Technical Focus

Considerations for the Evaluation of Plus Stutter for AmpFℓSTR® PCR Amplification Kits in Human Identification Laboratories

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AmpFℓSTR® PCR Amplification kits from Life Technologies use PCR (Polymerase Chain Reaction) to amplify short tandem repeat (STR) loci in multiplex assays. The STR fragments are visualized via capillary electrophoresis on Applied Biosystems Genetic Analyzers.

A new generation of AmpFℓSTR® kits (e.g. the Identifiler® Plus, Identifiler® Direct, NGM™ kits) has been developed to address requirements for more capable multiplex systems. These kits use modified PCR cycling conditions for enhanced sensitivity, a new buffer formulation to improve performance with inhibited samples and an improved process for DNA synthesis and purification of the amplification primers to deliver a much cleaner electrophoretic background.

Peaks other than the desired target alleles may be detected on the electropherogram and these are clearly described in the relevant AmpFℓSTR® kit User Guides. Examples of extra peaks include incomplete 3', a nucleotide addition (at the n-1 position), dye artifacts and stutter products. This article will only address stutter products and the current understanding of their formation and significance in commonly used AmpFℓSTR® kits.

STR motif structure and stutter type variation

To understand the mechanism of stutter formation further, we review the basic repeat motif classifications of STR loci, several types of which have been described[1]. In general terms, repeats may be classified as Simple, Compound or Complex motifs.

Simple: A single repeating sequence where the allele designation results directly from the number of repeats found in a relatively conserved four nucleotide motif. HUMTH01 is characterized by a repetitive series of [TCAT] sequences[1], however, some alleles may exhibit a loss of the leading thymine at a single repeat [TCAT]^4 CAT [TCAT]^5.

Compound: Two or more simple repeats that combine to create the repeat structure. An example of the vWA repeat motif has been reported[1] as the combination of primarily three simple repeats [ATCT]^2 [GTCT]$^{3-4}$ [ATCT]$^{9-13}$.

Complex: Repeats that may be comprised of variable length and/or sequence that combine to create the structure. An example of the D21S11 repeat motif has been reported[1] as a mixture of di-, tri-, tetra- and hexanucleotides repeat sequences.

Introduction to stutter products

The slipped strand mispairing model, the primary mechanism for the formation of stutter products in STR sequences, was first reported more than 20 years ago and offered as a theory for genetic evolution[2,3,4]. For Human Identification analysis, understanding the characteristics of stutter peaks is particularly relevant when samples contain DNA mixtures[5,6,7]. A stutter product is a well-characterized PCR artifact that appears as a minor peak one or more repeat units upstream or downstream from a parent allele in the electropherogram. The primary stutter for tetranucleotide repeat products occurs at a position one repeat unit smaller (n-4) than the major STR product[8]. Other stutter products, including smaller or larger fragments, may be observed including n-8, n+4, n+8, etc.[9] but these are more rare and may not always be detectable by current capillary electrophoresis instruments.

Figure 1

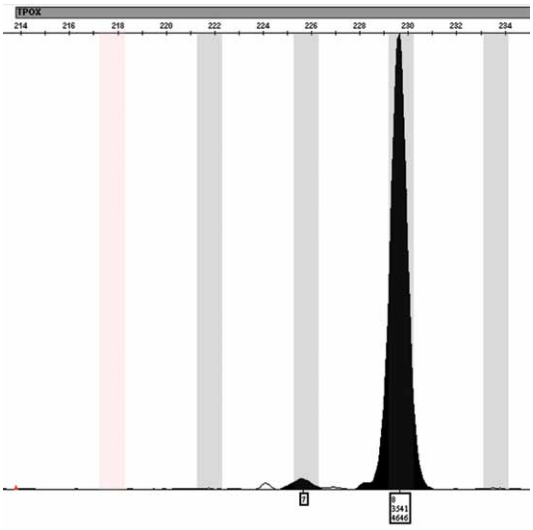


Figure 1. Demonstration of a stutter peak in relation to the parent allele (8) at the TPOX locus.

