0.66 (825)
3 kV/5 s	583	168 rfu	175 rfu	49%	80 rfu	0.73 (419)

[a] Based on 30 amplifications with an input of 20, 25 or 30 pg pristine DNA007 each (*n* = 90).
[b] 99% of single heterozygous alleles were below this value.

height ratio becomes better with shorter injection times, although far fewer loci (419 instead of 1048) remain to calculate this ratio. This is probably caused by the fact that the peak heights are generally lower for shorter injection times, which reduces the efficacy of detecting both alleles at a heterozygous locus. Thereby, especially heterozygous loci with good peak height balance will remain for peak height ratio calculation. Overall, the 3 kV/15 s CE settings show the most complete DNA profiles. Nevertheless, due to the repeatedly encountered pull-up peaks in profiles for routine casework and the additional time needed for re-running and re-analysing these samples, we have chosen to use the 3 kV/5 s CE injection settings for routine DNA analysis, with the possibility to rerun the samples at 3 kV/15 s (or to use a LT technique) when needed.

Stutter ratios

Stutter ratios were determined based on the DNA profiles from the reference set of 2085 Dutch blood donors. For all 15 STR markers -1 repeat unit stutter ratio thresholds were determined and the +1 stutter ratio threshold for D22S1045, as well. For the various loci, between 1279 and 3119 data points were obtained.

Locus-specific -1 repeat stutter

In order to determine whether the locus-specific -1 stutter ratios were normally distributed, the data were categorised in intervals of one percent and plotted against the number of observations per category. A normal distribution was plotted in the same graph, as is shown in Fig. 2 for the -1 stutter ratios of three loci (D10S1248, VWA and D16S539). Visual inspection suggests that the data are not normally distributed, which is confirmed by a Kolmogorov–Smirnov (K–S), a Lilliefors and a Shapiro–Wilk W test (Fig. 2). Similar results were obtained for the other loci (results not shown). Consequently, the stutter ratio thresholds could not be calculated using the average plus 2 or 3 standard deviations from the mean (to comprise 95.45 or 99.73 % of the data points, respectively) without

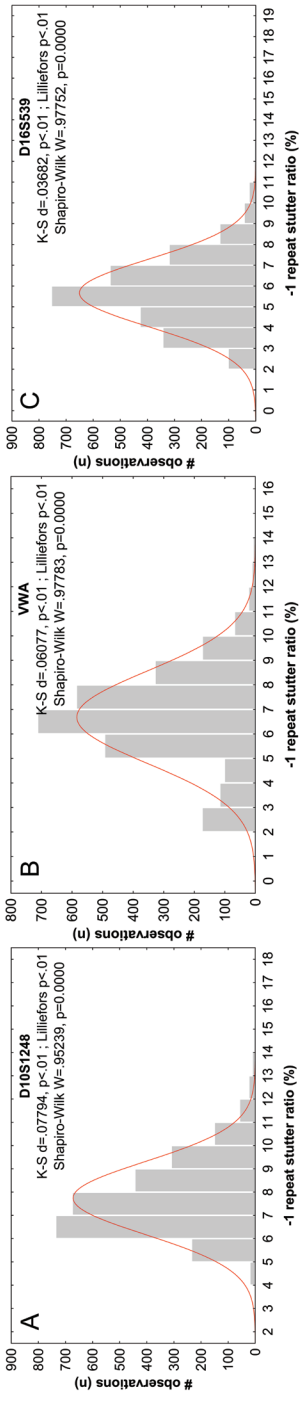

Fig. 2 Minus 1 repeat stutter ratio (in categories of 1 % intervals) plotted against the number of observations (grey bars) and compared to the normal distribution (curve) for locus D10S1248 (A), VWA (B) and D16S539 (C). Normal distribution test results by a Kolmogorov–Smirnov (K–S), a Lilliefors and a Shapiro–Wilk W test are shown in the upper right-hand corner.

normalisation, and we preferred to determine these thresholds empirically.

