CHAPTER

7

Low-Level DNA and Complex Mixtures

"The limits of each DNA typing procedure should be understood, especially when the DNA sample is small, is a mixture of DNA from multiple sources, or is contaminated with interfering substances."

NRC I, 1992, p. 8

"For the complex DNA profile, there is no predominant or overarching standard interpretation method."

Peter Gill (Gill et al. 2012, report to the UK Forensic Science Regulator, p. 18)

INTERPRETATION CHALLENGES WITH LOW TEMPLATE DNA

Short tandem repeat (STR) typing works best when using optimal amounts of DNA. The multiplex polymerase chain reaction (PCR) for most STR kits typically is designed by manufacturers to work best with DNA input amounts in the range of 0.5 ng to 1.5 ng. Larger multiplexes (e.g. 16plex) appear to have a tighter optimal DNA quantity range than smaller multiplexes (e.g. 3plex). While DNA quantitation can help make decisions on how best to proceed with processing a specific sample, in the end the resulting capillary electrophoresis (CE) electropherogram after PCR and CE provides the most useful metric of the amount of DNA per allele, per locus, and per contributor if a mixture is present (Gill et al. 2012). In addition, examining the STR profile as a whole will provide an indication of potential DNA degradation effects.

Complex mixtures, which are defined here as biological samples containing DNA with three or more contributors, exhibit several significant challenges. First, *allele sharing will occur* at many of the loci tested, making it challenging to unambiguously discern the full genotypes of the mixture contributors. Second, complex mixtures are *likely to contain low-template DNA* (LTDNA) for one or more of the contributors since PCR reactions are usually run with 1 ng or less of total DNA. Each additional contributor to a mixture means a dilution of one or more of the contributors into the stochastic danger zone where allele drop-out is more likely. Concepts developed for two-person mixtures like stochastic thresholds will not always be applicable with mixtures containing three or more contributors, largely because of the possibility of allele sharing.

With more contributors, there will be more allele sharing and thus more stacking of allelic contribution at each observed allele. In the hypothetical example shown in Figure 7.1, four contributor genotypes (10,11 & 10,12 & 10,10 & 11,12) combine to produce only three observed alleles. Allele 10 is the highest peak in the example data illustrated in Figure 7.1(a).

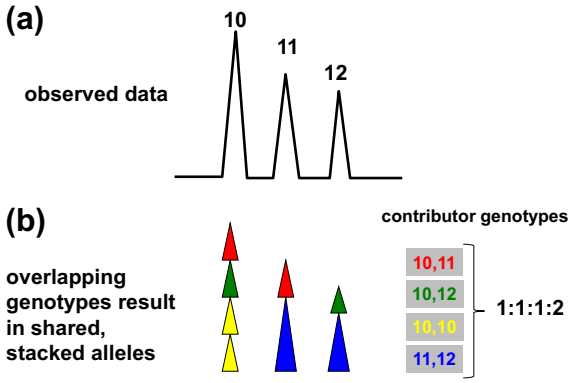

FIGURE 7.1 Illustration of (a) hypothetical observed data from a complex mixture for a single STR locus exhibiting alleles 10, 11, and 12 and (b) allele stacking from a set of contributor genotypes present in a 1:1:1:2 ratio.

Depending on the relative peak heights of allele 10 compared to alleles 11 and 12, a poorly designed mixture interpretation protocol might inappropriately consider the 10 allele as the major contributor in the mixture without considering the impact of allele stacking from multiple contributor genotypes.

Of course, in actual casework, the full identity of all contributor genotypes and mixture ratios is not known and must be inferred based on assessing the evidentiary DNA profile as a whole. As a general principle, the greater the number of contributors to a mixture, the lower the probability that the complete DNA profiles creating the mixture can be deciphered.

When lower amounts of DNA are PCR-amplified, there is a higher degree of stochastic variation. Thus, the relative ratios of the heterozygous alleles illustrated by different pairs of colored triangles in Figure 7.1(b) may become more skewed. When more stochastic variation occurs in heterozygote peak height ratios and stutter ratios, it becomes increasingly difficult to pair alleles into genotypes from individual contributors.

In an evaluation of low copy number (LCN) DNA profiling results in New Zealand, scientists from the Institute of Environmental Science and Research (ESR) evaluated 20 DNA samples that were fully heterozygous at the 10 STR loci in the SGM Plus kit (Petricevic et al. 2010). Lower heterozygote balance (Hb) and higher stutter ratios were observed with 12.5 pg or 25 pg examined at 34 PCR cycles versus 1 ng examined at 28 PCR cycles (Figure 7.2).

Figure 7.3 illustrates how the overlap of high stutter and low heterozygote balance with stochastic variation makes the use of peak height information for restricting genotype combinations more difficult from low-level DNA results. DNA results from high-signal two-person mixtures containing discernable major/minor profiles can exploit the gap between the stutter ratio and heterozygote balance (Figure 7.3(a)) and enable mixture devolution through restricting possible genotype combinations.

However, when one or more of the contributors fall into the stochastic range (e.g. <100 pg amplified for that particular contributor's DNA), higher levels of stochastic variation can lead to more variability in peak height ratios of heterozygotes and more significant stutter products (Figure 7.3(b)). This variation leads to a lower confidence in appropriately associating allele pairs into individual contributor genotypes. Quoting the 2010 ESR

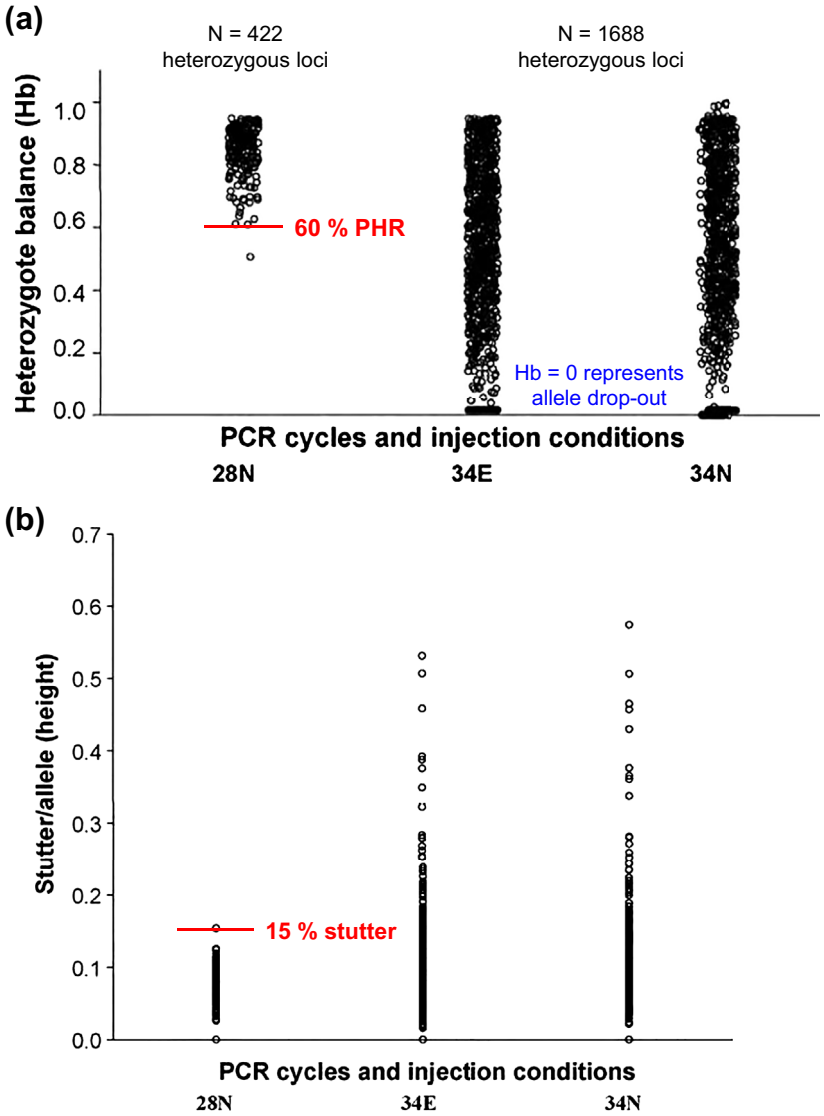

FIGURE 7.2 (a) Heterozygote balance across 10 STR loci in 20 fully heterozygous samples tested with SGM Plus at the ESR Laboratory in New Zealand compared among three sets of conditions: 28N = 1 ng target DNA tested with 28 PCR cycles and normal CE injection (10s @ 3kV); 34E = 12.5 pg and 25 pg target DNA tested in duplicate with 34 PCR cycles and enhanced CE injection (15 s @ 5 kV); 34N = 12.5 pg and 25 pg target DNA tested in duplicate with 34 PCR cycles and normal CE injection. (b) Stutter ratio data under the same conditions. Adapted from Petricevic et al. (2010).

study: "Because of the greater stochastic effects, the interpretation of mixed LCN profiles should utilize models that place much less emphasis on peak heights/area differences. … However in some instances it is still possible to infer a strong major component of mixtures" (Petricevic et al. 2010).

