CHAPTER 6

BIOLOGY OF STRs: STUTTER PRODUCTS, NON-TEMPLATE ADDITION, MICROVARIANTS, NULL ALLELES AND MUTATION RATES

> The most humbling aspect of the Human Genome Project so far has been the realization that we know remarkably little about what the vast majority of human genes do.
>
> (James Watson, *DNA: The Secret of Life*, 2003, p. 217)

During polymerase chain reaction (PCR) amplification of short tandem repeat (STR) alleles, a number of artifacts can arise that may interfere with the clear interpretation and genotyping of the alleles present in the DNA template. In this chapter, we will focus on those PCR products that give rise to additional peaks besides the true, major allele peak(s). These artifacts include stutter products and non-template nucleotide addition. Other factors that impact STR typing, including microvariants, tri-allelic patterns, allele dropout, and mutations will also be covered.

STUTTER PRODUCTS

A close examination of electropherograms containing STR data typically reveals the presence of small peaks several bases shorter than each STR allele peak (Figure 6.1). These 'stutter product' peaks result from the PCR process when STR loci are copied by a DNA polymerase. In the literature, this stutter product has also been referred to as a shadow band or a DNA polymerase slippage product (Hauge and Litt 1993).

Sequence analysis of stutter products from the tetranucleotide repeat locus VWA has shown that they contain one repeat unit less than the corresponding main allele peak (Walsh *et al.* 1996). Stutter products that are larger in size by one repeat unit than the corresponding alleles are only rarely observed in commonly used tetranucleotide repeat STR loci.

Stutter products have been reported in the literature since STRs (microsatellites) were first described. The primary mechanism that has been proposed to explain the existence of stutter products is slipped-strand mispairing (Hauge and Litt 1993, Walsh *et al.* 1996). In the slipped-strand mispairing model, a region of primer-template complex becomes unpaired during primer extension allowing slippage of either primer or template strand such that one repeat forms a non-base-paired loop (Hauge and Litt 1993). The consequence of this

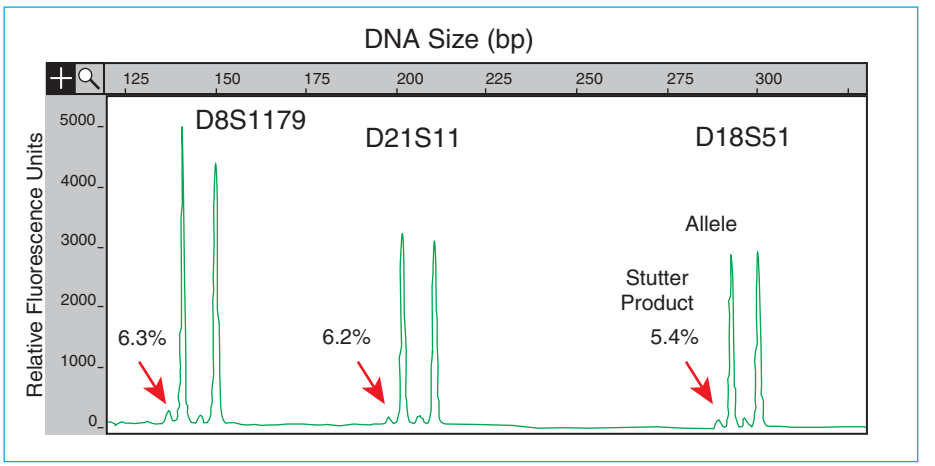

Figure 6.1
STR alleles shown with stutter products (indicated by arrows). Only the stutter percentage for the first allele from each locus is noted.

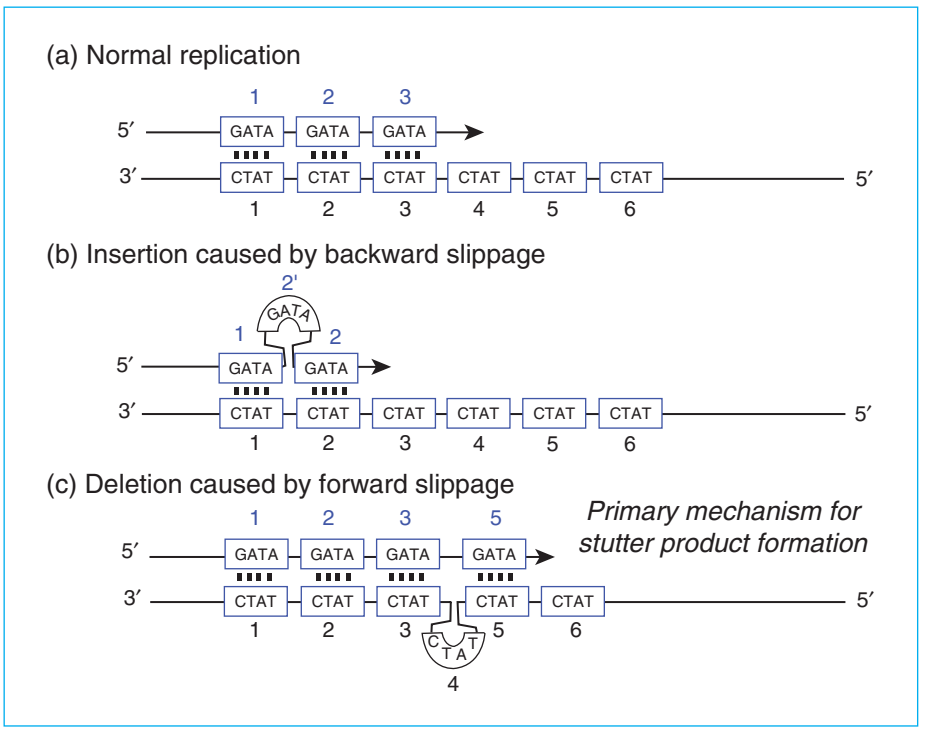

Figure 6.2
Illustration of slipped-strand mispairing process that is thought to give rise to stutter products. (a) During replication the two DNA strands can easily come apart in the repeat region and since each repeat unit is the same, the two strands can re-anneal out of register such that the two strands are off-set by a single repeat unit. (b) If a repeat unit bulges out on the new synthesized strand during extension then an insertion results in the next round of amplification. (c) If on the other hand, the repeat unit bulge occurs in the template strand, then the resulting synthesized strand is one repeat unit shorter than the full length STR allele. The frequency at which this process occurs is related to the flanking sequence, the repeat unit, and the length of the allele being amplified. Generally for tetranucleotide STR loci, stutter occurs less than 15% of the time and is observed as a small peak one repeat shorter than the STR allele.

one repeat loop is a shortened PCR product that is less than the primary amplicon (STR allele) by a single repeat unit (Figure 6.2).

IMPACT OF STUTTER PRODUCTS ON DATA INTERPRETATION

Stutter products impact interpretation of DNA profiles, especially in cases where two or more individuals may have contributed to the DNA sample (see Chapter 7).