To determine the -1 stutter ratio threshold empirically, for each locus the -1 stutter ratio data points were sorted in increasing order. After exclusion of the 1 % highest data points, the maximum value was chosen as the empirical locus-specific stutter ratio threshold. Table 2 shows a comparison between the empirically determined stutter ratio thresholds (Supplementary Table S1) and the thresholds provided by Applied Biosystems. For thirteen of the NGM™ STRs, the stutter ratio thresholds could be lowered by 0.46 % to 1.79 %; a slight elevation by 0.04 % and 0.06 % was needed for two loci (D19S433 and TH01, respectively). The number of stutters on which the stutter ratio thresholds are based differs per locus (Table 2), which is due to aspects such as different rates of homozygous or heterozygous donors for the loci, or more or less alleles with stutter peaks below the detection threshold of 25 rfu. The finding that most of the -1 stutter ratio thresholds could be lowered is especially interesting to assist minor contributor allele detection in unequal mixtures with low template DNA components. Although lowering the stutter ratio filters may result in slightly more stutters to be designated as alleles, maintaining stutter filters that are set relatively high may prevent the minor component(s) of unequal mixtures from being called. In our experience with unequal mock casework mixtures (for which the donors are known), the gain of additional alleles from the minor component(s) is more valuable than the drawback of a sporadically called stutter peak [16] (data not shown).

A noteworthy observation on locus TH01 was the presence of different stutter products

Table 2
Locus-specific −1 repeat stutter ratio thresholds.

Locus	D10S1248	VWA	D16S539	D2S1338	D8S1179	D21S11	D18S51	D22S1045	D19S433	TH01[c]	FGA	D2S441	D3S1358	D1S1656	D12S391
n[a]	2629	2756	2658	3119	2891	2865	2903	2641	2760	1279	2966	2681	2748	3119	2953
emp[b] (%)	12.12	11.08	10.11	12.55	10.13	10.91	12.66	16.30	11.10	5.32	11.82	7.68	12.12	13.21	14.82
AB (%)	12.89	11.82	10.57	13.55	10.82	11.40	13.89	17.99	11.06	5.26	12.61	9.47	13.77	14.16	15.84
emp – AB (%)	−0.77	−0.74	−0.46	−1.00	−0.69	−0.49	−1.23	−1.69	+0.04	+0.06	−0.79	−1.79	−1.65	−0.95	−1.02

[a] Number of −1 stutters >25 rfu.
[b] Empirically determined −1 stutter ratio threshold comprising 99% of the stutters.
[c] Allele 9.3 on locus TH01 showed stutter products on position 8.3 (*n* = 191), but also on position 9 (*n* = 52) or on both (*n* = 30); only the 8.3 stutter products were used to determine the stutter ratio threshold.

for allele 9.3. The most frequently occurring stutter peak appeared on position 8.3 (n = 191), but also on position 9 (n = 52) or on both positions (n = 30) stutter products occurred (Fig. 3A–C). The general repeat sequence for locus TH01 is $[AATG]_n$. For allele 9.3 a deletion of one adenosine in the seventh repeat is reported: $[AATG]_6ATG[AATG]_3$ [17,18]. According to the slipped strand displacement model, stutter peaks are formed when the DNA polymerase dissociates from the template during extension and the template strand loops out followed by (out-of-register) re-annealing and further extension of the fragment [8]. A possible explanation for the different stutter products preceding allele 9.3 is that looping out occurs not only for full tetra-nucleotide repeats, but also for the tri-nucleotide repeat number 7.

When following this hypothesis, the occurrence of the two -1 stutter products is independent of the amplification kit employed. For the determination of the -1 repeat length stutter ratios, only the stutters on position 8.3 were taken into account. When analysing a DNA profile with allele 9.3 on TH01, however, one has to realise that a stutter peak on position 9 may be present.

All the analyses described in this paper have been performed using the early version of the NGM™ kit (AB). In the meantime, AB has replaced this version with a new version of NGM™ containing primer adjustments for amelogenin, D2S441 and D22S1045 to avoid a number of known null alleles. AB has not adjusted the stutter filters for this updated version of NGM™. In order to analyse whether the new primers for D2S441 and D22S1045 influence the stutter ratios in our hands, we evaluated the genotyping results from 776 reference samples that were typed with the new version of NGM™ for the Dutch national DNA database. The results did not require changes to be made in the stutter ratio thresholds for the new version of NGM™. Thus, we continue to use the -1 and +1 repeat stutter ratios as determined in this study for use in both database and casework DNA analysis.