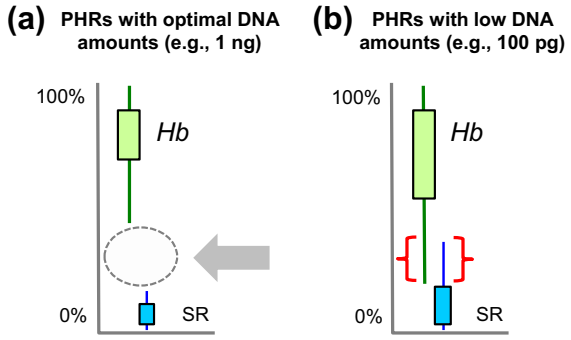

FIGURE 7.3 Impact of template DNA amount on variation in peak height ratios (PHRs). The observed spread in stutter ratio (SR) and heterozygous balance (*Hb*) are illustrated using box and whisker plots with (a) optimal versus (b) lower levels of DNA template being amplified. The gap between the SR and *Hb* (noted with the large gray arrow) is what enables mixture deconvolution through assuming restricted genotype combinations. The larger box and longer whiskers in the low-level DNA plot (b) reflect a larger variance causing the potential ranges of Hb and SR to overlap (noted by red brackets) and therefore greater uncertainty exists in reliably associating alleles into genotypes due to inherent stochastic effects during PCR.

Potential DNA Amounts in Mixtures

Table 7.1 compares the quantity of DNA and theoretical cell counts with single-source, two-person, three-person, and four-person mixtures with selected DNA mixture ratios. Studies with single-source samples have shown that stochastic effects such as elevated stutter and allele drop-out occur at around 15 to 20 cells or 100 pg to 125 pg (Butler & Hill 2010). Thus, the minor component in a 0.5-ng two-person mixture at a 4:1 ratio may experience adverse stochastic effects. Likewise, all four contributors in a 0.5-ng four-person mixture at a 1:1:1:1 ratio could potentially experience allele drop-out. Some amount of allele drop-out is highly likely when amplifying 0.25 ng total DNA containing a three-person or a four-person mixture with *any* mixture ratio.

Enhanced detection methods, such as the 34 PCR cycles used in the ESR study (Petricevic et al. 2010), may enable more alleles to be detected, but lead to greater variability in heterozygote balance and increased stutter product formation as shown in Figure 7.2.

Limitations of SWGDAM 2010 Guidelines

In January 2010, the FBI Laboratory's Scientific Working Group on DNA Analysis Methods (SWGDAM) approved interpretation guidelines for autosomal STR analysis (SWGDAM 2010). The SWGDAM 2010 guidelines, which discuss among other topics the use of a stochastic threshold, were primarily designed to cover single-source samples and two-person mixtures. The Frequently Asked Questions (FAQs) section of the SWGDAM website (SWGDAM FAQs 2014) states:

> ***Question:*** *Are the 2010 SWGDAM Interpretation Guidelines applicable to all DNA mixtures?*
>
> ***SWGDAM Response****: These guidelines were written with single-source samples and two-person mixtures in mind, and are not intended to replace a laboratory's previously validated mixture interpretation guidelines and/or*

TABLE 7.1 Comparison of the Quantity of DNA and Theoretical Cell Counts with Various Numbers of Contributors and DNA Mixture Ratios

Amount of DNA in PCR	Single-source	Two-person mixture	Three-person mixtures		Four-person mixtures	
		4:1	1:1:1	5:2:1	1:1:1:1	5:2:2:1
1 ng	1	0.8 + 0.2	0.33 × 3	0.6 + 0.25 + 0.13	0.25 × 4	0.5 + 0.2 + 0.2 + 0.1
	150	120 + 30	50 × 3	94 + 38 + 19	38 × 4	75 + 30 + 30 + 15
0.5 ng	0.5	0.4 + 0.1	0.16 × 3	0.31 + 0.12 + 0.06	0.125 × 4	0.25 + 0.1 + 0.1 + 0.05
	75	60 + 15	24 × 3	47 + 18 + 9	19 × 4	38 + 15 + 15 + 7
0.25 ng	0.25	0.2 + 0.05	0.08 × 3	0.15 + 0.06 + 0.03	0.062 × 4	0.12+0.05+0.05+0.02
	38	30 + 7	12 × 3	23 + 9 + 4	9 × 4	18 + 7 + 7 + 3
0.1 ng	0.1	0.075 + 0.025	0.03 × 3	0.062+0.02+0.01	0.025 × 4	0.05+0.02+0.02+0.01
	15	11 + 4	5 × 3	10 + 3 + 1	4 × 4	7 + 3 + 3 + 1
0.05 ng	0.05	0.04 + 0.01	0.016 × 3	0.03+0.012+0.006	0.0125 × 4	0.025+0.01+0.01+0.005
	7	6 + 1	2 × 3	5 + <2 + <1	2 × 4	4 + 1 + 1 + <1

Under each amount of DNA, the top row shows the approximate amount of DNA per person in nanograms (ng) and the bottom row provides the approximate number of cells assuming 6 picograms (pg) per diploid cell. Information courtesy of Dr. Charlotte Word.

policy. The basic concepts *outlined in the 2010 SWGDAM Mixture Interpretation Guidelines hold true as they relate to DNA mixtures of three or more contributors, low-level DNA samples, and mixtures containing biologically related individuals. However, there are nuances and limitations to the interpretation of these more complex mixtures, which are not fully explored in the 2010 guidelines. The Autosomal STR Interpretation Committee is tasked with reviewing and revising these SWGDAM guidelines. Laboratories are encouraged to perform additional validation studies of complex mixtures to further their understanding of the issues related to these challenging samples.*

Understanding the original designed scope of the 2010 SWGDAM guidelines is important as well to discussions on possible statistical approaches for coping with more complex mixtures that will be covered in Chapter 13.

Limitations of a Stochastic Threshold

Chapter 4 briefly discussed methods for establishing a stochastic threshold, which is used to assess the possibility of allele drop-out. When allelic peaks are seen below a stochastic threshold with a two-person mixture, then the possibility exists that a sister allele may not be detected because it was not amplified to a detectable level. Thus, a stochastic threshold is intended as a warning indicator of possible allele drop-out.

However, the potential of allele stacking, especially with more than two contributors (see Figure 7.1), can limit the usefulness of a stochastic threshold. Just because allelic peaks at a locus are above an established stochastic threshold does not mean that no allele drop-out has occurred

in a complex mixture. Of course, if peaks are observed below a stochastic threshold in a complex mixture, then allele drop-out of a sister allele is possible as with simple two-person mixtures and the detected allele could be a false homozygote.

Validation Needs to Match Sample Types

If a laboratory desires to develop appropriate protocols that will enable reliable interpretation of DNA from low-level DNA or mixtures involving three or more contributors, then validation studies need to be performed with known samples that mimic the amounts of DNA and complexity of profiles where stochastic effects and allele dropout are expected. In short, three- or four-person mixtures of known genotypes should be mixed at specific ratios and amplified multiple times. Then these complex mixture profiles should be subjected to interpretation approaches to see if a true contributor is appropriately associated with the mixture and if non-contributors are appropriately excluded.

In my opinion, a laboratory cannot run a single two-person mixture series (e.g. 9:1, 5:1, 3:1, 1:1, 1:3, 1:5, and 1:9) and feel confident that minimum requirements for "mixture validation" have been met. This type of a limited validation may simply be able to help determine that a minor contributor can be detected down to a certain level. Determining that a mixture exists is not the same as fully interpreting a mixture. Developing robust interpretation protocols will require considering more samples – especially ones that go beyond a cursory combination of control samples 9947A and 9948 (D.N.A. Box 7.1).

D.N.A. BOX 7.1

LIMITATIONS OF CREATING MIXTURES WITH STANDARD CONTROL SAMPLES 9947A AND 9948

Cell lines 9947A and 9948 have been used since the mid-1990s as control samples for forensic DNA testing (Fregeau et al. 1995). Genomic DNA from these cell lines were included in NIST Standard Reference Material (SRM) 2391, 2391a, and 2391b as well as positive controls in STR typing kits for a number of years. Many forensic laboratories have, for the sake of convenience and availability, used the STR kit positive control 9947A in their heterozygous balance studies and mixed 9947A and 9948 together for simple mixture validation studies. Unfortunately, due to the large number of homozygotes (with five of the original 13 CODIS core loci), allele drop-out cannot be effectively measured across all loci with these samples. For example, because TPOX, D5S818, D8S1179, D13S317, and D21S11 are homozygous in 9947A, it is not possible to examine heterozygote balance or to look for missing sister alleles at these loci when replicate low-level, serial dilutions are studied. Validation studies involving fully heterozygous samples are more effective at studying allele drop-out across all loci. Likewise, there is a great deal of allele sharing between 9947A and 9948 making them less than ideal candidates for studying detection of minor components in 2-person mixtures. The table below shows the observed alleles at 9947A and 9948 as well as the number of alleles seen when these two DNA samples are mixed together.