Sequence analysis of n-4 stutter products at tetranucleotide STR loci has revealed that the stutter product is missing a single tetranucleotide core repeat unit relative to the main allele[8]. The proportion of the stutter product relative to the main allele (stutter percentage) can be measured by dividing the height of the stutter peak by the height of the parent allele peak.

The types and levels of stutter seen within a profile are dependent upon many factors[10] including:

Repeat motif: Shorter repeat motifs (e.g. dinucleotide repeats GA_n) generally show higher levels of stutter than longer repeats (e.g. tetranucleotide repeats $GTCA_n$). Shorter repeats are also more likely to demonstrate multiple types of stutter (e.g. n-2, n-4, n+2, n+4).

Allele length: Alleles containing long, uninterrupted repeat sequences display higher levels of stutters than shorter alleles at the same locus. Long alleles containing interruptions to the repeat sequence will demonstrate lower than expected levels of stutter[8].

Reaction conditions: The specific balance of reagents within the reaction and the amount of template DNA used can affect the level of stutter observed. For example, higher levels of magnesium chloride are known to increase the amount of stutter formed[10].

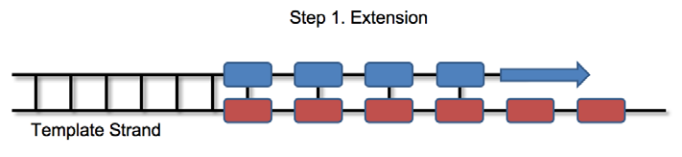
As a result, each individual locus exhibits a different stutter profile. It is also important to remember that stutter percentage cannot be measured accurately for allele peaks that are off-scale. Stutter may appear unusually high relative to the main peak when data is off-scale due to an underestimation of the quantity of the parent allele.

The cause of stutter

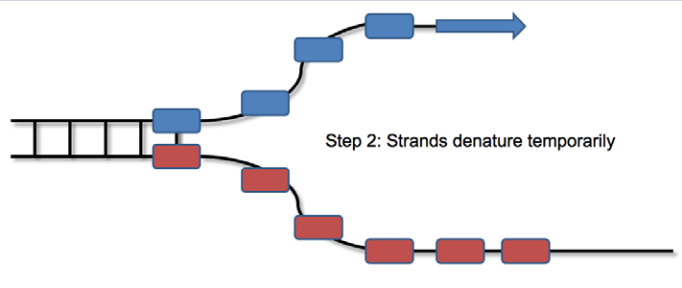
The predominant theory of stutter production is the strand slippage model[8,11]. During amplification, several enzyme molecules are required to form a single PCR product due to the limited processivity (number of bases added) of each enzyme molecule. As one enzyme molecule becomes exhausted and dissociates from the newly forming PCR product, the DNA strands are thought to separate. As a new enzyme molecule takes over, the DNA strands re-associate but due to the presence of repeated sequences, the strands may misalign creating PCR products which are either shorter or longer than the true allele by one or more repeat units[8].

The formation of plus or minus stutter is thought to depend upon which strand loops out during misalignment. Looping of the template strand results in minus stutter (generally observed to be the most common form of stutter), looping of the product strand results in plus stutter.

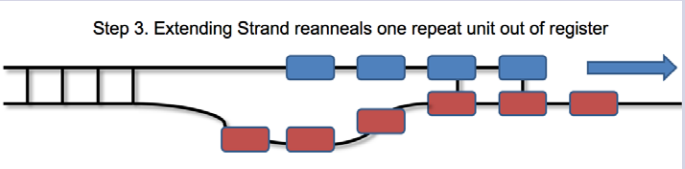
Step 1: The DNA polymerase has extended through four repeat units represented by the blue boxes.



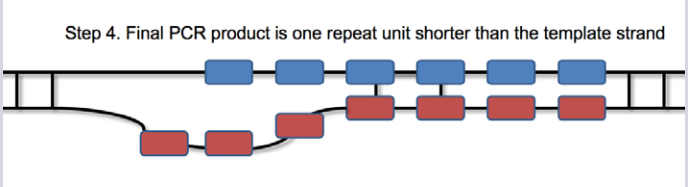
Step 2: Enzyme dissociates allowing the template and product strands to separate.