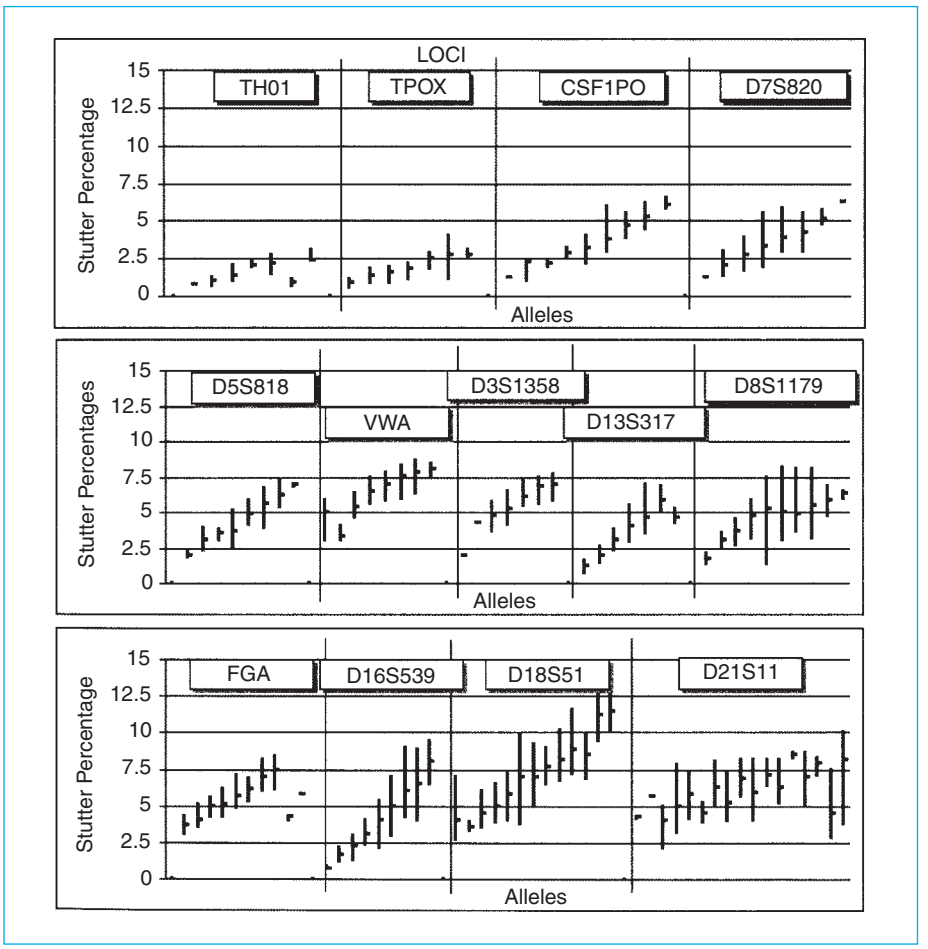

Figure 6.3

Stutter percentages for 13 CODIS STR loci (data adapted from AmpFlSTR manuals). Alleles for each STR locus are shown from smallest to largest. Each locus has a different average stutter percentage but all loci show the trend of increasing stutter with larger alleles (longer number of repeats).

Because stutter products are the same size as actual allele PCR products, it can be challenging to determine whether a small peak is a real allele from a minor contributor or a stutter product of an adjacent allele.

Mixture interpretation requires a good understanding of the behavior of stutter products in single source samples. Often a laboratory will quantify the percentage of stutter product peak heights compared to their corresponding allele peak heights. The percentage of stutter product formation for an allele is determined simply by dividing the stutter peak height by the corresponding allele peak height.

A plot of the alleles from each of the 13 standard STR loci reveals variation in the percentage of stutter for each locus as well as alleles from the same locus (Figure 6.3). Such a plot illustrates several important principles. First, each locus has a different amount of stutter product formation. Second, longer alleles for a STR locus exhibit a greater degree of stutter than smaller alleles for the same locus. Third, stutter percentage with the standard tetranucleotide

repeats is generally less than 15% for all 13 CODIS core STR loci under standard amplification conditions.

REDUCED STUTTER PRODUCT FORMATION

The amount of stutter product formation may be reduced when using STR markers with longer repeat units, STR alleles with imperfect repeat units, and DNA polymerases with faster processivity. Several pentanucleotide repeat loci have been developed in an effort to produce STR markers that exhibit low amounts of stutter products to aid in mixture interpretation (Bacher and Schumm 1998). The first seven loci have been labeled Penta A through Penta G. Penta E has been incorporated in the GenePrint® PowerPlex™ 2.1 system and reportedly exhibits an average stutter percentage of less than 1% (Bacher *et al.* 1999). Both Penta D and Penta E are part of the PowerPlex® 16 kit (Krenke *et al.* 2002).

Alleles for a STR locus that contain variations on the common repeat motif exhibit a smaller amount of stutter product formation. For example, the common repeat motif for the STR marker TH01 is AATG. However, with allele 9.3, there is an ATG nucleotide sequence present in the middle of the repeat region (Puers *et al.* 1993). When the core repeat sequence has been interrupted, stutter product formation is reduced compared to alleles that are similar in length but possess uninterrupted core repeat sequences. This fact has been demonstrated with sequencing results from several VWA alleles (Walsh *et al.* 1996).

The amount of stutter may be related to the DNA polymerase processivity, or how rapidly it copies the template strand. Stutter products have been shown to increase relative to their corresponding alleles with a slower polymerase (Walsh *et al.* 1996). If thermal stable DNA polymerases become available in the future that are faster than the current 50–60 base processivity of *Taq* DNA polymerase, then it may be possible to reduce the stutter product formation. A faster polymerase would be able to copy the two DNA strands before they could come apart and re-anneal out of register during primer extension (see Figure 6.2).

A summary of stutter product formation is listed below:

- Primarily one repeat unit smaller than corresponding main allele peak;
- Typically less than 15% of corresponding allele peak height;
- Quantity of stutter depends on locus as well as PCR conditions and polymerase used;
- Propensity of stutter decreases with longer repeat units (pentanucleotide repeats<tetra-<tri-<dinucleotides);
- Quantity of stutter is greater for large alleles within a locus;
- Quantity of stutter is less if sequence of repeats is imperfect.

NON-TEMPLATE ADDITION

DNA polymerases, particularly the *Taq* polymerase used in PCR, often add an extra nucleotide to the 3′-end of a PCR product as they are copying the template strand (Clark 1988, Magnuson *et al.* 1996). This non-template addition is most often adenosine and is therefore sometimes referred to as 'adenylation' or the '+A' form of the amplicon. Non-template addition results in a PCR product that is one base pair longer than the actual target sequence.

Addition of the 3′ A nucleotide can be favored by adding a final incubation step at 60°C or 72°C after the temperature cycling steps in PCR (Clark 1988, Kimpton *et al.* 1993). However, the degree of adenylation is dependent on the sequence of the template strand, which in the case of PCR results from the 5′-end of the reverse primer (Figure 6.4). If the forward primer is labeled with