Locus	9947A	9948	Mixture of 9947A & 9948	#alleles
Amelogenin	**X,X**	X,Y	male & female	–
CSF1PO	10,12	10,11,12	10,11,12	3
FGA	23,24	24,26	23,24,26	3
TH01	8,9.3	6,9.3	6,8,9.3	3
TPOX	**8,8**	8,9	8,9	2
vWA	17,18	**17,17**	17,18	2
D3S1358	14,15	15,17	14,15,17	3
D5S818	**11,11**	11,13	11,13	2
D7S820	10,11	**11,11**	10,11	2
D8S1179	13,13	12,13	12,13	2
D13S317	**11,11**	**11,11**	11	1
D16S539	11,12	**11,11**	11,12	2
D18S51	15,19	15,18	15,18,19	3
D21S11	**30,30**	29,30	29,30	2
Penta D	**12,12**	8,12	8,12	2
Penta E	12,13	**11,11**	11,12,13	3
D2S1338	19,23	23,23	19,23	2
D19S433	14,15	13,14	13,14,15	3
D1S1656	**18.3,18.3**	14,17	14,17,18.3	3
D2S441	10,14	11,12	10,11,12,14	4
D10S1248	13,15	12,15	12,13,15	3
D12S391	18,20	18,24	18,20,24	3
D22S1045	11,14	16,18	11,14,16,18	4
SE33	19,29.2	23.2,26.2	19,23.2,26.2,29.2	4
D6S1043	12,18	**12,12**	12,18	2

Life Technologies (formerly as Applied Biosystems) has supplied a control DNA sample "007" with many of its kits since the launch of the AmpFlSTR SGM Plus kit in 1999 largely because the 007 sample was fully heterozygous at all 11 tested SGM Plus kit. In 2012, Promega began using as its positive STR kit control a sample labeled "2800M Control DNA," which is heterozygous at 21 of the 24 STR loci shown here (the three homozygotes are CSF1PO, TPOX, and D22S1045).

It is well known that low amounts of DNA template (e.g. 50 pg) do not behave as consistently as optimal DNA target quantities (e.g. 1 ng) and that additional measures which account for potential allele drop-out are necessary (see Butler 2012, Gill et al. 2012). A meaningful and reliable protocol to benefit work conducted with complex mixtures requires developing an understanding of allele drop-out and the impact of potential allele sharing.

Assessing allele drop-out can begin with replicate amplifications of low-level DNA from a dilution series of single-source samples possessing fully heterozygous genotypes. For example, five or ten replicates from single-source DNA samples containing 100 pg, 50 pg, 30 pg, and 10 pg will enable gathering data in a range where allele drop-out is expected (NIST Low Template DNA Testing 2014). Protocols should be regularly queried with controls (i.e. samples with known genotypes). Results obtained with these controls and a specific method should be carefully examined to see whether the expected alleles and genotypes are obtained.

Laboratories cannot adequately understand performance characteristics of low-template, complex DNA mixtures from having run a few high-template, simple DNA mixtures such as a few mixtures of 9947A and 9948. Attempts at *validation extrapolation*, where a simple two-person mixture study is expected to provide guidance for proper interpretation of less optimal mixtures, will not enable creation of robust protocols that provide consistent, reliable results. Every DNA interpretation protocol should be based on validation data, the scientific literature, and experience (SWGDAM 2010). Empirical data are always needed to establish limitations for a technique.

Some Complex Mixture Studies

Several published studies have examined complex DNA mixtures with controlled validation experiments. For laboratories pursuing their own STR validation studies involving more than two contributors, reviewing these and other articles would be a good starting point.

The Netherlands Forensic Institute (NFI) assessed four mock cases involving LTDNA containing two, three, or four contributors in which some of the contributors were brothers (Benschop et al. 2012). Four DNA mixtures were amplified in quadruplicate and reviewed by eight different NFI reporting officers with known samples being examined after profile interpretation. Likelihood ratio calculations were performed with a probabilistic genotyping tool LRmix (Gill & Haned 2013). The results from the eight reporting officers were then classified into one of four categories used at NFI: (a) exclusion, (b) match with statistical evaluation, (c) match without statistical evaluation, and (d) cannot be included or excluded (i.e. inconclusive). Because reporting officers provided conclusions similar to those expected for known samples, this study "gives the impression that the evaluation of LTDNA profiles is feasible" (Benschop et al. 2012). The use of pooled samples has also been found to aid complex LTDNA mixture interpretation (Benschop et al. 2013, Budimliga & Caragine 2012).

A German team from Institutes of Legal Medicine in Ulm and Hannover studied two types of degraded samples with DNA amounts as little as 50 pg and 100 pg per contributor (Pfeifer et al. 2012). They selected six contributors and combined these DNA samples into ten mixtures with up to four contributors for study. Different STR kits were evaluated as were the composite and consensus models (see below) for combining information from replicate PCR amplifications (Pfeifer et al. 2012).

Genotype Possibilities with Three or More Contributors

With each additional contributor to a mixture, the number of possible genotype combinations increases. A single-source sample (one contributor) has two possible genotype combinations: either a homozygote (PP) or a heterozygote (PQ). Two-person mixtures (two contributors) have 14 different genotype combinations. DNA mixtures consisting of three contributors have 150 possible combinations of genotypes at each locus and mixtures with four contributors have more than 600 possible genotype combinations at each locus (D.N.A. Box 7.2).

D.N.A. BOX 7.2

NUMBER OF POSSIBLE GENOTYPE COMBINATIONS

Evaluation of numbers of possible genotype combinations with specific numbers of alleles (vertical) and number of contributors (horizontal). Bruce Heidebrecht (Maryland State Police Forensic Laboratory) helped check some of this information.

	1 contributor *either hom or het*	#	2 contributors *7 classes*	#	3 contributors *23 classes*	#	4 contributors *41 classes*	#
		2		14		150		>600
1 allele (1 hom, 0 het)	homozygote (hom)	1	(a) 2 hom, 1 shared	1	(a) 3 hom, 1 shared	1	(a) 4 hom, 1 shared	1
2 alleles (2 hom, 1 het)	heterozygote (het)	1	(a) hom + hom, 0 shared (b) hom + het, 1 shared (c) hom + het, 2 shared	1 2 1	(a) 2 hom + hom, 0 shared (b) 2 hom + het, 1 shared (c) hom + hom + het, 2 shared (d) hom + 2 het, 2 shared (e) 3 het, 2 shared	2 2 1 2 1	(a) 3 hom + hom, 1 shared (b) 2 hom + 2 hom, 2 shared (c) 3 hom + het, 1 shared (d) 2 hom + hom + het, 2 shared (e) 2 hom + 2 het, 2 shared (f) hom + hom + 2 het, 2 shared (g) hom + 3 het, 2 shared (h) 4 het, 2 shared	2 1 2 2 1 1 2 1
3 alleles (3 hom, 3 het)	– *possible tri-allele?*		(a) hom + het, 1 shared (b) het + het, 1 shared	3 3	(a) hom + hom + hom, 0 shared (b) hom + hom + het, 0 shared (c) hom + hom + het, 1 shared (d) hom + 2 het, 2 shared (2:2:2) (e) hom + het + het, 2 shared (4:1:1) (f) hom + het + het, 2 shared (3:2:1) (g) het + het + het, 2 shared (3:2:1) (h) het + het + het, 3 shared (2:2:2)	1 3 6 3 3 6 6 1	(a) 2 hom + hom + hom, 1 shared (b) 3 hom + het, 1 shared (c) hom + hom + hom + het, 2 shared (d) hom + hom + het + het, 2 shared (e) hom + 2 het + het, 2 shared (f) hom + het + het + het, 3 shared (g) 3 het + het, 2 shared (h) 2 het + 2 het, 3 shared (i) 2 het + het + het, 3 shared	3 3 3 5 9 3 6 2 3
4 alleles (4 hom, 6 het)	–		(a) het + het, 0 shared	3	(a) hom + hom + het, 0 shared (b) hom + het + het, 1 shared (3:1:1:1) (c) hom + het + het, 1 shared (2:2:1:1) (d) 2 het + het, 2 shared (2:2:1:1) (e) het + het + het, 1 shared (3:1:1:1) (f) het + het + het, 2 shared (2:2:1:1)	6 12 12 6 4 12	(a) hom + hom + hom + hom, 0 shared (b) 2 hom + hom + het, 1 shared (4:2:1:1) (c) hom + hom + hom + het, 1 shared (3:2:2:1) (d) hom + hom + 2 het, 2 shared (2:2:2:2) (e) hom + hom + het + het, 2 shared (3:2:2:1) (f) het + het + het + het, 4 shared (2:2:2:2) (g) het + het + het + het, 3 shared (3:2:2:1) (h) 2 hom + het + het, 2 shared (4:2:1:1) (i) hom + het + het + het, 3 shared (3:2:2:1) (j) 2 het + het + het, 3 shared (3:2:2:1) (k) 2 het + het + het, 3 shared (4:2:1:1) (l) 2 het + het + het, 2 shared (3:3:1:1)	1 12 12 6 24 2 8 12 24 24 12 24
5 alleles (5 hom, 10 het)	–		–		(a) hom + het + het, 0 shared (b) het + het + het, 1 shared	15 30	(a) hom + hom + hom + het, 0 shared (b) hom + hom + het + het, 1 shared (c) hom + het + het + het, 2 shared (d) het + het + het + het, 3 shared (2:2:2:1:1) (e) 2 het + het + het, 2 shared (3:2:1:1:1)	10 30 30 >4 >11
6 alleles (6 hom, 15 het)	–		–		(a) het + het + het, 0 shared	15	(a) hom + hom + het + het, 0 shared (b) hom + het + het + het, 1 shared (c) het + het + het + het, 2 shared	30 48 >13
7 alleles (7 hom, 21 het)	–		–		–		(a) hom + het + het + het, 0 shared (b) het + het + het + het, 1 shared	70 >35
8 alleles (8 hom, 28 het)	–		–		–		(a) het + het + het + het, 0 shared	105

The different groupings of genotype combinations for a three-person mixture are shown in Figure 7.4. If we designate detected alleles as capital letters in sequential fashion starting with P, then we could have alleles P, Q, R, S, T, or U. Working all of these potential genotype combinations out by hand becomes increasingly difficult without a computer program.