Locus-specific +1 repeat stutter

Tri-nucleotide repeat loci, like D22S1045, are known to show higher -1 and +1 stutters than tetra-nucleotide repeat loci [9,11]. This is illustrated in Fig. 3D, where allele 15 is followed by a stutter product of 4.3 %, while the +1 stutter ratios for other loci did not

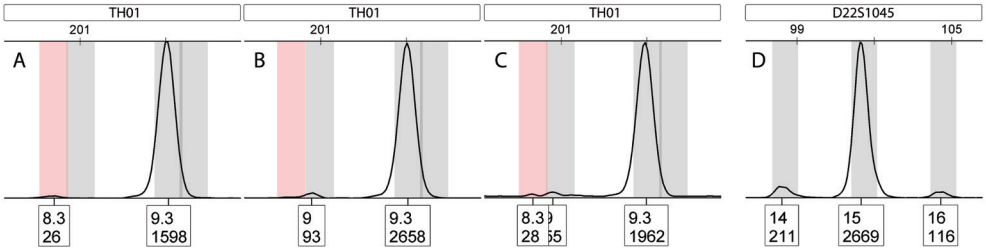

Fig. 3 Examples of stutter products for locus TH01 allele 9.3 on position 8.3 (A), 9 (B) or both (C), and a random example of -1 and +1 stutter on locus D22S1045 (D). In the boxes below the peaks, the upper value represents the allele call and the lower value the peak height in rfu.

exceed 2.50 % in over 99 % of the cases. We determined the empirical +1 stutter ratio threshold for D22S1045 on 2153 data points in the reference DNA profiles. Based on these results, a +1 stutter ratio threshold of 7.27 % is appropriate to comprise 99 % of the +1 stutter products.

Allele-specific -1 repeat stutter

Next to locus-specific stutter ratio thresholds, we evaluated the possibility to use allele-specific -1 stutter ratio thresholds. The empirically determined -1 stutter ratios are plotted per allele for each locus, and Fig. 4 shows an example for three loci (D10S1248, D21S11 and D2S441). For most loci, a rise in stutter ratio is seen with increasing numbers of "full" repeats (Fig. 4A and B). For x.1, x.2 or x.3 microvariants, the stutter ratios are lower than expected based on their length alone, but also they increase with increasing length (Fig. 4B). For these microvariants, the series of repeats is interrupted by a modified repeat. The length of uninterrupted stretches of repeats affects the occurrence of stutters and stutter ratio; the longer the stretches are, the higher the ratio of stutter products [10,11]. This explains the lower stutter ratios for the microvariants. The full alleles at locus D2S441 do not follow the general trend of increasing stutter ratio with higher allele number. Actually, a double pattern of increasing allele-specific stutter ratio seems to be present: one from alleles 10 to 13, and the other from alleles 12 to 16, with two clusters of stutter ratios for alleles 12 and 13. We hypothesise that two different repeat sequences underlie these differences in stutter ratio. This could be tested by sequencing (a number of) these samples, but that is beyond the scope of this study. Interestingly, Phillips et al. [3] did sequence a number of DNA samples for D2S441 and found single nucleotide polymorphisms (SNPs; predominantly in repeat number 6) for various alleles and populations. Such SNPs do interrupt the repeat sequence and shorten the number of uninterrupted repeats significantly, and their results thus support our hypothesis.