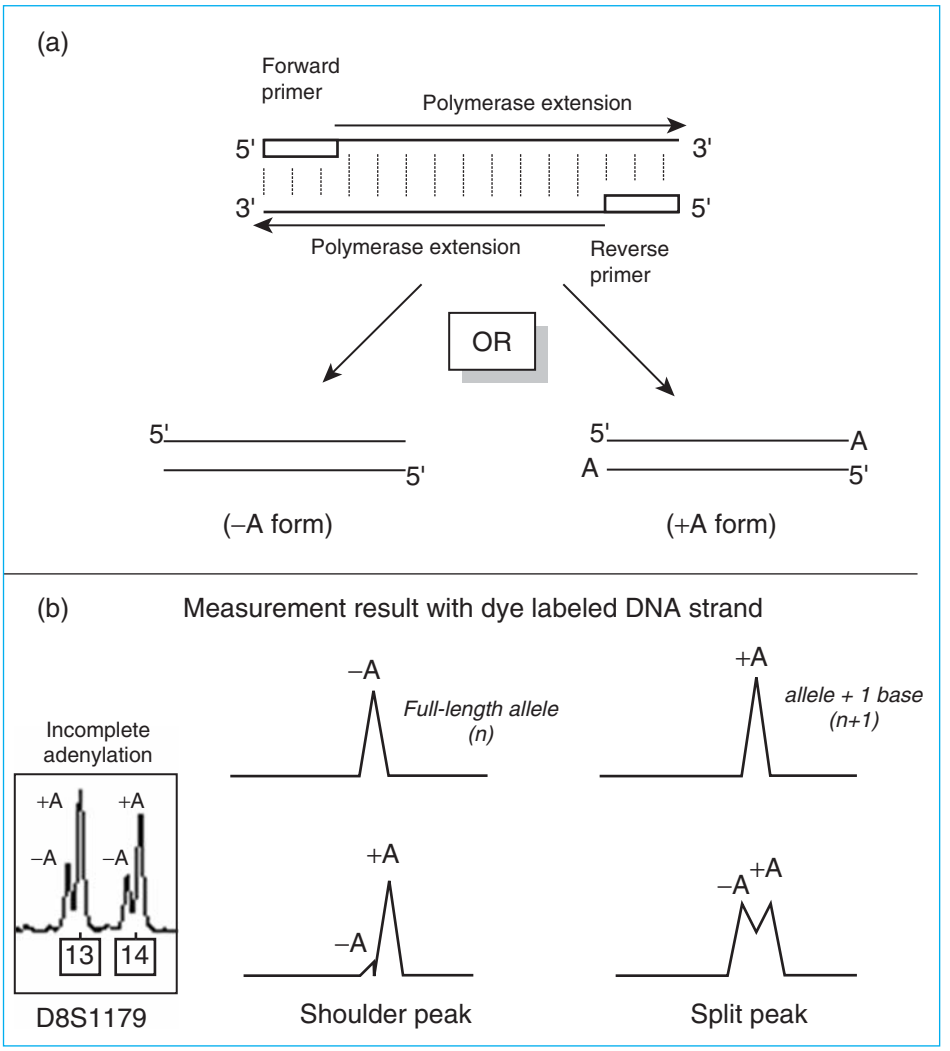

Figure 6.4

Schematic of non-template nucleotide addition shown (a) with illustrated measurement result (b). DNA polymerases add an extra nucleotide beyond the 3′-end of the target sequence extension product. The amount of non-template addition is dependent on the sequence of the 5′-end of the opposing primer. In the case of dye labeled PCR products where the fluorescent dye is on the forward primer, the reverse primer sequence is the critical one.

a fluorescent dye to amplify the STR allele, then only the top strand is detected by the fluorescent measurement. Since the sequence at the 3′-end of the top (labeled) strand serves as a template for polymerase extension, the terminal nucleotide of the labeled strand is determined by the 5′-end of the reverse primer used in generating the complementary unlabeled strand (Magnuson *et al.* 1996). One study found that if the 5′-terminus of the primer is a guanosine, then a complete addition is favored by the polymerase (Brownstein *et al.* 1996). Thus, every locus will have slightly different adenylation properties because the primer sequences differ.

Now why is all of this important? From a measurement standpoint, it is better to have all of the molecules as similar as possible for a particular allele. Partial adenylation, where some of the PCR products do not have the extra adenine (i.e., –A peaks) and some do (i.e., +A peaks), can contribute to peak broadness if the separation system's resolution is poor. Sharper peaks improve the likelihood that a system's genotyping software can make accurate calls. In addition, variation in the adenylation status of an allele across multiple samples can have an impact on accurate sizing and genotyping potential microvariants. For example, a non-adenylated TH01 10 allele would be the same size as a fully adenylated TH01 9.3 allele because they contain an identical number of base pairs. Therefore, it is beneficial if all PCR products for a particular amplification are either +A or –A rather than a mixture of +/–A products. Table 6.1 lists some of the methods that have been used to convert PCR products into either the –A or +A form.

Table 6.1
Ways to convert STR allele peaks to either –A or +A forms.

Method	Result	Reference
Conversion to fully adenylated products (+A form)		
Final extension at 60°C or 72°C for 30–45 minutes	Promotes full adenylation of all products	Kimpton *et al*. 1993, Applied Biosystems 1999
Addition of sequence GTTTCTT on the 5′-end of reverse primers ('PIG-tailing')	Promotes nearly 100% adenylation of the 3′ forward strand	Brownstein *et al*. 1996
Conversion to blunt-ended products (–A form)		
Restriction enzyme site built into reverse primer	Makes blunt end fragments following restriction enzyme digestion	Edwards *et al*. 1991
Enzymatic removal of one base overhang	Exonuclease activity of *Pfu* or T4 DNA polymerase removes +A	Ginot *et al*. 1996
Use of modified polymerase without terminal transferase activity	Polymerase does not add 3′ A nucleotide	Butler and Becker 2001

During PCR amplification most STR protocols include a final extension step to give the DNA polymerase extra time to completely adenylate all double-stranded PCR products. For example, the standard AmpFℓSTR® kit amplification parameters include a final extension at 60°C for 45 minutes at the end of thermal cycling (Applied Biosystems 1999). In order to make correct genotype calls, it is important that the allelic ladder and the sample have the same adenylation status for a particular STR locus. For all commercially available STR kits, this means that the STR alleles are all in the +A form.

Amplifying higher quantities of DNA than the optimal amount suggested by the manufacturer's protocols can result in incomplete 3′ A nucleotide addition and therefore split peaks. The addition of 10 ng of template DNA to a PCR reaction with AmpFℓSTR Profiler Plus results in split peaks compared to using only 2 ng of the same template DNA (Figure 6.5). Thus, quantifying the amount of DNA prior to PCR and adhering to the manufacturer's protocols will produce improved STR typing results with using commercial STR kits.

MICROVARIANTS AND 'OFF-LADDER' ALLELES

Rare alleles are encountered in the human population that may differ from common allele variants at tested DNA markers by one or more base pairs. Sequence variation between STR alleles can take the form of insertions, deletions, or nucleotide changes. Alleles containing some form of sequence variation compared to more commonly observed alleles are often referred to as *microvariants* because they are only slightly different from full repeat alleles. Because microvariant alleles often do not size the same as consensus alleles present in the reference allelic ladder, they can be referred to as 'off-ladder' alleles.

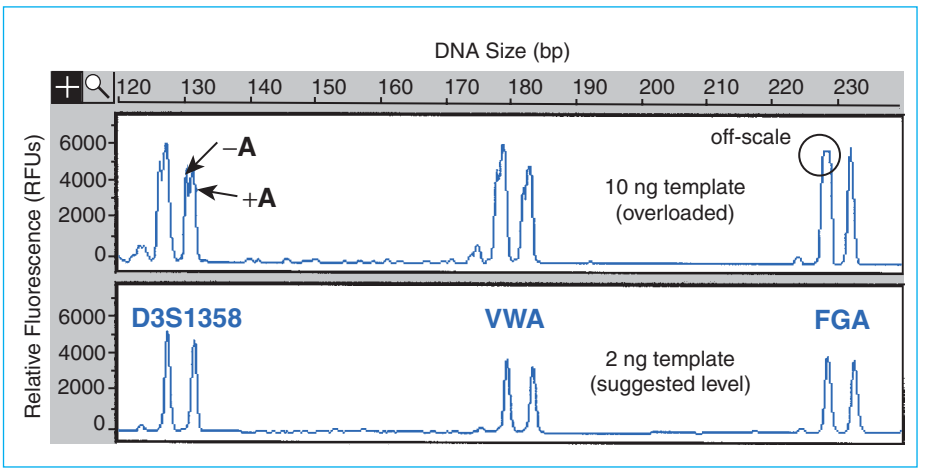

Figure 6.5

Incomplete non-template addition with high levels of DNA template. In the top panel, partial adenylation (both −A and +A forms of each allele) is seen because the polymerase is overwhelmed due to an abundance of DNA template. Note also that the peaks in the top panel are off-scale and flat-topped in the case of the smaller FGA allele. When the suggested level of DNA template is used, all alleles are fully adenylated (bottom panel).