Step 3: Strands re-anneal but the template strand has looped out and the extending strand aligns out-of-register by one repeat unit.



Step 4: Final stutter product is formed.



Source: Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. Walsh PS, Fildes NJ, Reynolds R. Nucleic Acids Res. 1996 Jul 15;24(14):2807-12.

Whether the two strands re-anneal according to their original complement or out of register position may be influenced by the ability of either strand to accommodate the formation of the required loop. Therefore, the probability of occurrence of a slippage event should increase proportionally with repeat length (i.e. 15 repeat units would have a higher likelihood of slippage than 10 repeat units). Overall amplicon length (i.e. base pairs) has a minimal influence on the formation of the loop, as enzyme complex dissociation outside of the repeat cluster would not be expected to produce stutter[11].

Trinucleotide motifs in human identification testing

While most loci used in Human Identification testing utilize tetranucleotide repeat sequences, some AmpF*ℓ*STR® PCR Amplification kits utilize trinucleotide repeat sequences and valuable information regarding stutter products has been gained by performing investigations on these markers.

DYS392

The DYS392 locus is a simple trinucleotide [TAT_n] repeat locus on the Y chromosome and is contained in the Yfiler® kit. Amplification of the DYS392 locus typically results in three distinct detectable PCR products: the true allele product (n), a stutter product three bases smaller (n-3) and a reproducible low-level product three bases larger (n+3). Sequence analysis of the n+3 product demonstrated that its sequence is one TAT repeat longer than the true allele product. Experiments demonstrated that the quantity of both the n-3 and n+3 stutter product increased as the allele number increased. The stutter percentage also increased as magnesium concentration increased, and when the amount of input DNA decreased[10].

D22S1045

D22S1045 is an example of an autosomal trinucleotide locus and is used in the NGM™ and NGM SElect™ kits. It produces a higher level of both plus and minus stutter than tetranucleotide markers, and generally requires the use of n+3 stutter filters during data analysis. Filters for plus stutter are not generally required for tetranucleotide markers (for more information on the D22S1045 locus please refer to the *AmpF*ℓ*STR® NGM Select™ PCR Amplification Kit User Guide PN 4458841)*[12].

Comparing the stutter levels of the Identifiler®, Identifiler® Direct and Identifiler® Plus kits

One of the major improvements made to our Next Generation AmpF*ℓ*STR® systems (e.g. Identifiler® Direct, Identifiler® Plus, NGM™ and NGM SElect™ kits), is the redevelopment of the buffer system which included a re-optimization of the magnesium concentration. Magnesium is required to enable the amplification

Figure 2

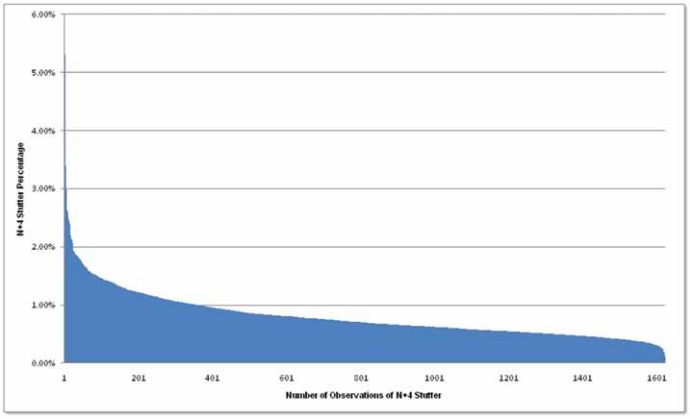


Figure 2. Distribution of observations of peaks in the n+4 stutter position for 200 samples amplified using the Identifiler® Plus kit.