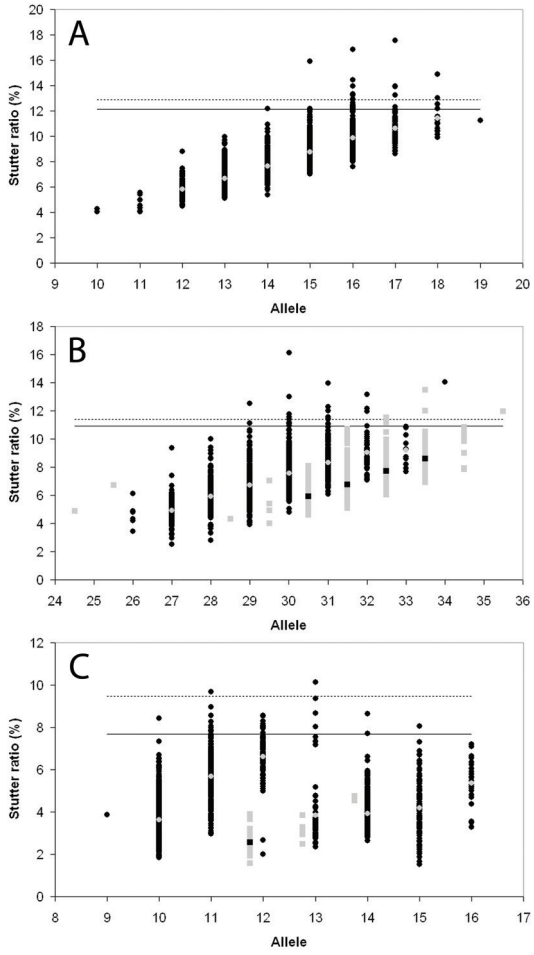

Fig. 4 Empirically determined allele-specific stutter ratios plotted per allele for locus D10S1248 (A), D21S11 (B) and D2S441 (C). Black dots represent allele-specific stutter ratios for "full" alleles, with grey dots as their average when at least 10 data points were present. Grey squares correspond to allele-specific stutter ratios for "x.2" (B) or "x.3" (C) alleles, with black squares as their average. The horizontal lines represent the locus-specific stutter ratio thresholds provided by AB (dotted) or empirically determined (solid).

The use of allele-specific -1 repeat stutter ratio thresholds could aid the analysis of unequal mixtures when peaks at stutter position have a peak height around the locus-specific stutter ratio threshold. To determine allele-specific stutter ratio thresholds that comprise at least 99 % of the stutters (our empirical approach), per allele 100 data points are needed as a minimum. In our data set this is achieved only for the most frequent alleles. Since the stutter data are not normally distributed, extrapolation of the data to values for less frequent alleles is not appropriate. Another disadvantage of the allele-specific stutter ratio thresholds is that they cannot be entered into GeneMapper® ID-X (standard in many laboratories) and can therefore not be applied automatically during DNA profile analysis.

Low template DNA analysis

To determine which LT DNA technique is most fit for use with NGM™, we increased the number of PCR cycles from 29 to 29 + 5 or the CE injection settings from 3 kV/15 s to 9 kV/10 s. A dilution series with an input range from 63 to 8 pg DNA was used. For the method with 29 + 5 PCR cycles, the STR profiles with

Table 3
NGM performance for LT DNA analyses; dilution series of pristine DNA007 ($n=6$).

Input	Average percentage detected alleles			Average number of drop-ins/profile								
	3 kV/15 s	9 kV/10 s	29+5 cycles	3 kV/15 s			9 kV/10 s			29+5 cycles		
				−1	+1	[a]	−1	+1	[a]	−1	+1	[a]
63 pg	99%	100%	n.a.[b]	0.0	0.0	0.0	1.0	0.8	0.0	n.a.	n.a.	n.a.
31 pg	90%	92%	n.a.	0.5	0.0	0.0	0.3	0.7	0.0	n.a.	n.a.	n.a.
16 pg	66%	78%	79%	0.0	0.0	0.0	0.3	0.0	0.0	0.2	2.0	1.0
8 pg	41%	57%	57%	0.0	0.0	0.0	0.7	0.0	0.2	0.2	1.0	0.0

[a] Drop-ins at other positions than −1 and +1 repeat stutter distance.
[b] Not analysed due to over-loaded profiles.

an input of 63 or 31 pg DNA were highly overloaded and could not be analysed (Table 3); the profiles with a PCR input of 16 or 8 pg DNA for this method were analysed with 1.5 times the stutter ratio thresholds (see details below). The average number of drop-ins, resulting from contaminating alleles, elevated stutters, or other artefacts [12], was calculated per profile. The position on which the drop-ins occurred was divided into three categories: -1 stutter position, +1 stutter position, or other position. Our standard method shows the lowest number, while the method with 29 + 5 PCR cycles relatively shows the highest number of drop-ins per profile. The method with CE at 9 kV/10 s shows a number of drop-in alleles in-between the other two methods. For all methods, drop-ins occur predominantly at -1 or +1 stutter position, thereby most likely representing elevated stutter artefacts (also termed as stutter drop-ins [6]).