One example of a common microvariant is allele 9.3 at the STR locus TH01. The repeat region of TH01 allele 9.3 contains nine full repeats (AATG) and a partial repeat of three bases (ATG). The 9.3 allele differs from the 10 allele by a single base deletion of adenine in the seventh repeat (Puers *et al.* 1993).

Microvariants exist for most STR loci and are being identified in greater numbers as more samples are being examined around the world. In a recent study, 42 apparent microvariants were seen in over 10 000 samples examined at the CSF1PO, TPOX, and TH01 loci (Crouse *et al.* 1999). Microvariants are most commonly found in more polymorphic STR loci, such as FGA, D21S11, and D18S51, that possess the largest and most complex repeat structures compared to simple repeat loci, such as TPOX and CSF1PO (see Appendix I).

DETERMINING THE PRESENCE OF A MICROVARIANT ALLELE

Suspected microvariants can be fairly easily seen in heterozygous samples where one allele lines up with the fragment sizes in the allelic ladder and one does not (Figure 6.6). In the example shown here, the sample contains a peak that lines up with allele 25 from the FGA allelic ladder and a second peak that is labeled

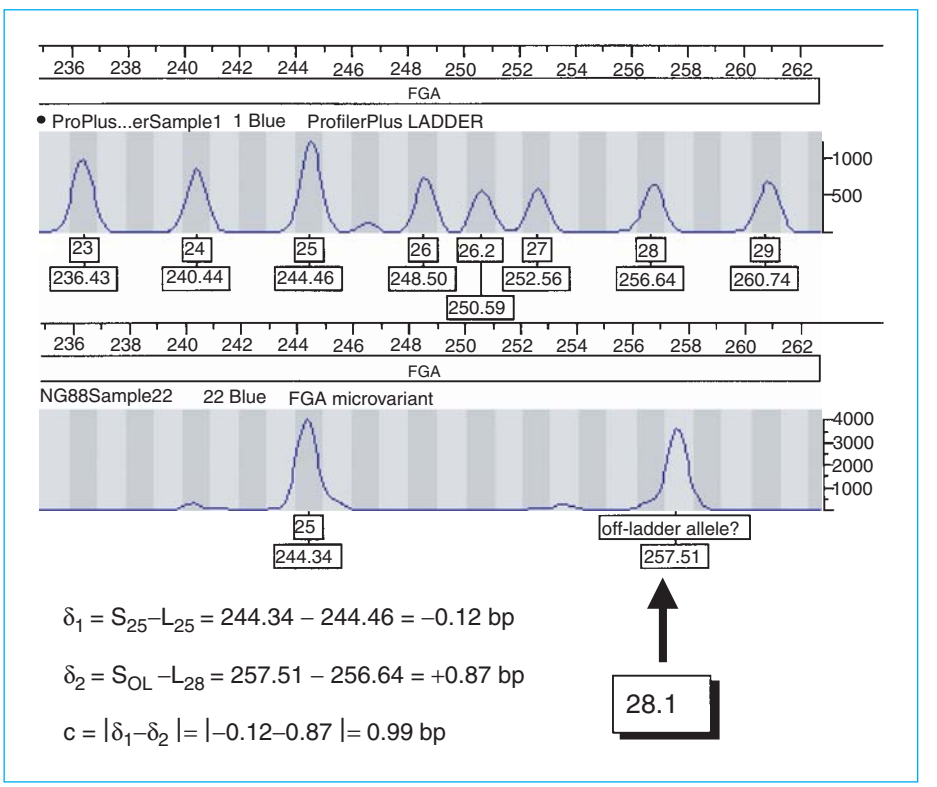

Figure 6.6

Detection of a microvariant allele at the STR locus FGA. The sample in the bottom panel is compared to the allelic ladder shown in the top panel using Genotyper 2.5 software. Peaks are labeled with the allele category and the calculated fragment sizes using the internal sizing standard GS500-ROX.

as an 'off-ladder allele' and lines up between the 28 and 28.2 shaded virtual bins created by the ladder. Each peak is labeled with its calculated size in base pairs determined by reference to the internal GS500 sizing standard (see Chapter 15). The relative size difference between the questioned sample and an allelic ladder marker run under the same electrophoretic conditions is then used to determine if the allele is truly a microvariant (Gill *et al.* 1996).

In Figure 6.6, the size difference between the sample allele 25 and the ladder allele 25 is –0.12 bp (δ_1) while the 'off-ladder allele' differs from the ladder allele 28 by +0.87 bp (δ_2). The relative peak shift between the two alleles in this heterozygous sample is 0.99 bp ($|\delta_1 - \delta_2|$) and therefore the 'off-ladder' allele is 1 bp larger than allele 28 making it a true 28.1 microvariant at the FGA locus.

The presence of a STR microvariant at a particular locus usually becomes evident following a comparison to an allelic ladder made up of characterized alleles for that locus. However, not all alleles (particularly rare microvariant alleles) can be incorporated into the standard allelic ladder used for genotyping STR markers. Therefore, interpolation of data from peaks that migrate between two characterized alleles or extrapolation of data from peaks that fall outside the expected allele range may be performed. Caution is in order though if 'off-ladder' alleles are more than a one or two repeats away from the nearest allele in the ladder since tetranucleotide repeats do not always size exactly 4.0 bp apart (see Gill *et al.* 1996, Applied Biosystems 1999, Butler *et al.* 2004).

If an allele peak falls in between the nominal alleles present in the allelic ladder, the sample may be designated by the allele number followed by a '.*x*' (Crouse *et al.* 1999). For example, the larger FGA allele shown in Figure 6.6 would be designated as a '28.*x*' allele. However, it is more common to label variant alleles by their calculated repeat content (e.g., 28.1). If an allele migrates above or below the defined allelic ladder, the allele is described as '>' or '<' than the nearest allele (Crouse *et al.* 1999).

SAME LENGTH BUT DIFFERENT SEQUENCE ALLELES

Complex repeat sequences, such as those found in D21S11, can contain variable repeat blocks in which the order is switched around for alleles that are the same length. For example, the STR locus D21S11 has four alleles that are all 210 bp when amplified with the Profiler Plus™ kit (Appendix I). While these alleles would be sized based on overall length to be 'allele 30', they contain repeat blocks of 4-6-CR-12, 5-6-CR-11, 6-5-CR-11, and 6-6-CR-10 for the pattern [TCTA]-[TCTG]-constant region (CR)-[TCTA]. In such cases, variant alleles would only be detectable with complete sequence analysis.

It is important to realize that from an operational point of view internal allele variation is not significant. In the end a match is being made against many loci not just one, such as D21S11, with possible internal sequence variation.

Most of the STR loci used in human identity testing have not exhibited internal sequence variation (see Appendix I), particularly the simple repeat loci TPOX, CSF1PO, D5S818, D16S539, TH01, D18S51, and D7S820. Remember that we are essentially binning alleles based on measured size anyway with STR typing since sequence analysis of individual alleles is too time consuming and would rarely reveal additional information because STR variation is primarily size-based.