Figure 3

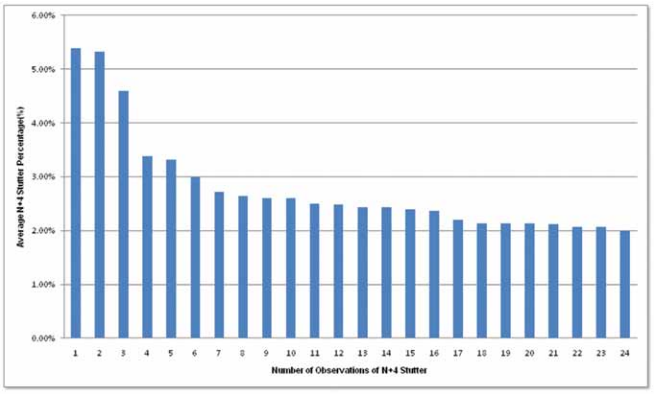


Figure 3. The 24 highest observed n+4 stutter position peaks, out of 1621 total, amplified using the Identifiler® Plus kit. This represents all instances where the n+4 stutter exceeded 2%.

Figure 4

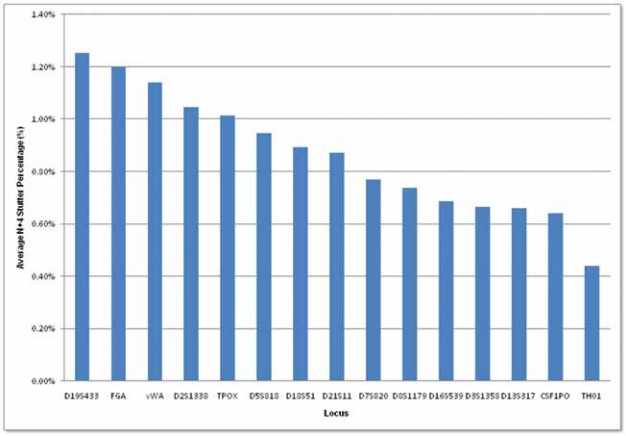


Figure 4. Average n+4 stutter percentage results per locus for peaks observed in the n+4 stutter position for 200 samples amplified using the Identifiler® Plus kit.

to function correctly but the concentration needs to balance the promotion of maximum amplification efficiency while minimizing the negative effects of high concentration such as reduced specificity. While the Identifiler® Direct and Identifiler® Plus kits have the same primer binding sequences as the Identifiler® kit, they contain a higher concentration of magnesium. In general, higher concentrations of magnesium result in higher stutter percentages thought to result from a lowered binding stringency allowing more efficient extension of misaligned DNA strands after strand slippage events. In our next generation AmpF*ℓ*STR® products, we balance the magnesium concentration to gain maximum improvement in amplification efficiency while minimizing the increase in stutter. However, due to the higher concentration of magnesium used, it is expected that all our next generation kits are expected to produce slightly higher levels of stutter than older kits such as the Identifiler® or SGM Plus® kits.

Identifiler® Plus kit stutter study

While the occurrence of stutter is extremely well characterized and documented, this section will describe data analysis performed on a small data set of anonymous donors to demonstrate the various traits exhibited by stutter at tetranucleotide loci in STR multiplex assays.

Materials and methods

Genomic DNA from 200 anonymous donors was amplified using the Identifiler® Plus kit targeting an input DNA concentration of 1 ng for 28 cycles. Electrophoresis was carried out according to manufacturer recommendations on a 3130*xl* Genetic Analyzer and data analyzed using GeneMapper® *ID-X* Software v1.2 with a peak detection threshold of 10 RFU to capture low level stutter products. Data was exported into Microsoft® Excel® software and screened for samples showing peaks in the n+4 stutter position. Data was then screened to remove examples where the peak could be attributed to, or contain contributions from, electrophoresis artifacts such as pull-up, instrument noise or other profile anomalies. The subsequent population of peaks was submitted for further analysis.