Stutter ratios tend to increase when performing additional PCR cycles, such as 28 + 6 cycles for SGM Plus™ [13] or 29 + 5 cycles for NGM™. In order to determine the magnitude of this increase, a comparison was made between the (previously mentioned) DNA007 profiles with a PCR input of 8 and 16 pg that were each replicated 6 times with 29 + 5 cycles for NGM™, and 29 cycles NGM™ amplifications with an input of 250 or 500 pg DNA007 in 6 replicates each, as these two sets showed comparable peak heights. Stutters were determined in the same way as described in the Materials and methods section on Stutter ratios. In Supplementary Table S2 is shown that, in total, 198 stutters were obtained for DNA profiles after 29 + 5 cycles and 375 stutters after 29 cycles. For each allele in the DNA007 profiles for which it was possible to determine a stutter ratio, we calculated the average stutter ratio over the 12 DNA profiles that were amplified with either 29 + 5 or 29 PCR cycles. Next, the ratio between them was determined by dividing the average stutter ratio for example for allele 12 on D10S1248 after 29 + 5 cycles by the average stutter

ratio for the same allele after 29 PCR cycles. Such a ratio could be determined for 24 alleles in the DNA007 profiles and the ratios ranged between 1.0 and 1.5. When analysing SGM Plus™ DNA profiles after 28 + 6 PCR cycles, we used to multiply the stutter ratio thresholds by 1.5, and, based on the results, it is appropriate to use this factor of 1.5 for the analysis of 29 + 5 cycles NGM™ DNA profiles, as well.

We do not use a stochastic threshold for any of the LT DNA techniques as we always consider that allele drop-out may have occurred. For these LT methods, it is needed to perform multiple PCR amplifications of the same DNA extract in combination with a consensus method [6]. In our laboratory, the *n*/2 method is used, for which an allele is included in the consensus when it is designated in at least half of the replicates (with $n = 3$ or $n = 4$ as optimal replicate number) [6].

Conclusion

Our conclusions and decisions for practical use are summed up below.

- The stochastic threshold is dependent on the injection settings used; for 3 kV/5 s 175 rfu, for 3 kV/10 s 300 rfu, and for 3 kV/15 s 400 rfu is appropriate.
- Since the stochastic threshold includes 99 % of the single alleles on heterozygous loci, some may remain un-flagged. The maximum observed peak height in this data set is 613 rfu.
- Stutters are not normally distributed and stutter ratio thresholds are best determined empirically.
- Thirteen -1 stutter ratio thresholds are lowered by up to 1.79 % compared to the ones provided by AB; two are elevated slightly (with a maximum of 0.06 %). This will assist allele calling of the minor contributor in unequal mixtures.
- Especially for the tri-nucleotide repeat locus D22S1045 a +1 stutter ratio threshold is needed, which is set at 7.27 %.
- Allele-specific -1 stutter ratio thresholds can only be determined for the most frequent alleles and cannot be entered into GeneMapper® ID-X. Consequently, they will not (yet) be applied.
- Low template DNA analysis can be performed with 9 kV/10 s CE injection settings; only for the very low ranges (<31 pg) the use of 29 + 5 PCR cycles is recommended.

In conclusion, when introducing a new STR kit for routine use, we recommend in-house validation of several aspects, such as the stochastic threshold, -1 and +1 repeat stutter ratio thresholds and low template DNA analysis methods. These parameters will optimise the analyses of complex mixtures and low template DNA samples.

Acknowledgements

This study was supported by a grant from the Netherlands Genomics Initiative/ Netherlands Organization for Scientific Research (NWO) within the framework of the Forensic Genomics Consortium Netherlands. The authors are grateful for the voluntary cooperation of the DNA donors for the Dutch reference set. We would like to thank Ankie van Gorp and Corina Benschop for critically reading the manuscript.