With a three-person mixture, genetic loci exhibiting only a single allele would be comprised of PP homozygotes from all three contributors (Figure 7.4). Loci with two alleles could be composed of one

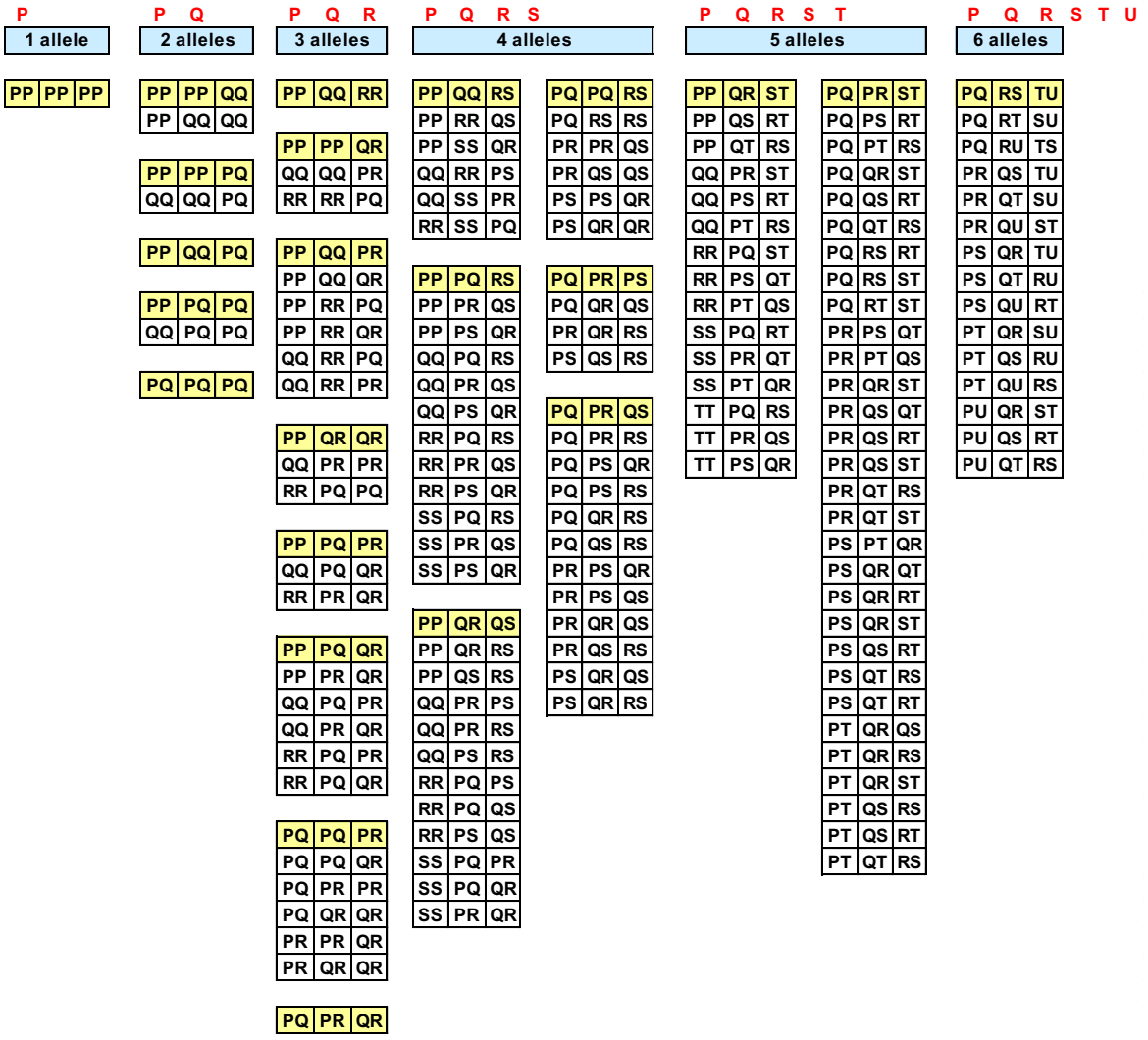

FIGURE 7.4 Display of 150 potential genotype combinations with a three-person mixture organized by the number of detected alleles. There are 23 different classes or groupings of genotype combinations with the first one in each group being highlighted in yellow. Adapted from Overson (2009) *(allele nomenclature changed from ABCDEF to PQRSTU).*

of eight possible combinations from five different "families" or classes of genotype combinations, which include: (1) two homozygous genotypes (PP + PP + QQ or PP + QQ + QQ), (2) a homozygous + a heterozygous combination sharing one allele (PP + PP + PQ or QQ + QQ + PQ), (3) two individual homozygotes coupled with a heterozygote sharing one allele (PP + QQ + PQ), (4) a homozygote with two heterozygotes sharing one allele (PP + PQ + PQ or QQ + PQ + PQ), or (5) three homozygotes sharing both alleles (PQ + PQ + PQ).

Three allele loci have eight families of genotype combinations, four allele loci have six families, five allele loci have two families, and six allele loci possess a single family of genotype combinations. There are 15 different combinations of heterozygotes with non-overlapping alleles (e.g. PQ + RS + TU or PQ + RT + SU, and so forth). The probability of observing a particular number of alleles at each locus in a mixture is related to the allele frequencies for each locus. More polymorphic loci such as D18S51 or D2S1338 will exhibit a greater number of alleles than a less polymorphic locus such as TPOX.

Difficulties with Estimating the Number of Contributors

The most common approach to estimating the number of contributors in a mixture is through using a maximum allele count. If contributor genotypes are heterozygous at a locus and there is no overlap in the alleles observed, then there would be twice as many alleles at an examined locus as there are contributors to the mixture. Thus, seeing four alleles at one or more loci would be an indication that two people contributed to the mixture whereas observing six alleles at one or more loci would suggest that three individuals contributed to the mixed DNA sample.

Unfortunately it is not this easy. Some alleles are fairly common and thus homozygotes occur as well as overlapping heterozygous alleles. This allele sharing makes accurately deducing the number of contributors challenging − and the challenge only grows with each additional contributor to a DNA mixture. Use of a maximum likelihood estimator that incorporates allele frequency information with the maximum allele count can help somewhat with improving the accuracy of estimating the number of contributors (Haned et al. 2011a,b). A Bayesian network approach has also been used (Biedermann et al. 2012). The most recent approach is a computational tool named NOC*It* that calculates the probability distribution for the number of contributors to a DNA sample (Swaminathan et al. 2013).

The examination of mixtures with more STR loci does help with estimating the number of contributors, particularly when using loci that have a high heterozygosity rate such as D18S51, D12S391, or SE33. Table 7.2 provides a summary of the benefit from adding nine additional STR loci to the 13 core STR loci used with the U.S. Combined DNA Index System (CODIS).

While it is fairly unlikely to mistake a four-person mixture as a single-source sample (with 13 STRs, the probability in Table 7.2 is 7×10^{-25}), as the number of contributors increases so does the potential for overlap with alleles between the contributors. For example, a five-person mixture has a 41% chance of being mistaken as a three-person mixture due to having at most six alleles at any of the 13 STRs examined. By using the 22 STR loci available in the GlobalFiler STR kit, this possibility of mistaking a five-person mixture for a three-person mixture drops to a probability of occurring a mere 0.48% of the time. Note, however, that a six-contributor DNA profile (based on the maximum allele count approach) would appear like a five-person mixture 99.9% of the time using 13 STRs and only drop to 86% with 22 STRs.