Table 1

Locus	Ave	Std Dev	Count	Max	Min
D19S433	1.25	1.28	19	5.38	0.28
FGA	1.20	0.49	87	3.32	0.46
vWA	1.14	0.76	39	4.59	0.32
D2S1338	1.05	1.61	9	5.31	0.22
TPOX	1.01	0.49	38	1.92	0.24
D5S818	0.95	0.34	142	2.60	0.41
D18S51	0.89	0.42	101	2.63	0.40
D21S11	0.87	0.30	224	2.50	0.39
D7S820	0.77	0.39	122	2.72	0.36
D8S1179	0.74	0.30	207	2.12	0.35
D16S539	0.69	0.29	162	2.13	0.24
D3S1358	0.66	0.36	151	2.01	0.19
D13S317	0.66	0.24	164	1.70	0.13
CSF1PO	0.64	0.25	129	1.93	0.30
TH01	0.44	0.17	27	0.75	0.12

Table 1. Average, Standard Deviation, Count and Min/Max stutter percentage values for peaks observed in the n+4 stutter position for 200 samples amplified using the Identifiler® Plus kit.

Note: The methods used to calculate n-4 stutter percentages used as filters in the GeneMapper® *ID* and GeneMapper® *ID-X* Software changed with the next-generation kit advancements and values presented in the Identifiler® kit User Guide are not directly comparable to the values presented in the Identifiler® Direct and Identifiler® Plus kit User Guides.

Results

Following data screening, 1,621 peaks in the n+4 position were evaluated for stutter percentage. The following figures represent the expected performance of n+4 stutter using the Identifiler® Plus kit.

Discussion

Only 1.4% (24 out of 1,691) of the data points produced peaks in the n+4 stutter position greater than 2% that were not the result of other known artifacts such as pull up. A n+4 stutter of 2% represents a 100 RFU stutter peak for a corresponding parent peak of 5,000 RFU, which is below many commonly used stochastic or analytical thresholds. Proper characterization of the levels of n+4 stutter through internal laboratory validation studies should allow laboratories to interpret plus stutter in the same way as minus stutter is handled currently and should not pose significant disruption to the data analysis and interpretation process.

Conducting a Stutter Study: Data Analysis Considerations

When analyzing data for any form of stutter, it is important to consider artifacts and anomalies which could artificially inflate the stutter percentage observed. Some common pitfalls that may be encountered and suggested remedies are:

- Contribution of pull up to the height of a peak in the stutter position
- Examine other dye channels to see if the questioned peak is in line with signal from another dye set in the same sample
- Overall baseline noise
- Examine the data to see if the peak shape is consistent with the expected Gaussian distribution generally observed with high-quality data
- Expecting the stutter percentage to be the same for both alleles in a heterozygote
- Examine your known data and find examples where one allele shows a level of stutter that is significantly different from the corresponding allele in a heterozygote

Example A. Contribution of pull up to the height of a peak in the n+4 stutter position

In this example, upon first examination, it appears that the 14.2 allele has generated a high amount of n+4 stutter (10.77%) at the D19S433 locus.

Figure 5

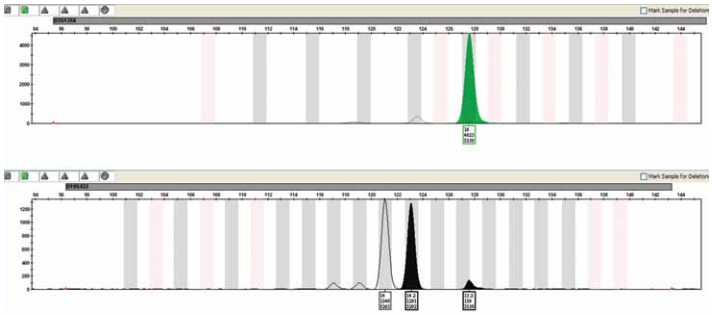


Figure 5. Example of inflated signal in the yellow dye channel due to high signal in the green dye channel at D19S433.

However, upon closer examination, it is evident that the height of this peak is artificially inflated due to a peak in the same position in the green dye channel. Therefore, the true peak height of the actual n+4 artifact is unknown.