Supplementary data

Supplementary Table S1
Insight into the composition of the -1 stutter dataset based on the 6-FAM-channel of NGM DNA profiles from 2085 Dutch reference samples. Data are presented based on the parent allele peak height and on the stutter peak height. When less than 100 data points are available, the values are shown in grey, and the highest stutter ratio becomes the stutter ratio filter value.

		parent peak height (rfu)				stutter peak height (rfu)		
		>400[a]	<400[b]	400-1000	>1000	>25[a]	>50	25-50
D10S1248	*n*	2629	21	211	2418	2629	2562	67
D10S1248	emp.thres.	12.12	18.13	13.95	12.00	12.12	12.16	11.38
D10S1248	avg	7.74	10.32	8.11	7.71	7.74	7.77	6.72
D10S1248	stdev	1.56	2.80	1.99	1.51	1.56	1.55	1.49
vWA	*n*	2756	14	376	2380	2756	2487	269
vWA	emp.thres.	11.08	14.37	12.88	10.91	11.08	11.10	9.82
vWA	avg	6.68	10.79	7.12	6.61	6.68	6.91	4.62
vWA	stdev	1.88	2.55	1.90	1.87	1.88	1.73	1.98
D16S539	*n*	2658	6	336	2322	2658	2297	361
D16S539	emp.thres.	10.11	14.53	10.82	9.85	10.11	10.23	9.07
D16S539	avg	5.67	10.18	6.20	5.59	5.67	5.87	4.37
D16S539	stdev	1.63	2.52	1.80	1.59	1.63	1.54	1.58
D2S1338	*n*	3119	51	888	2231	3119	2833	286
D2S1338	emp.thres.	12.55	19.00	13.50	12.15	12.55	12.63	10.42
D2S1338	avg	7.86	10.15	8.17	7.73	7.86	7.97	6.77
D2S1338	stdev	1.78	2.62	1.94	1.70	1.78	1.78	1.44

[a] The empirically determined -1 stutter thresholds that are presented in the Results and discussion section on Locus-specific -1 repeat stutter, are based on parent peaks >400 rfu and stutter peaks >25 rfu.
[b] Parent peaks <400 rfu (representing LT DNA) show relatively high stutter thresholds based on small numbers of stutter peaks and were left out of the overall stutter threshold determination; for LT DNA multiple independent amplifications should be performed in combination with a consensus method to accommodate stochastic amplification effects, such as elevated stutters.

Supplementary Table S2
Ratio between the average stutter ratio per allele for DNA007 after 29+5 (8 and 16 pg DNA input, 6 replicates each) or 29 (250 and 500 pg DNA input, 6 replicates each) NGM PCR cycles.

		29+5 cycles		29 cycles		29+5/29
marker	allele	average stutter ratio	*n*	Average stutter ratio	*n*	ratio
D10	12	6.66	8	5.80	16	1.15
	15	10.58	8	8.58	16	1.23
D12S391	18	7.17	8	6.82	16	1.05
	19	9.88	2			
D16S539	9	3.85	9	3.65	16	1.06
	10	4.65	3			
D18S51	12	5.39	6	4.98	16	1.08
	15	8.92	6	7.32	16	1.22
D19S433	14	7.91	10	6.35	16	1.25
	15	7.56	2			
D1S1656	13	7.61	5	6.89	16	1.10
	16	12.45	10	9.98	16	1.25
D21S11	28	7.37	10	5.87	16	1.26
	31	10.48	8	8.01	16	1.31
D22	11	4.36	9	2.91	16	1.50
	16	11.01	10	9.53	16	1.15
D2S1338	20	9.24	9	8.80	16	1.05
	23	8.51	7	8.28	16	1.03
D2S441	14	5.60	7	3.75	16	1.49
	15	4.17	1			
D3S1358	15	8.94	9	7.57	16	1.18
	16	8.42	1			
D8S1179	12	8.47	10	6.67	16	1.27
	13	8.96	2			
FGA	24	8.79	7	7.74	16	1.14
	26	9.64	7	9.54	16	1.01
TH01	7	3.38	8	2.23	15	1.52
	9.3	2.17	1	1.47	8	1.48
VWA	14	3.74	8	3.11	16	1.20
	16	8.05	7	6.07	16	1.33
n total			198		375	24

References

1. P. Gill, L. Fereday, N. Morling, P.M. Schneider, The evolution of DNA databases – recommendations for new European STR loci, Forensic Sci. Int. 156 (2006) 242–244.
2. P. Gill, C.H. Brenner, J.S. Buckleton, A. Carracedo, M. Krawczak, W.R. Mayr, N. Morling, M. Prinz, P.M. Schneider, B.S. Weir, DNA commission of the International Society of Forensic Genetics: recommendations on the interpretation of mixtures, Forensic Sci. Int. 160 (2006) 90–101.