TABLE 7.2 Allele Sharing Impacts Number of Contributors Determination

		1	2	3	4	5
		2 alleles	*4 alleles*	*6 alleles*	*8 alleles*	*10 alleles*
6	**CODIS 13**	1.75×10^{-40}	6.34×10^{-9}	0.161	0.946	0.999
	22 STRs	0 ($<10^{-99}$)	9.59×10^{-21}	5.32×10^{-5}	0.188	0.860
5	**CODIS 13**	9.78×10^{-33}	2.10×10^{-6}	0.414	0.990	
	CODIS22	6.36×10^{-61}	7.01×10^{-15}	0.00484	0.610	
4	**CODIS 13**	7.02×10^{-25}	0.00052	0.786		
	22 STRs	3.50×10^{-46}	3.49×10^{-9}	0.165		
3	**CODIS 13**	8.42×10^{-17}	0.05949			
	22 STRs	5.77×10^{-31}	0.00043			
2	**CODIS 13**	1.70×10^{-8}				
	22 STRs	2.05×10^{-15}				

There is a limited ability to reliably decipher the number of contributors to a mixture based on a maximum allele count observed at any STR loci tested. As more loci are evaluated, the chance of incorrectly deciphering the number of contributors is reduced. The 13 Combined DNA Index System (CODIS) STR loci are compared to the 22 STRs discussed in the possible U.S. core expansion set (Hares 2012). The values in this table were determined using U.S. Caucasian allele frequencies (Hill et al. 2013, see Appendix 1) and reflect the probability of incorrectly assigning a specific number of contributors based on the maximum number of observed alleles at one or more loci (listed across the top) compared to the true number of contributors (listed down the left side). For example, four contributors would look like three contributors (with a maximum of 6 alleles at one or more STR locus) about 79% of the time when examining results from the 13 CODIS STRs. However, adding 9 STR loci reduces the probability of four contributors appearing as three contributors from 79% to 16%. This maximum allele count method does not consider any relative peak height imbalances in DNA mixtures. Data kindly provided by Mike Coble based on work by James Curran, Jo Bright, Mike Coble, and John Buckleton.

As illustrated in Table 7.2, mixture *detectability* will improve with use of more STR loci. However, mixture *interpretation* will take longer with the larger PCR multiplexes since there is more data to review. Also the chance of some or most of the contributors being in the LTDNA stochastic realm increases as the number of mixture contributors increases.

LOW-QUANTITY AND LOW-QUALITY DNA

Low-quantity and low-quality DNA templates are subject to stochastic processes during PCR amplification, which can skew the normal stutter product amounts and heterozygote balance (Butler & Hill 2010, Butler 2012). Generally an analyst will know that the DNA profile they are evaluating has components that are in the stochastic range based on allele peak heights. However, there

is no clear distinction between LTDNA and conventional DNA profiles. Peter Gill and colleagues have written: "Because [stochastic] effects increase progressively as the amount of DNA decreases, there is no *natural* delineator that can be used to differentiate between *conventional* and *low-level* DNA profiles" (Gill et al. 2012).

Degraded DNA may exhibit both low-level DNA characteristics with the longer STR loci in a multiplex and conventional performance at the low relative molecular mass end of the electropherogram (Gill et al. 2012). Although there may be sufficient total DNA molecules available to render a DNA quantitation value in the conventional target range (e.g. 500 pg), a degraded DNA sample may have a limited number of intact molecules for the larger molecular mass STR markers. Thus, the larger STR marker targets may only be present at a sub-optimal level (e.g. 20 pg).

Replicate Testing to Form a Consensus or a Composite Profile

Results from replicate PCR amplifications can be combined to form either consensus or composite profiles (Figure 7.5). The requirement for repeated observations of an allele with the consensus method has made it a typical method of choice (Gill et al. 2000).

Replicate testing helps eliminate problems with random allele drop-in events but consumes more DNA sample (Cowen et al. 2011). One study found that consensus profiling did eliminate any drop-in spurious alleles from the final profile, but the act of subdividing an already low-quantity sample into multiple aliquots increased the total amount of allele and locus drop-out (Grisedale & van Daal 2012). There is a balance that must be struck between running as much DNA as possible in a single PCR versus splitting the sample to have replicate results, albeit from smaller amounts of DNA that could exhibit more variation and individual amplification ambiguity.

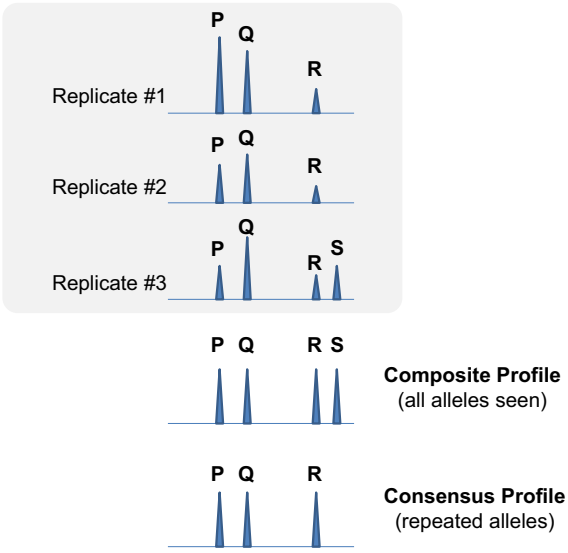

FIGURE 7.5 Replicate amplifications are often performed with low-template DNA (LTDNA) samples to aid overall result reproducibility. Detected alleles are labeled P, Q, R, and S. A composite profile contains all detected alleles (P, Q, R, and S) whereas a consensus profile only includes alleles that occur in more than one replicate amplification (P, Q, and R).

Combining the composite and consensus approaches, a team from Belgium created what they call a "Bracket" method (Bekaert et al. 2012). Clearly identifiable dominant profiles are separated from minor alleles that are enclosed in either soft () or hard [] brackets depending on their reproducibility in the replicate amplifications tested. This group typically refrains from attempting to determine minor DNA profiles due to the possibility of multiple minor contributors or extensive allele drop-out.

Challenges with Allele Drop-Out

At a particular locus, an evidentiary sample may possess one or more alleles that fail to be amplified during the PCR process due to stochastic effects. Thus, a reference sample from the suspect might contain a heterozygous genotype of PQ while the evidentiary sample only displays a P. This nonconcordance may be the result of the Q allele being lost due to allele drop-out (the prosecution's hypothesis) or the real perpetrator is homozygous PP and the evidence does not have a Q allele because the PQ suspect did not commit the crime (the defense's hypothesis).

There are several other possible explanations as well that include: (a) the real perpetrator is a heterozygote PF where F represents another allele besides Q that has dropped out; in this situation, P is considered a true allele and not due to drop-in; (b) the real perpetrator is a homozygote FF (not Q), but the F allele has dropped out, and the P allele is due to allele drop-in; or (c) the real perpetrator is a heterozygote FF′ but these alleles have dropped out and the P allele is due to allele drop-in (Gill et al. 2012).

To address these possible explanations, the probability of drop-out, or Pr(D), and the probability of drop-in due to contamination, or Pr(C), have to be determined. It should also be demonstrated that Pr(D) and Pr(C) are consistent over time if constant values are going to be used in probabilistic approaches. One of the principles advocated by Peter Gill in his 2012 report to the UK Regulator is that "interpretation methodology should incorporate a probabilistic consideration of drop-out and additional alleles, such as drop-in, stutters, gross contamination and additional contributors" (Gill et al. 2012).

Assessing the Probability of Drop-Out

The Pr(D) is an estimate of how often alleles are expected to fail to amplify under certain PCR amplification conditions typically involving low-template DNA amounts. The Forensic Science Service introduced the concepts of allele drop-out and allele drop-in in their 2000 landmark LCN paper (Gill et al. 2000). However, almost a decade went by before statistical methods and interpretation approaches began to put these concepts into practice (Gill et al. 2009, Gill & Buckleton 2010). An important point in determining and using the Pr(D) concept is that alleles with lower peak heights have a greater chance of a sister allele in the allele pair failing to amplify due to stochastic effects if a heterozygous genotype is truly present.

Torben Tvedebrink and colleagues from the University of Copenhagen demonstrated a useful method for assigning the probability of drop-out on a per locus basis using logistical regression and the heights of the surviving sister allele in a heterozygote (Tvedebrink et al. 2009). Table 7.3 lists the allele drop-out probabilities with various STR loci at specific peak height levels.

TABLE 7.3 Average Peak Heights (in Relative Fluorescence Units) for Various Allele Drop-Out Probabilities [Pr(D)] across 10 STR Loci Present in the SGM Plus Kit

Pr(D)	D3S1358	vWA	D16S539	D2S1338	D8S1179	D21S11	D18S51	D19S433	TH01	FGA	Overall
0.0001	556	577	622	562	558	461	531	722	723	692	648
0.0005	384	399	430	388	385	318	367	499	499	478	439
0.001	327	340	366	331	328	271	313	425	426	407	371
0.005	226	235	253	228	226	187	216	293	294	281	251
0.01	192	200	215	194	193	159	184	250	250	239	212
0.05	132	137	147	133	132	109	126	171	171	164	142
0.1	111	115	124	112	111	92	106	144	144	138	119
0.2	92	95	103	93	92	76	88	119	120	114	98
0.3	81	84	91	82	81	67	78	105	106	101	86
0.4	73	76	82	74	74	61	70	95	95	91	77
0.5	67	69	75	68	67	55	64	87	87	83	70
0.6	61	63	68	62	61	50	58	79	79	76	63
0.7	55	57	62	56	55	46	53	71	71	68	57
0.8	49	50	54	49	49	40	46	63	63	60	50
0.9	40	42	45	41	40	33	39	52	52	50	41
0.95	34	35	38	34	34	28	32	44	44	42	34
0.99	23	24	26	23	23	19	22	30	30	29	23

Based on the data used to produce this model of allelic behavior, if a D18S51 peak was detected at 126 RFU, there is about a 5% chance of the sister allele in a heterozygote dropping-out (i.e. being below a 50 RFU detection threshold). Data reproduced with permission from Tvedebrink et al. (2009).