PEAKS OUTSIDE THE ALLELIC LADDER RANGE AND THREE-BANDED PATTERNS

Occasionally new rare alleles may fall outside the allele range spanned by the locus allelic ladder. If these peaks fall between two STR loci in a multiplex set, they can be challenging to assign to a particular locus unless testing is performed with individual locus-specific primer sets or a different multiplex. These extreme 'off-ladder' alleles can be confirmed with singleplex amplification of the two loci in the multiplex bracketing the new allele. Alternatively the sample could be amplified again using a separate multiplex where the loci are present in a different order. For example, if a PCR product was observed between the typical VWA and D16S539 allele ranges when using the SGM Plus kit, then it could be either a large VWA allele or a small D16S539 allele, which is doubtful because allele 5 is the smallest run on the D16S539 ladder. A different kit, such as PowerPlex 16, could be used on this same sample to help address the source of the new allele since the loci are put together in a different combination. With PowerPlex 16 a large VWA allele would appear between the VWA and D8S1179 expected allele ranges, but a small D16S539 allele would fall between D7S820 and D16S539 (see Figure 5.4).

Three-banded or tri-allelic patterns are sometimes observed at a single locus in a multiplex STR profile. These extra peaks are not a result of a mixture but are reproducible artifacts of the sample. Extra chromosomal occurrences or primer point mutations have been known to happen and result in a three-banded pattern (Crouse *et al.* 1999). For example, three-banded patterns have been observed in the 9948 cell line with CSF1PO and in the K562 cell line with D21S11 (see D.N.A. Box 7.1).

The three peaks or bands seen at a particular locus may or may not be equal in intensity. While the TPOX three-banded patterns reported by Crouse and co-workers (1999) were approximately equal in intensity (similar to Figure 6.7a), there are also occasions when tri-allelic patterns occur with peaks of unequal intensity. In Figure 6.7b, a sum of two of the alleles is approximately equivalent in amount as the third allele as seen in Figure 6.7b for D18S51. The peak heights for alleles 14 and 15 when added together are similar to amount as the D18S51 allele 22. Thus, it is likely that the 14 and 15 alleles came from one parent while the 22 repeat allele came from the other. There is probably some

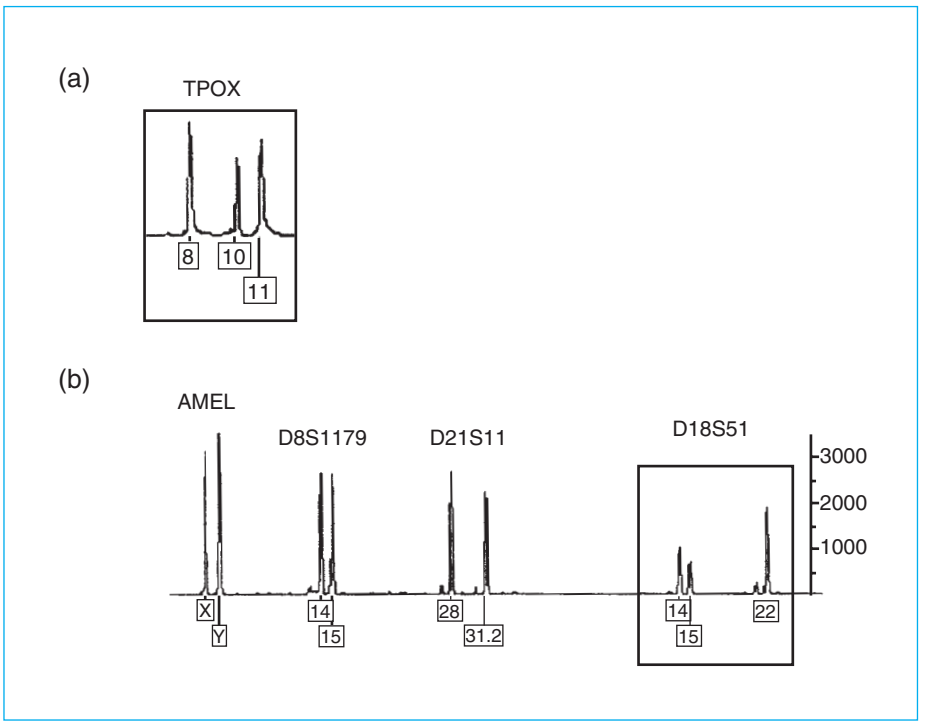

Figure 6.7
Tri-allelic patterns observed at (a) TPOX and (b) D18S51 from different samples. Allele calls are listed underneath each peak. (a) The TPOX result was obtained with the Identifiler STR kit and run on the ABI 3100. (b) This DNA sample was amplified with the Profiler Plus™ STR kit, separated on the ABI Prism 310 Genetic Analyzer and viewed with Genotyper software. Only the green dye-labeled PCR products are shown here for simplicity's sake.

kind of chromosome duplication for the region surrounding the D18S51 marker in this individual. Note that in this example the other STR loci besides D18S51 have two peaks of similar intensity, which suggests that a sample mixture is not likely (see Chapter 7).

More than 50 different tri-allelic patterns have been reported at all 13 CODIS STR loci with most of them being seen at TPOX and FGA. A frequently updated listing of tri-allelic patterns may be found on the STRBase web site: http://www.cstl.nist.gov/biotech/strbase/var_tab.htm.

ALLELE DROPOUT AND NULL ALLELES

When amplifying DNA fragments that contain STR repeat regions, it is possible to have a phenomenon known as *allele dropout*. Sequence polymorphisms are known to occur within or around STR repeat regions. These variations can occur in three locations (relative to the primer binding sites): within the repeat region, in the flanking region, or in the primer-binding region (Figure 6.8).

If a base pair change occurs in the DNA template at the PCR primer binding region, the hybridization of the primer can be disrupted resulting in a failure to amplify, and therefore failure to detect an allele that exists in the template DNA. More simply, the DNA template exists for a particular allele but fails to