3. C. Phillips, L. Fernandez-Formoso, M. Garcia-Magarinos, L. Porras, T. Tvedebrink, J. Amigo, M. Fondevila, A. Gomez-Tato, J. varez-Dios, A. Freire-Aradas, A. Gomez-Carballa, A. Mosquera-Miguel, A. Carracedo, M.V. Lareu, Analysis of global variability in 15 established and 5 new European Standard Set (ESS) STRs using the CEPH human genome diversity panel, Forensic Sci. Int. Genet. 5 (2011) 155–169.
4. R. Green, J. Mulero, R. Lagace, W. Norona, C. Chang, N. Oldroyd, L. Hennessy, Developmental Validation of the AmpF*l*STR® NGM™ Kit, a Robust and Highly Discriminatory STR Multiplex, 2009, https://www3.appliedbiosystems.com/cms/groups/applied_markets_marketing/documents/generaldocuments/cms_073984.pdf.
5. A.S. Matai, J. Harteveld, T. Sijen, The Next Generation Multiplex (NGM™ Kit) in a Forensic Setting, Forensic News, 2010, http://marketing.appliedbiosystems.com/images/All_Newsletters/Forensic_0710/pdfs/Customer-Corner/nextGeneration. pdf?bcsi_scan_B0A38A178AE5B708=0&bcsi_scan_filename=nextGeneration.pdf.
6. C.C. Benschop, C.P. van der Beek, H.C. Meiland, A.G. van Gorp, A.A. Westen, T. Sijen, Low template STR typing: effect of replicate number and consensus method on genotyping reliability and DNA database search results, Forensic Sci. Int. Genet. 5 (2011) 316–328.
7. P. Gill, R. Puch-Solis, J. Curran, The low-template-DNA (stochastic) threshold—its determination relative to risk analysis for national DNA databases, Forensic Sci. Int. Genet. 3 (2009) 104–111.
8. P.S. Walsh, N.J. Fildes, R. Reynolds, Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA, Nucleic Acids Res. 24 (1996) 2807–2812.
9. A.J. Gibb, A.L. Huell, M.C. Simmons, R.M. Brown, Characterisation of forward stutter in the AmpF*l*STR SGM Plus PCR, Sci. Justice 49 (2009) 24–31.
10. C. Brookes, J.A. Bright, S. Harbison, J. Buckleton, Characterising stutter in forensic STR multiplexes, Forensic Sci. Int. Genet. 6 (2012) 58–63.
11. M. Klintschar, P. Wiegand, Polymerase slippage in relation to the uniformity of tetrameric repeat stretches, Forensic Sci. Int. 135 (2003) 163–166.
12. R.A. van Oorschot, K.N. Ballantyne, R.J. Mitchell, Forensic trace DNA: a review, Investig. Genet. 1 (2010) 14.
13. A.D. Kloosterman, P. Kersbergen, Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci, J. Soc. Biol. 197 (2003) 351–359.
14. A.A. Westen, J.H. Nagel, C.C. Benschop, N.E. Weiler, B.J. de Jong, T. Sijen, Higher capillary electrophoresis injection settings as an efficient approach to increase the sensitivity of STR typing, J. Forensic Sci. 54 (2009) 591–598.
15. N. Oldroyd, R. Green, J. Mulero, L. Hennessy, J. Tabak, Development of the AmpF*l*STR® NGM SElect™ Kit: New Sequence Discoveries and Implications for Genotype Concordance, Forensic News, 2011, http://www3.appliedbiosystems.com/cms/groups/applied_markets_marketing/documents/generaldocuments/cms_090694.pdf.
16. N.E. Weiler, A.S. Matai, T. Sijen, Extended PCR conditions to reduce drop-out frequencies in low template STR typing including unequal mixtures, Forensic Sci. Int. Genet. 6 (2012) 102–107.
17. C. Puers, H.A. Hammond, L. Jin, C.T. Caskey, J.W. Schumm, Identification of repeat sequence heterogeneity at the polymorphic short tandem repeat locus HUMTH01[AATG]n and reassignment of alleles in population analysis by using a locus-specific allelic ladder, Am. J. Hum. Genet. 53 (1993) 953–958.
18. J.M. Butler, STRBase, 2011, http://www.cstl.nist.gov/biotech/strbase/str_TH01.htm. Accessed July 2011.