Monitoring Allele Drop-In Rates

Increased sensitivity with newer STR kits means that contamination risks are real. Sometimes consumables such as pipet tips and PCR tubes are not fully DNA-free (Gill et al. 2010). Allele drop-in relates to one or two extra alleles that cannot be attributed to the known reference sample whereas gross contamination refers to the transfer or a partial or even a full DNA profile from another source (Gill et al. 2012).

The probability of drop-in, Pr(C), is commonly estimated through detection of extra alleles in negative controls where it is expected that no DNA is present. A laboratory should maintain some kind of record of any spurious alleles noted while examining negative control data in order to record drop-in and gross contamination events (Gill & Kirkham 2004, Gill et al. 2012). This record not only serves as a monitor over time but can also be used to provide data for probabilistic models.

PROBABILISTIC GENOTYPING

Given that allele drop-out and allele drop-in are real issues faced with low-template DNA and complex mixtures, movement is underway towards *probabilistic genotyping* that involves calculating the probability of potential genotype combinations at each STR locus in an effort to best explain DNA mixture results (Kelly et al. 2014). Probabilistic genotyping is often divided into two general approaches: (1) semi-continuous model and (2) fully continuous model. Both of these approaches can incorporate Pr(D) and Pr(C).

Under the *semi-continuous* approach, the presence or absence of alleles is used to make decisions about potential genotype combinations. Peak height information is not considered. The *fully continuous* model utilizes allele and peak height information along with parameters such as heterozygote balance, mixture ratios, and stutter ratios to ascertain what contributor genotypes may be present. Statistical aspects of probabilistic genotyping and software programs that utilize the semi-continuous or fully continuous models are discussed in Chapter 13.

HIGH-DENSITY SNP ARRAYS?

In August 2008, a group of scientists led by David Craig at the Translational Genomics Research Institute in Phoenix, Arizona, published an article entitled "Resolving individuals contributing trace amounts of DNA to highly complex mixtures using high-density SNP genotyping microarrays" (Homer et al. 2008). These researchers claimed that by examining 500,000 single nucleotide polymorphisms (SNPs) using high-density SNP genotyping microarrays they could identify the presence of genomic DNA of specific individuals within highly complex mixtures, including mixtures where an individual contributes less than 0.1% (1 part in a thousand) of the total genomic DNA. Their high-density SNP array method utilizes differences of individual alleles from a reference set of population allele frequencies to infer whether or not a specific individual is present in the mixture.

The authors of this paper (Homer et al. 2008) boldly claimed the utilities of their approach for identifying individual trace contributors within a forensic mixture – although the test required hundreds of nanograms of pristine DNA. Given the potential for allele drop-out at numerous loci when testing forensically relevant amounts of DNA for a few nanograms or less, the initial theoretical claims of Homer et al. (2008) have yet to be substantiated. A number of follow-up studies discussed the approach taken and its potential limitations (Visscher & Hill 2009, Braun et al. 2009, Jacobs et al. 2009, Sampson & Zhao 2009, Clayton 2010).

In a follow-up study focused on addressing potential forensic applications, Thore Egeland and several Norwegian colleagues found that it was not possible to reliably infer the presence of minor contributors to mixtures following the approach described by David Craig's team in 2008 (Egeland et al. 2012). Egeland and his colleagues prepared 25 mixtures consisting of two, three, four, or five contributors with mixture proportions ranging from 0.01 to 0.99 and examined them in a blinded experiment with 360 SNPs (rather than the 500,000 SNPs studied by Homer et al. 2008).

The Norwegian study found that major contributors and all samples with mixture proportions exceeding 0.33 could be correctly identified (Egeland et al. 2012). Of 39 people not in a tested mixture, the SNP method always correctly excluded them. However, in 11% of the cases examined, low-level mixture contributors were incorrectly assigned as non-contributors. In addition, an inconclusive

result was obtained in 24% of the cases studied. The Norwegians conclude that "contrary to Homer et al. 2008, it is not possibl[e] to accurately infer the presence of contributors to unbalanced mixtures following the suggested approach" (Egeland et al. 2012).

Dr. Kevin McElfresh, a forensic research scientist formerly of LifeCodes and Bode Technology Group, first spoke on the topic of high-density SNP typing for forensic mixtures at the October 2009 International Symposium on Human Identification (ISHI) meeting (McElfresh et al. 2009). He presented further SNP typing work with a poster presentation at the 2011 ISHI meeting (McElfresh et al. 2011) and in 2013 started a new company to pursue SNP typing, The Center for Advanced Forensic DNA Analysis™ (CAFDA 2014).

Two Israeli scientists have proposed using a panel of 1,000 to 3,000 SNPs with each SNP having a relatively low minor allele frequency in the target population (Voskoboinik & Darvasi 2011). They predict that this approach can differentiate even brothers in a mixture composed of up to 10 contributors. While, as noted in this section, there have been several theoretical studies claiming benefits of high-density SNP typing, substantiating data has been slow to be released. Only time will tell what kind of role high-density SNP typing will play in handling complex forensic DNA mixtures of the future.

MORE POOR-QUALITY SAMPLES BEING SUBMITTED

Due to the success of DNA testing and its value to the criminal justice system, many laboratories are seeing an increasing number of poor quality/quantity samples being submitted. For example, at the International Society of Forensic Genetics (ISFG) 2009 meeting, Sabine Michel from the Regional Center of Identification in Gosselies, Belgium reported that the number of samples with <100 pg of DNA had increased from 19% in 2004 to 45% in 2008 (Michel 2009). Laboratories in the United States are processing more "touch evidence" samples since the 2008 National Institute of Justice funded study on the value of using DNA to help solve burglary cases (Roman et al. 2008). This mentality of swab first and ask questions (i.e. try to interpret results) later has led to what Ted Hunt, a prosecutor from Kansas City, Missouri, terms "swab-a-thons" by the police looking for any useful DNA results (Hunt 2014).

Many laboratories have not adopted stringent case acceptance policies and are being drowned in a tsunami of sample submissions. At the same time, the ability to recover information from low-level DNA samples has increased as the sensitivity of DNA testing methods has improved. Sensitivity is in many ways a two-edge sword − although more information can be recovered, interpretation becomes more challenging and time-consuming. Contamination and potential secondary or tertiary transfer becomes a more significant issue when working to recover low-level DNA (see Chapter 16). Depending on the case context, recovering DNA information from the equivalent of a few cells may not be considered probative.

From 2008 to 2010, the Swedish National Laboratory for Forensic Science collected 2,033 samples containing low template DNA in 417 cases (Dufva & Nilsson 2011). The results were grouped into four categories: (1) a useful DNA profile relevant to the case, (2) a useful DNA profile but not relevant to the case, (3) complex, and (4) weak. A "successful" case was defined as having at least one sample with results in category 1. This Swedish study found an overall success rate of 38%. Results recovered from knives provided a useful DNA profile relevant to the case about 19% of the time (Dufva & Nilsson 2011).

At the American Academy of Forensic Sciences (AAFS) meeting in February 2014, two presentations discussed poor success rates seen in their laboratories with swabs from gun cases and other touch evidence (Bitner et al. 2014, Samples et al. 2014). Only about 10% of profiles obtained from over 9,500 touch evidence swabs received from 2007 to 2011 produced usable DNA results in New York City (Samples et al. 2014). Improving the swab collection training for the evidence collection teams and altering laboratory DNA extraction procedures have helped improve recovery in recent years, but overall their laboratory continues to see more inconclusive results than not for many types of touch evidence cases (Samples et al. 2014). A review of touch DNA items processed by the Allegheny County Office of the Medical Examiner Forensic Laboratory from 2008 to 2013 grouped sample types into firearms, magazines, baseball caps, masks, car door handles, doorknobs, shirts, and gloves (Bitner et al. 2014).

No formal analysis of the collection of DNA evidence has been performed and reported in recent years. However, based on feedback the author has received from discussions with forensic laboratories around the world, the number of complex mixtures (those containing three or more contributors) appears to have risen significantly. It is important to keep in mind that increased complexity in sample results can lead to decreased confidence in the interpretation of the evidence. Uninterpretable results lead to reporting inconclusive findings. Laboratories may want to consider increasing the stringency of their case/sample acceptance policy in order to avoid a "garbage in, garbage out" situation.

A COMPLEXITY THRESHOLD?