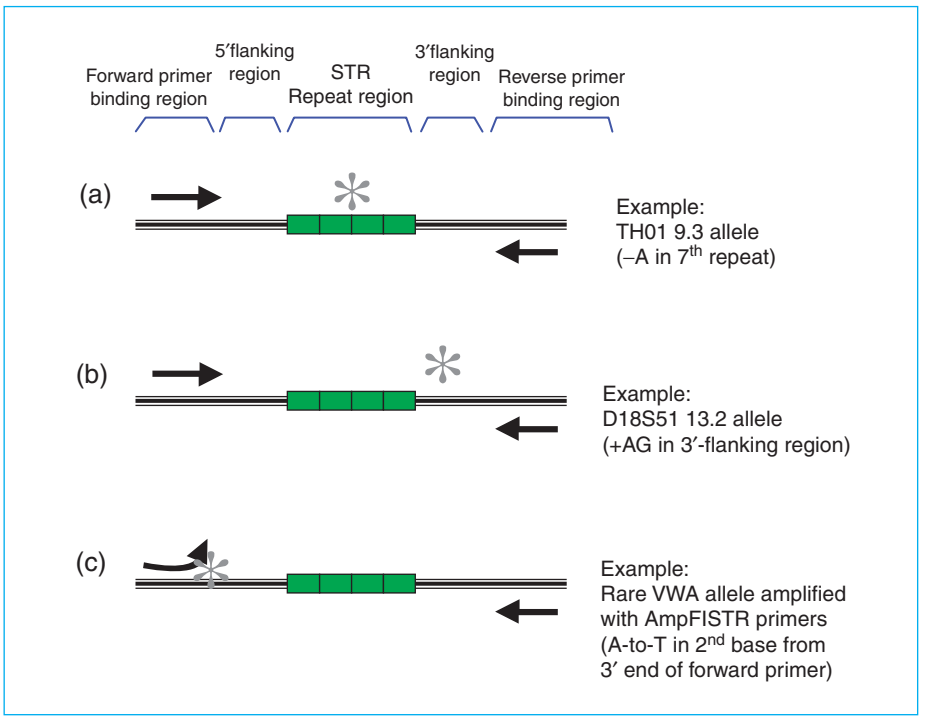

Figure 6.8

Possible sequence variation in or around STR repeat regions and the impact on PCR amplification. The asterisk symbolizes a DNA difference (base change, insertion or deletion of a nucleotide) from a typical allele for a STR locus. In situation (a), the variation occurs within the repeat region and should have no impact on the primer binding and the subsequent PCR amplification (although the overall amplicon size may vary slightly). In situation (b), the sequence variation occurs just outside the repeat in the flanking region but interior to the primer annealing sites. Again, PCR should not be affected although the size of the PCR product may vary slightly. However, in situation (c) the PCR can fail due to a disruption in the annealing of a primer because the primer no longer perfectly matches the DNA template sequence.

amplify during PCR due to primer hybridization problems. This phenomenon results in what is known as a *null allele*. Fortunately null alleles are rather rare because the flanking sequence around STR repeats is fairly stable and consistent between samples.

DISCOVERY OF NULL ALLELES

Null alleles have been 'discovered' by the observation of different typing results when utilizing independent STR primer sets. During a comparison of STR typing results on 600 population samples at the VWA locus, one sample typed 16,19 with Promega's PowerPlex kit and 16,16 with Applied Biosystem's AmpF*l*STR® Blue kit (Kline *et al.* 1998). In this case, VWA allele 19 dropped out with the AmpF*l*STR® VWA primer set due to a sequence polymorphism near the 3′-end of the forward primer (Walsh 1998).

Allele dropout may occur due to mutations (variants) at or near the 3′-end of a primer and thus produce little or no extension during PCR. In this case, the VWA allele 19 was present in the sample but failed to be amplified by one of the primer sets. It was later reported that the null allele resulted from a rare A–T nucleotide change in the DNA template at the second base from the 3′-end of the AmpF*l*STR® VWA forward primer (Walsh 1998).

Potential null alleles resulting from allele dropout can be predicted by statistical analysis of the STR typing data. The observed number of homozygotes can be compared to the expected number of homozygotes based on Hardy–Weinberg equilibrium (Chakraborty *et al.* 1992). An abnormally high level of homozygotes would indicate the possible presence of null alleles. Thus, each set of population data should be carefully examined when new STR markers are being tested in a forensic DNA laboratory (see Chapter 20).

A number of primer concordance studies have been conducted in the past few years as use of various STR kits has become more prevalent. An examination of over 2000 samples comparing the PowerPlex® 16 kit to the Profiler Plus™ and COfiler™ kit results found 22 examples of allele dropout due to a primer mismatch at seven of the 13 core STR loci: CSF1PO, D8S1179, D16S539, D21S11, FGA, TH01, and VWA (Budowle *et al.* 2001, Budowle and Sprecher 2001). Table 6.2 contains a summary of findings from some concordance studies that identified allele dropout with a particular primer pair.

SOLUTIONS TO NULL ALLELES

If a null allele is detected at a STR locus, there are several possible solutions. First, the problem PCR primer could be redesigned and moved away from the problematic site. This approach was taken early in the development of the D7S820 primers for the Promega PowerPlex® 1.1 kit (Schumm *et al.* 1996) and more recently with a D16S539 flanking region mutation (Nelson *et al.* 2002, see Chapter 5). However, this solution could result in the new primer interfering with another one in the multiplex set of primers or necessitate new PCR reaction optimization experiments. Clearly this solution is undesirable because it is time-consuming and labor intensive.

A second solution is to simply drop the STR locus from the multiplex mix rather than attempting to redesign the PCR primers to avoid the site. This approach is only desirable when early in the development cycle of a multiplex STR assay. The Forensic Science Service dropped the STR locus D19S253 from consideration in their prototype second-generation multiplex when a null allele was discovered (Urquhart *et al.* 1994).

A third, and more favorable, solution is to add a 'degenerate' primer that contains the known sequence polymorphism. This extra primer will then amplify alleles containing the problematic primer binding site sequence variant. This approach was taken with the AmpF*l*STR® kits for the D16S539 mutation mentioned previously (Holt *et al.* 2002, see Chapter 5). However, if the sequence variation at the primer binding site is extremely rare, it may not be worth the effort to add an additional primer to the multiplex primer mix.

Table 6.2

Summary of discordant results observed with STR kits as reported in the literature due to various concordance studies with different PCR primer sets. These discrepancies arise due to polymorphic nucleotides or insertions/deletions that occur in the tested DNA templates near the 3′-end of a primer binding site that disrupt proper primer annealing and result in allele dropout upon PCR amplification. STR kits reported here include: PowerPlex 1.1 (PP1.1), PowerPlex 16 (PP16), Identifiler (ID), Profiler Plus (ProPlus), COfiler, and SGM Plus.

Locus	STR Kits/Assays Compared	Results	Reference
VWA	PP1.1 *vs* ProPlus	Loss of allele 19 with ProPlus; fine with PP1.1	Kline *et al.* (1998)
VWA	PP16 *vs* ProPlus	Loss of alleles 15 and 17 with ProPlus; fine with PP16	Budowle *et al.* (2001)
VWA	ID *vs* miniplexes	Loss of alleles 12, 13, and 14 with miniplex assay; fine with ID	Drabek *et al.* (2004)
VWA	SGM *vs* SGM Plus	Loss of allele 17 with SGM Plus; fine with SGM	Clayton *et al.* (2004)
D5S818	PP16 *vs* ProPlus	Loss of alleles 10 and 11 with PP16; fine with ProPlus	Alves *et al.* (2003)
D5S818	ID *vs* miniplexes	Loss of allele 12 with miniplex assay; fine with ID	Drabek *et al.* (2004)
D13S317	ID *vs* miniplexes	Shift of alleles 10 and 11 due to deletion outside of miniplex assay but internal to ID	Drabek *et al.* (2004)
D16S539	PP1.1 *vs* PP16 *vs* COfiler	Loss of alleles with PP1.1; fine with PP16 and COfiler	Nelson *et al.* (2002)
D16S539	PP16 *vs* COfiler	Loss of allele 12 with PP16; fine with COfiler	Budowle *et al.* (2001)
D8S1179	PP16 *vs* ProPlus	Loss of alleles 15, 16, 17, and 18 with ProPlus; fine with PP16	Budowle *et al.* (2001)
D8S1179	SGM *vs* SGM Plus	Loss of allele 16 with SGM Plus; fine with SGM	Clayton *et al.* (2004)
FGA	SGM *vs* SGM Plus	Loss of allele 26 with SGM Plus; weak amp of same allele with SGM	Cotton *et al.* (2000)
FGA	PP16 *vs* ProPlus	Loss of allele 22 with ProPlus; fine with PP16	Budowle and Sprecher (2001)
D18S51	SGM *vs* SGM Plus	Loss of alleles 17, 18, 19, and 20 with SGM Plus (in Kuwaiti individuals); fine with SGM	Clayton *et al.* (2004)
CSF1PO	PP16 *vs* COfiler	Loss of allele 14 with COfiler; fine with PP16	Budowle *et al.* (2001)
TH01	PP16 *vs* COfiler	Loss of allele 9 with COfiler; fine with PP16	Budowle *et al.* (2001)
TH01	SGM *vs* SGM Plus	Loss of allele 6 with SGM Plus; fine with SGM	Clayton *et al.* (2004)
D21S11	PP16 *vs* ProPlus	Loss of allele 32.2 with PP16; fine with ProPlus	Budowle *et al.* (2001)
D19S433	SGM *vs* SGM Plus	Loss of allele 11 with SGM Plus; fine with SGM	Clayton *et al.* (2004)

A fourth possible solution to correct for allele dropout that will work for some problematic primer binding sites is to re-amplify the sample with a lower annealing temperature and thereby reduce the stringency of the primer annealing. If the primer is only slightly destabilized, as detected by a peak height imbalance with a heterozygous sample (Figure 6.9, middle panel), then the peak height imbalance may be able to be corrected by lowering the annealing temperature during PCR.