Example B. Overall baseline noise

While Example A shows the contribution to stutter from another dye channel, in other circumstances the effect may be more subtle and simply result in deformation of the expected peak shape. While the peak may be labeled, and may fall in a stutter position, users should be cautious if the peak shape is amorphous. A good guide would be to examine the peak shape of the other stutter peaks for comparison. In the following example, although the FGA 21 allele (red) falls within the n+4 stutter region, it has a deformed appearance when compared to the n-4 stutter from the same allele.

Figure 6

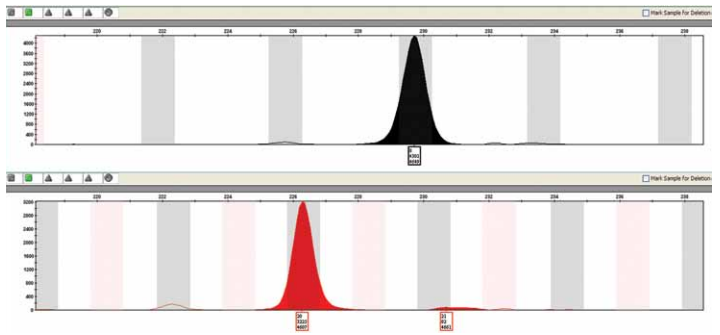


Figure 6. Example of peak deformation at the n+4 stutter position possibly due to high signal in the yellow dye channel at FGA.

Example C. Expecting the stutter percentage to be the same for both alleles in a heterozygote

This example demonstrates the level of variation that can be observed when examining stutter ratios.

At the D21S11 locus, the 30 allele produces a peak in the n+4 stutter position at approximately 2.5% while the associated n+4 stutter corresponding with the 31.2 allele is at a lower level of only 0.77%.

Figure 7

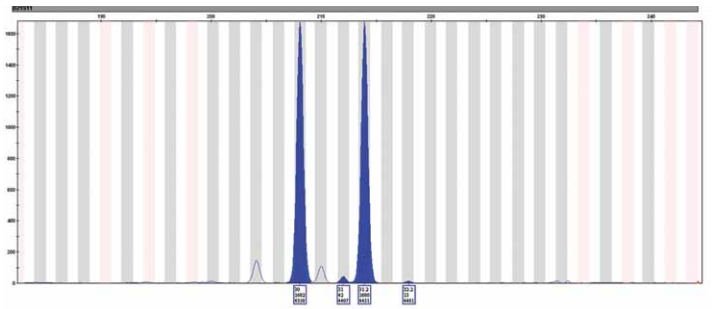


Figure 7. Example of variation in the relative n+4 stutter percentages for heterozygotes at D21S11.

Stutter anomalies

Although stutter peaks generally conform to a well documented set of characteristics, there may be exceptions to the accepted norm. For example, during testing of a subset of our population samples, a particular pattern was recognized in a sample of Korean descent.

When amplified with the Identifiler® Plus kit, the sample produced an n+4 stutter of approximately 5.3% for allele 17 at the D2S1338 locus. The partner allele of the heterozygote (24) generated no detectable n+4 stutter product (Figure 8).

Figure 8

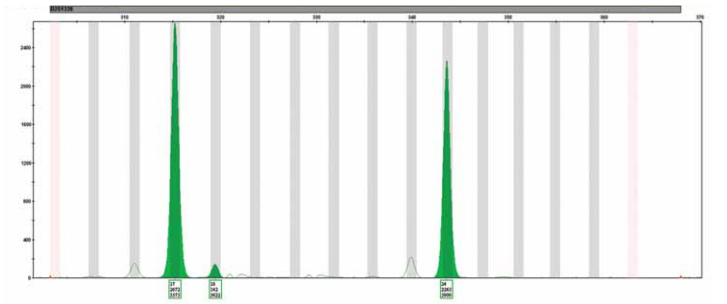


Figure 8. Example of the variation in the relative n+4 stutter percentages for a Korean population sample for allele 17 at the D2S1338 locus with the AmpFℓSTR® Identifiler® Plus PCR Amplification kit.