Some DNA mixtures will be too complex to solve. Laboratories may benefit from developing criteria for when to stop working on a sample or on a case based on a preliminary analysis of samples received. This might be termed a "complexity threshold" (Rudin & Inman 2012). One idea for creating a complexity threshold is the use of receiver operator characteristics (ROC) curves that correlate the number of false positives and false negatives under certain conditions (Gordon 2012, Grgicak 2012). For example, simulations can be run and visualized via ROC curves to determine how many non-concordant results (i.e. missing alleles in the evidence sample) are permitted before there is a chosen probability of falsely including an innocent person (Gordon 2012).

In one of their complex mixture studies, NFI proposed to develop criteria for assessing the peak heights, position of allele calls (such as in potential stutter positions), the consistency of allele calls among replicates, and a maximum number of allele drop-outs that could be considered for non-concordance (Benschop et al. 2012). Presumably studying the variability of these parameters in validation studies with known mixture contributors could lead to an effective complexity threshold.

In April 2012, an international conference was held in Rome, Italy, entitled "The hidden side of DNA profiles: artifacts, errors and uncertain evidence" (Pascali & Prinz 2012). Peter Schneider, a forensic DNA researcher from Cologne, Germany, shared his thoughts on what to do when evidence becomes too complex to reliably interpret: "If you cannot explain your evidence to someone that is not from the field (like a judge) — and you need a lot of technical excuses to report something — then the result is not good. You should leave it on your desk and not take it to court. This is a very common sense approach to this problem" (Rome 2012).

Low-level DNA and complex mixtures are challenging to interpret. Not every DNA result can or should be interpreted. When there is a high degree of interpretation uncertainty from an evidentiary

sample, it makes little sense to try and draw conclusions (either inclusion *or* exclusion of reference samples) — and expect those conclusions to be reliable.

With stochastic effects ever-present in low-level DNA PCR amplifications, allele drop-out and potential allele sharing from multiple contributors lead to greater uncertainty in the specific genotype combinations that can be reliably assumed. Furthermore, stochastic thresholds often lose their value and meaning when allele sharing is possible with three or more contributors to a DNA mixture. Probabilistic genotyping approaches (see Chapter 13) using computer simulations that estimate relative contributions of potential contributors are being developed to try and explain observed data.

Another approach may be to develop a complexity threshold in order to halt efforts on poor quality data. Sensitive DNA detection technology has the potential to outpace reliable interpretation. The forensic DNA community needs to be vigilant in efforts to appropriately interpret challenging evidence without pushing too far.

Reading List and Internet Resources

Interpretation Challenges

Anjos, M. J., et al. (2006). Low copy number: interpretation of evidence results. *Progress in Forensic Genetics 10, ICS, 1288*, 616–618.

Anslinger, K., & Bayer, B. (2011). The effect of increased cycle numbers using the Quantifiler Duo DNA Quantitation Kit (AB) on the detection of minute amounts of male DNA in mixtures and its application in routine case work. *Forensic Science International: Genetics Supplement Series, 3*, e277–e278.

Balding, D. J., & Buckleton, J. (2009). Interpreting low template DNA profiles. *Forensic Science International: Genetics, 4*, 1–10.

Budowle, B., et al. (2009). Validity of low copy number typing and applications to forensic science. *Croatian Medical Journal, 50*, 207–216.

Butler, J. M., & Hill, C. R. (2010). Scientific issues with analysis of low amounts of DNA. *Profiles in DNA, 13*(1). Available at http://www.promega.com/profiles/1301/1301_02.html. Accessed March 24, 2014.

Butler, J. M. (2012). Chapter 11 — Low-Level DNA testing: issues, concerns, and solutions. In *Advanced Topics in Forensic DNA Typing: Methodology* (pp. 311–346). San Diego: Elsevier.

Caragine, T., et al. (2009). Validation of testing and interpretation protocols for low template DNA samples using AmpFlSTR Identifiler. *Croatian Medical Journal, 50*(3), 250–267.

Carracedo, A., et al. (2012). Focus issue — Analysis and biostatistical interpretation of complex and low template DNA samples. *Forensic Science International: Genetics, 6*(6), 677–678.

Cowen, S., et al. (2011). An investigation of the robustness of the consensus method of interpreting low-template DNA profiles. *Forensic Science International: Genetics, 5*, 400–406.

Decorte, R., et al. (2011). An automated approach for generating consensus profiles from low template STR typing results. *Forensic Science International: Genetics Supplement Series, 3*, e435–e436.

Forster, L., et al. (2008). Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle 'low copy number' (LCN) method for analysis of trace forensic DNA samples. *Forensic Science International: Genetics, 2*, 318–328.

Fregeau, C. J., et al. (1995). Characterization of human lymphoid cell lines GM9947 and GM9948 as intra- and interlaboratory reference standards for DNA typing. *Genomics, 28*, 184–197.

Gill, P., et al. (2000). An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Science International, 112*, 17–40.

Gill, P., et al. (2006). DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Science International, 160*, 90–101.

Gill, P., et al. (2007). LoComatioN: a software tool for the analysis of low copy number DNA profiles. *Forensic Science International, 166*, 128–138.

Gill, P., et al. (2008). Interpretation of complex DNA profiles using empirical models and a method to measure their robustness. *Forensic Science International: Genetics, 2*, 91–103.

Gill, P., & Buckleton, J. (2010). A universal strategy to interpret DNA profiles that does not require a definition of *low-copy-number*. *Forensic Science International: Genetics, 4*, 221–227.
Gill, P., et al. (2012). The interpretation of DNA evidence (including low-template DNA). Available at http://www.homeoffice.gov.uk/publications/agencies-public-bodies/fsr/interpretation-of-dna-evidence. Accessed March 24, 2014.
Gross, T., et al. (2009). A review of low template STR analysis in casework using the DNA SenCE post-PCR purification technique. *Forensic Science International: Genetics Supplement Series, 2*, 5–7.
Kloosterman, A. D., & Kersbergen, P. (2003a). Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci. *Journal de la Société de Biologie, 197*, 351–359.
Kloosterman, A. D., & Kersbergen, P. (2003b). Efficacy and limits of genotyping low copy number DNA samples by multiplex PCR of STR loci. *Progress in Forensic Genetics 9, ICS1239*, 795–798.
Michel, S., et al. (2009). Interpretation of low-copy-number DNA profile after post-PCR purification. *Forensic Science International: Genetics Supplement Series, 2*, 542–543.
NIST Low Template DNA Testing: Available at http://www.cstl.nist.gov/strbase/LTDNA.htm. Accessed March 24, 2014.
Noël, J., et al. (2009). Searching a DNA databank with complex mixtures from two individuals. *Forensic Science International: Genetics Supplement Series, 2*, 464–465.
Overson, T. L. (2009). System and method for the deconvolution of mixed DNA profiles using a proportionately shared allele approach. *US Patent Application 2009/0270264 A1*.
Pedersen, L. M. I., et al. (2011). Purification and concentration of PCR products leads to increased signal intensities with fewer allelic drop-outs and artifacts. *Forensic Science International: Genetics Supplement Series, 3*, e347–e348.
Petricevic, S., et al. (2010). Validation and development of interpretation guidelines for low copy number (LCN) DNA profiling in New Zealand using the AmpFlSTR SGM Plus multiplex. *Forensic Science International: Genetics, 4*, 305–310.
Roeder, A. D., et al. (2009). Maximizing DNA profiling success from sub-optimal quantities of DNA: a staged approach. *Forensic Science International: Genetics, 3*, 128–137.
Romanini, C., et al. (2011). A comparison of AmpFlSTR Identifiler kit versus AmpFlSTR Identifiler Plus kit in challenging bone samples by using normal and increased PCR cycle number. *Forensic Science International: Genetics Supplement Series, 3*, e514–e515.
Smith, P. J., & Ballantyne, J. (2007). Simplified low-copy-number DNA analysis by post-PCR purification. *Journal of Forensic Sciences, 52*, 820–829.
SWGDAM. (2010). *SWGDAM interpretation guidelines for autosomal STR typing by forensic DNA testing laboratories*. Available at http://www.swgdam.org. Accessed March 24, 2014.
SWGDAM FAQs. (2014). Available at http://swgdam.org/faq.html. Accessed March 24, 2014.
Westen, A. A., et al. (2009). Higher capillary electrophoresis injection settings as an efficient approach to increase the sensitivity of STR typing. *Journal of Forensic Sciences, 54*, 591–598.
Westen, A. A., et al. (2012). Assessment of the stochastic threshold, back- and forward stutter filters and low template techniques for NGM. *Forensic Science International: Genetics, 6*, 708–715.
Weusten, J., & Herbergs, J. (2012). A stochastic model of the processes in PCR based amplification of STR DNA in forensic applications. *Forensic Science International: Genetics, 6*, 17–25.
Whitaker, J. P., et al. (2001). A comparison of the characteristics of profiles produced with the AMPFlSTR SGM Plus multiplex system for both standard and low copy number (LCN) STR DNA analysis. *Forensic Science International, 123*, 215–223.
Word, C. J. (2011). Mixture interpretation: why is it sometimes so hard? *Profiles in DNA*. Available at http://www.promega.com/resources/profiles-in-dna/2011/mixture-interpretation-why-is-it-sometimes-so-hard/. Accessed March 24, 2014.