No primer set is completely immune to the phenomenon of null alleles. However, when identical primer sets are used to amplify evidence samples and suspect reference samples, full concordance is expected from biological materials originating from a common source. If the DNA templates and PCR conditions are identical between two samples from the same individual, then identical DNA profiles should result regardless of how well or poorly the PCR primers amplify the DNA template.

The potential of null alleles is not a problem within a laboratory that uses the same primer set to amplify a particular STR marker. However, with the emergence of national and international DNA databases, which store only the genotype information for a sample, allele dropout could potentially result in a false negative or incorrect exclusion of two samples that come from a common source.

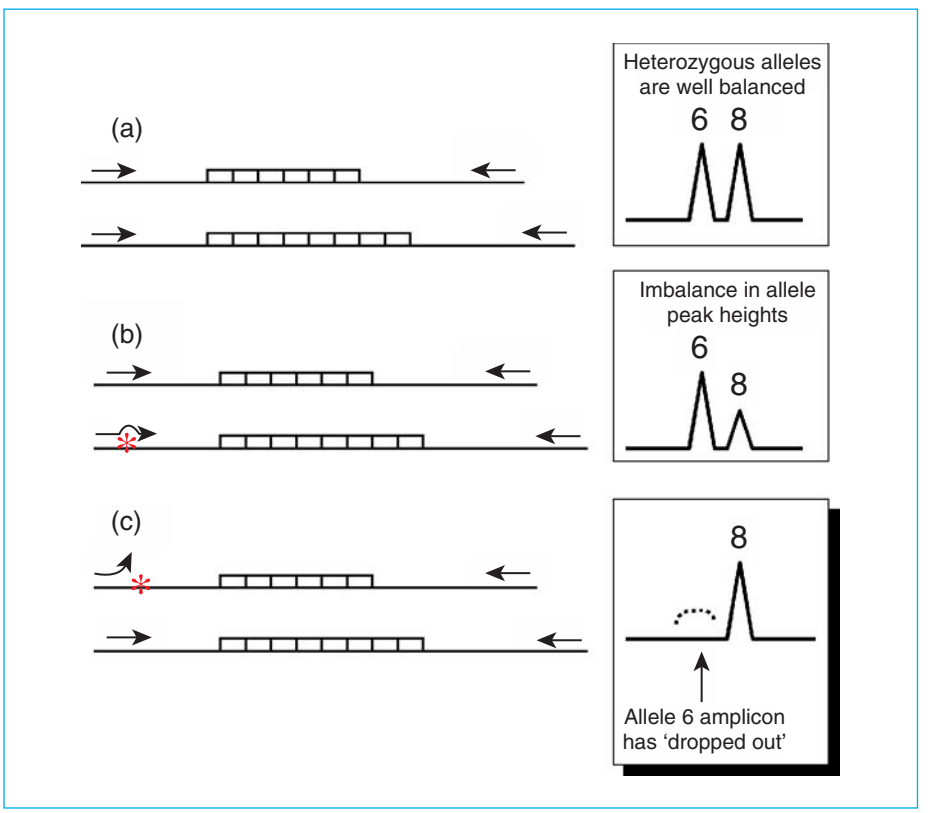

Figure 6.9

Impact of a sequence polymorphism in the primer binding site illustrated with a hypothetical heterozygous individual. Heterozygous allele peaks may be well-balanced (a), imbalanced (b), or exhibit allele dropout (c).

To overcome this potential problem, the matching criteria in database searches can be made less stringent when searching a crime stain sample against the DNA database of convicted offender profiles (see Chapter 18). That is, the database search might be programmed to return any profiles with a match at 25 out of 26 alleles instead of 26 out of 26.

When primers are selected for amplification of STR loci, candidate primers are evaluated carefully to avoid primer binding site mutations (Schumm *et al.* 1996, Wallin *et al.* 2002). Sequence analysis of multiple alleles is performed, family inheritance studies are conducted, within-locus peak signal ratios for heterozygous samples are examined, apparent homozygous samples are re-amplified with lower annealing temperatures, and statistical analysis of observed versus expected homozygosity is performed on population databases (Walsh 1998). It is truly a challenge to design multiplex STR primer sets in which primer binding sites are located in sequence regions that are as highly conserved as possible and yet do not interfere with primers amplifying other loci.

USE OF DEGENERATE PRIMERS IN COMMERCIAL KITS

The third solution mentioned above for solving potential allele dropout with primer binding site mutations is to add an additional PCR primer to the assay that can hybridize properly to the alternative allele when it exists in a sample. This has been the preferred solution for Applied Biosystems (e.g., Wallin *et al.* 2002) while Promega have moved their primers to overcome allele dropout problems (Nelson *et al.* 2002). According to their publications, Applied Biosystems has added an additional primer to correct for single point mutations in AmpF*l*STR® primer binding sites for D16S539 (Wallin *et al.* 2002), VWA (Lazaruk *et al.* 2001), and D8S1179 (Leibelt *et al.* 2003).

MUTATIONS AND MUTATION RATES

As with any region of DNA, mutations can and do occur at STR loci. By some not completely characterized mechanism, STR alleles can change over time (Ellegren 2004). Theoretically, all of the alleles that exist today for a particular STR locus have resulted from only a few 'founder' individuals by slowly changing over tens of thousands of years (Wiegand *et al.* 2000). The mutational event may be in the form of a single base change or in the length of the entire repeat. The molecular mechanisms by which STRs mutate are thought to involve replication slippage or defective DNA replication repair (Nadir *et al.* 1996, Ellegren 2004).

DISCOVERY OF STR ALLELE MUTATIONS

Estimation of mutational events at a DNA marker may be achieved by comparison of genotypes from offspring to those of their parents. Genotype data from

D.N.A. Box 6.1

Allele specific mutation rates

Until recently, only general information on STR mutation rates was reported – namely, how many mutations occurred relative to the number of meioses measured (see Table 6.3). The realization that certain alleles are more prone to mutation than others has prompted the American Association of Blood Banks (AABB) to carefully examine *which alleles* were mutating based on records from accredited parentage testing laboratories.

Appendix 5 in the AABB Annual Report Summary for Testing in 2002, prepared by the parentage testing program unit in November 2003, notes the number of paternal and maternal mutations by both locus and allele. For example, with the STR locus FGA an apparent change from allele 24 to 25 was observed 62 times (11.7%) out of 530 total paternal mutations seen in 2002, while an apparent change from allele 19 to 20 was seen only eight times (1.5%). In general longer alleles were seen to mutate more frequently.