The same sample was amplified with the NGM SElect™ kit (which also contains the D2S1338 locus). The result confirmed the presence of a peak in the n+4 position relative to the 17 allele and also showed the presence of a peak in the n+4 position for the 24 allele (Figure 9).

Figure 9

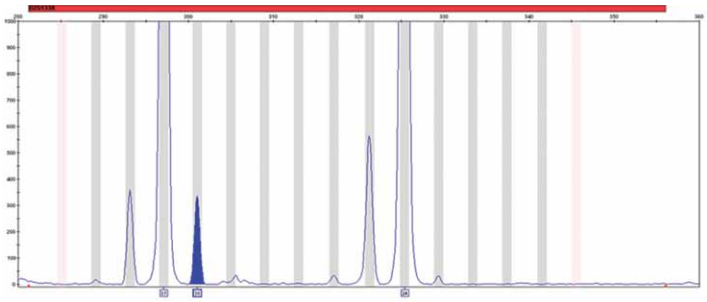


Figure 9. Example of the variation in the relative n+4 stutter percentages for a Korean population sample for allele 17 at the D2S1338 locus with the NGM SElect™ kit.

As described previously, the correlation of stutter products with magnesium concentration is well known. However, this sample was tested with multiple kits which employ different concentrations of magnesium chloride and the n+4 stutter percentages did not correlate to the known concentration of magnesium in the kits. The n-4 stutter percentages did demonstrate a direct correlation to magnesium chloride concentration.

Table 2

AmpFℓSTR® Kit	n+4 Stutter %	n-4 Stutter %
Identifiler® Kit	0.6	4.4
SGM Plus® Kit	3.7	4.9
SEfiler Plus™ Kit	5.7	5.6
MiniFiler™ Kit	4.3	6.1
Identifiler® Plus Kit	5.3	5.8
NGM SElect™ Kit	5.3	5.7

Table 2. Stutter percentage results at the D2S1338 locus from testing the questioned Korean Population sample using multiple STR kits.

All kits have the same primer sequences for the D2S1338 locus except the AmpFℓSTR® MiniFiler™ PCR Amplification kit which amplifies shorter amplicons and uses primers situated closer to the repeat region. The n-4 stutter percentage remained relatively constant while the percentage of the peak in the n+4 position varied across multiple assays, multiple magnesium concentrations and different primer sequences. The fact that the peak in the n+4 stutter position does not display expected stutter behavior suggests that this peak is not a genuine stutter product and is instead caused by another mechanism.

Further investigation supports the hypothesis that this artifact was not a stutter product from the 17 allele. Amplification of mini-preps prepared from the 17 and 24 alleles only demonstrated the artifact peak in the 18 position using the 24 allele as a template. It is possible that this peak originated due to somatic mutation or may be explained by anomalous migration of an alternate structure of the 24 allele, however the exact cause cannot be confirmed at this time.

Conclusions

Stutter products have been known to exist since STRs were first used in Human Identification testing in the mid 1990s. Laboratories have developed methods for characterizing stutter and incorporating the existence of stutter in standard operating procedures.

While advancements in STR technology produce more robust chemistries, a possible side effect may be a slight increase in the formation of stutter products. Many factors contribute to the level of stutter observed, several of which have been described here. Advancements in STR technology produce more robust chemistries but a possible side effect may be a slight increase in the formation of stutter products. Laboratories should therefore expect to see slightly higher levels of minus stutter and a slightly higher incidence of plus stutter in data generated with Next Generation STR kits. Rare mutations may occur that cause unexpectedly high or low levels of stutter for particular samples but careful investigations of the characteristics of the sample profile will identify such anomalies and distinguish them from samples exhibiting normal stutter behaviors.

Laboratories are encouraged to continue to perform stutter studies during internal validations to properly characterize and understand the position and percentage of stutter products commonly seen for the workflow specific to their laboratory.

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