The directionality of the mutation as either an expansion or a contraction of the repeat array can also vary significantly. For example, with paternal D16S539 mutations observed in 2002 there were 10 instances of allele 11 expanding to become allele 12 but only four examples of allele 11 contracting to allele 10. The process of expansion and contraction of the STR repeat regions probably occurs in a similar fashion as illustrated in Figure 6.2 for stutter product formation.

As this information continues to be collated in future studies, it should prove useful in refining mutation rates and aid in a better understanding of the process of STR origins and variability over human history.

Source:
American Association of Blood Banks Annual Report Summary for Testing in 2002 (see http://www.aabb.org/About_the_AABB/Stds_and_Accred/ptannrpt02.pdf)

paternity trios involving a father, a mother, and at least one child is examined. A discovery of an allele difference between the parents and the child is seen as evidence for a possible mutation (Figure 6.10). The search for mutations in STR loci involves examining many, many parent–child allele transfers because the mutation rate is rather low in most STRs.

The majority of STR mutations involve the gain or loss of a single repeat unit (see D.N.A. Box 6.1). Thus, a VWA allele with 14 repeats would show up as a 13

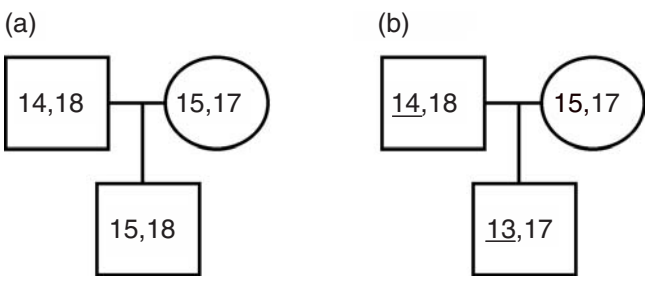

Figure 6.10

Mutational event observed in family trios. Normal transmission of alleles from a STR locus (a) is compared here to mutation of paternal allele 14 into the child's allele 13 (b).

or a 15 in the next generation following a mutational event (Figure 6.10). Paternal mutations appear to be more frequent than maternal ones for STR loci (Sajantila *et al.* 1999, Henke and Henke 1999). However, depending on the genotype combinations it can be difficult to ascertain from which parent the mutant allele was inherited.

MEASURING THE MUTATION RATE

Since the average mutation rate is below 0.1%, approximately 1000 parent–offspring allele transfers would have to be observed before one mutation would be seen in some STR markers (Weber and Wong 1993). Brinkman and co-workers (1998) examined 10 844 parent–child allele transfers at nine STR loci and observed 23 mutations. No mutations were observed at three of the loci (TH01, F13B, CD4). Sajantila *et al.* (1999) studied 29 640 parent–child allele transfers at five STRs and four minisatellites and observed only 18 mutational events (11 in three STR loci: D3S1359, VWA, and TH01). Two of the STRs, TPOX and FES/FPS, had no detectable mutations.

The mutation rates for the 13 core STR loci have been gathered from a number of studies in the literature and are summarized in Table 6.3. Most of these mutation rates are on the order of 1–5 mutations per 1000 allele transfers or generational events. The STR loci with the lowest observed mutation rates are CSF1PO, TH01, TPOX, D5S818, and D8S1179. Not surprisingly, the STR loci with the highest mutation rates – D21S11, FGA, D7S820, D16S539, and D18S51 – are among the most polymorphic and possess the highest number of observed alleles (Appendix I).

Table 6.3 (facing)

Observed mutation rates for the 13 core STR loci and other STR markers present in commercial kits. A total of 38 different paternity testing laboratories provided this STR mutation data, which is adapted from the American Association of Blood Banks (AABB) 2002 Annual Report issued in November 2003 that includes a compilation of multiple years (see http://www.aabb.org/About_the_AABB/Stds_and_Accred/ptannrpt02.pdf, p. 15). The reported mutations are divided into maternal or paternal sources or from either when the source of the mutation observed in a child could not be determined.

IMPACT OF MUTATION RATES ON PATERNITY TESTING

Low mutation rates are especially critical for paternity testing (see Chapter 23). This is because links are being made between the child and the alleged father based on the assumption that alleles remain the same when they are passed from one generation to the next. Parent–offspring allele transfer information tests for germ-line mutations. Additionally, genotypes from different kinds of tissues from the same individual are examined to demonstrate that no somatic mutations occur.

Mutations have practical consequences for paternity testing and mass disaster investigations (see Chapter 24) as well as population genetics where conclusions are being drawn from genetic data across one generation or many generations. In paternity testing situations, a high mutation rate for a STR marker could result in a false exclusion at that locus. With regards to population evolution studies, the mutation process must be subtracted from population demography and population history in order to accurately address the population

STR System	Maternal Meioses (%)	Paternal Meioses (%)	Number from either	Total Number of Mutations	Mutation Rate
CSF1PO	70/179 353 (0.04)	727/504 342 (0.14)	303	1 100/683 695	0.16%
FGA	134/238 378 (0.06)	1 481/473 924 (0.31)	495	2 110/712 302	0.30%
TH01	23/189 478 (0.01)	29/346 518 (0.008)	23	75/535 996	0.01%
TPOX	16/299 186 (0.005)	43/328 067 (0.01)	24	83/627 253	0.01%
VWA	133/400 560 (0.03)	907/646 851 (0.14)	628	1 668/1 047 411	0.16%
D3S1358	37/244 484 (0.02)	429/336 208 (0.13)	266	732/580 692	0.13%
D5S818	84/316 102 (0.03)	537/468 366 (0.11)	303	924/784 468	0.12%
D7S820	43/334 886 (0.01)	550/461 457 (0.12)	218	811/796 343	0.10%
D8S1179	54/237 235 (0.02)	396/264 350 (0.15)	225	675/501 585	0.13%
D13S317	142/348 395 (0.04)	608/435 530 (0.14)	402	1 152/783 925	0.15%
D16S539	77/300 742 (0.03)	350/317 146 (0.11)	256	683/617 888	0.11%
D18S51	83/130 206 (0.06)	623/278 098 (0.22)	330	1 036/408 304	0.25%
D21S11	284/258 795 (0.11)	454/306 198 (0.15)	423	1 161/564 993	0.21%
Penta D	12/18 701 (0.06)	10/15 088 (0.07)	21	43/33 789	0.13%
Penta E	22/39 121 (0.06)	58/44 152 (0.13)	55	135/83 273	0.16%
D2S1338	2/25 271 (0.008)	61/81 960 (0.07)	31	94/107 231	0.09%
D19S433	22/28 027 (0.08)	16/38 983 (0.04)	37	75/67 010	0.11%
F13A01	1/10 474 (0.01)	37/65 347 (0.06)	3	41/75 821	0.05%
FES/FPS	3/18 918 (0.02)	79/149 028 (0.05)	None reported	82/167 946	0.05%
F13B	2/13 157 (0.02)	8/27 183 (0.03)	1	11/40 340	0.03%
LPL	0/8 821 (<0.01)	9/16 943 (0.05)	4	13/25 764	0.05%
SE33 (ACTBP2)	0/330 (<0.30)	330/51 610 (0.64)	None reported	330/51 940	0.64%

genetic questions being asked. Any time a family reference sample is used to try and match recovered remains during a mass disaster or missing persons investigation, mutations become an important issue because an exact match cannot be made when a mutation is present.

High mutation rates help keep STR markers polymorphic and therefore useful in human identity testing. It is important to keep in mind that while mutations can potentially impact kinship reference samples they will not affect

direct matches between personal effects and victims or perpetrators and crime scene evidence since any mutation that occurs will be consistent over an individual's lifetime.

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