Low–Level Complex DNA Mixtures

Bekaert, B., et al. (2012). Automating a combined composite – consensus method to generate DNA profiles from low and high template mixture samples. *Forensic Science International: Genetics, 6*(5), 588–593.
Benschop, C. C., et al. (2011). Low template STR typing: effect of replicate number and consensus method on genotyping reliability and DNA database search results. *Forensic Science International: Genetics, 5*, 316–328.
Benschop, C. C. G., et al. (2012). Assessment of mock cases involving complex low template DNA mixtures: a descriptive study. *Forensic Science International: Genetics, 6*(6), 697–707.
Benschop, C. C. G., et al. (2013). Consensus and pool profiles to assist in the analysis and interpretation of complex low template DNA mixtures. *International Journal of Legal Medicine, 127*, 11–23.
Bright, J. A., et al. (2012). Composite profiles in DNA analysis. *Forensic Science International: Genetics, 6*, 317–321.

Buckleton, J. (2009). Validation issues around DNA typing of low level DNA. *Forensic Science International: Genetics, 3*, 255–260.
Budimlija, Z. M., & Caragine, T. A. (2012). Interpretation guidelines for multilocus STR forensic profiles from low template DNA samples. *Methods in Molecular Biology, 830*, 199–211 [Alonso, A. (ed.), *DNA Electrophoresis Protocols for Forensic Genetics*].
Haned, H., et al. (2012). Exploratory data analysis for the interpretation of low template DNA mixtures. *Forensic Science International: Genetics, 6*, 762–774.
Kelly, H., et al. (2012). The interpretation of low level DNA mixtures. *Forensic Science International: Genetics, 6*, 191–197.
Mitchell, A. A., et al. (2011). Likelihood ratio statistics for DNA mixtures allowing for drop-out and drop-in. *Forensic Science International: Genetics Supplement Series, 3*, e240–e241.
Mitchell, A. A., et al. (2012). Validation of a DNA mixture statistics tool incorporating allelic drop-out and drop-in. *Forensic Science International: Genetics, 6*, 749–761.
Pfeifer, C., et al. (2012). Comparison of different interpretation strategies for low template DNA mixtures. *Forensic Science International: Genetics, 6*(6), 716–722.
Prieto, L., et al. (2014). Euroforgen-NoE collaborative exercise on LRmix to demonstrate standardization of the interpretation of complex DNA profiles. *Forensic Science International: Genetics, 9*, 47–54.
Weiler, N. E. C., et al. (2012). Extended PCR conditions to reduce drop-out frequencies in low template STR typing including unequal mixtures. *Forensic Science International: Genetics, 6*, 102–107.
Wetton, J. H., et al. (2011). Analysis and interpretation of mixed profiles generated by 34 cycle SGM Plus amplification. *Forensic Science International: Genetics, 5*(5), 376–380.

Determining Number of Contributors

Biedermann, A., et al. (2012). Inference about the number of contributors to a DNA mixture: comparative analyses of a Bayesian network approach and the maximum allele count method. *Forensic Science International: Genetics, 6*, 689–696.
Buckleton, J. S., et al. (2007). Towards understanding the effect of uncertainty in the number of contributors to DNA stains. *Forensic Science International: Genetics, 1*, 20–28.
Egeland, T., et al. (2003). Estimating the number of contributors to a DNA profile. *International Journal of Legal Medicine, 117*, 271–275.
Haned, H., et al. (2011a). The predictive value of the maximum likelihood estimator of the number of contributors to a DNA mixture. *Forensic Science International: Genetics, 5*(5), 281–284.
Haned, H., et al. (2011b). Estimating the number of contributors to forensic DNA mixtures: does maximum likelihood perform better than maximum allele count? *Journal of Forensic Sciences, 56*(1), 23–28.
Hares, D. R. (2012). Expanding the CODIS core loci in the United States. *Forensic Science International: Genetics, 6*(1), e52–e54.
Hill, C. R., et al. (2013). U.S. population data for 29 autosomal STR loci. *Forensic Science International: Genetics, 7*, e82–e83.
Lauritzen, S. L., & Mortera, J. (2002). Bounding the number of contributors to mixed DNA stains. *Forensic Science International, 130*, 125–126.
Paoletti, D. R., et al. (2005). Empirical analysis of the STR profiles resulting from conceptual mixtures. *Journal of Forensic Sciences, 50*, 1361–1366.
Paoletti, D. R., et al. (2012). Inferring the number of contributors to mixed DNA profiles. *IEEE/ACM Transactions on Computational Biology and Bioinformatics, 9*(1), 113–122.
Perez, J., et al. (2011). Estimating the number of contributors to two-, three-, and four-person mixtures containing DNA in high template and low template amounts. *Croatian Medical Journal, 52*(3), 314–326.
Presciuttini, S., et al. (2003). Allele sharing in first-degree and unrelated pairs of individuals in the Ge. F.I. AmpFlSTR Profiler Plus database. *Forensic Science International, 131*, 85–89.
Swaminathan, H., et al. (2013). NOC*It* – a computational tool to infer the number of contributors to a forensic DNA sample. Available at. In *Proceedings of the 24th International Symposium on Human Identification*. http://www.promega.com/products/pm/genetic-identity/ishi-conference-proceedings/24th-ishi-oral-presentations/. Accessed March 24, 2014.
Tvedebrink, T., et al. (2012). Identifying contributors of DNA mixtures by means of quantitative information of STR typing. *Journal of Computational Biology, 19*(7), 887–902.
Tvedebrink, T. (2012). On the exact distribution of the numbers of alleles in DNA mixtures. *International Journal of Legal Medicine, 128*(3), 427–437.

Low-Quantity and Low-Quality DNA

Benschop, C. C. G., et al. (2010). Low template STR typing: effect of replicate number and consensus method on genotyping reliability and DNA database search results. *Forensic Science International: Genetics, 5*(4), 316–328.

Buckleton, J., et al. (2014). Utilising allelic dropout probabilities estimated by logistic regression in casework. *Forensic Science International: Genetics, 9*, 9–11.

Butler, J. M. (2012). Low-level DNA testing: issues, concerns, and solutions. Chapter 11. In *Advanced Topics in Forensic DNA Typing: Methodology* (pp. 311–346). San Diego: Elsevier Academic Press.

Gill, P., et al. (2000). An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Science International, 112*, 17–40.

Gill, P., & Kirkham, A. (2004). Development of a simulation model to assess the impact of contamination in casework using STRs. *Journal of Forensic Sciences, 49*, 485–491.

Gill, P., et al. (2009). The low-template-DNA (stochastic) threshold–its determination relative to risk analysis for national DNA databases. *Forensic Science International: Genetics, 3*, 104–111.

Gill, P., et al. (2010). Manufacturer contamination of disposable plastic-ware and other reagents – an agreed position statement by ENFSI, SWGDAM and BSAG. *Forensic Science International: Genetics, 4*, 269–270.

Grisedale, K. S., & van Daal, A. (2012). Comparison of STR profiling from low template DNA extracts with and without the consensus profiling method. *Investigative Genetics, 3*, 14.

Hedman, J., et al. (2011). Evaluation of three new forensic DNA profiling kits on PCR-inhibitory crime scene samples. *Forensic Science International: Genetics Supplement Series, 3*, e457–e458.

Tvedebrink, T., et al. (2009). Estimating the probability of allelic drop-out of STR alleles in forensic genetics. *Forensic Science International: Genetics, 3*, 222–226.

Tvedebrink, T., et al. (2011). Statistical model for degraded DNA samples and adjusted probabilities for allelic drop-out. *Forensic Science International: Genetics Supplement Series, 3*, e489–e491.

Tvedebrink, T., et al. (2012). Statistical model for degraded DNA samples and adjusted probabilities for allelic drop-out. *Forensic Science International: Genetics, 6*, 97–101.

Tvedebrink, T., et al. (2012). Allelic drop-out probabilities estimated by logistic regression – further considerations and practical implementation. *Forensic Science International: Genetics, 6*, 263–267.

Probabilistic Genotyping Approach

Ballantyne, J., Hanson, E. K., & Perlin, M. W. (2012). DNA mixture genotyping by probabilistic computer interpretation of binomially-sampled laser captured cell populations: combining quantitative data for greater identification information. *Science & Justice, 53*(2), 103–114.

Cowell, R. G., et al. (2008). Probabilistic modelling for DNA mixture analysis. *Forensic Science International: Genetics Supplement Series, 1*, 640–642.

Cowell, R. G., et al. (2011). Probabilistic expert systems for handling artifacts in complex DNA mixtures. *Forensic Science International: Genetics, 5*(3), 202–209.

Curran, J. M. (2008). A MCMC method for resolving two-person mixtures. *Science & Justice, 48*, 168–177.

Gill, P., & Buckleton, J. (2010). Commentary on: Budowle B, Onorato AJ, Callaghan TF, Della Manna A, Gross AM, Guerrieri RA, Luttman JC, McClure DL. Mixture interpretation: defining the relevant features for guidelines for the assessment of mixed DNA profiles in forensic casework. *J Forensic Sci 2009, 54*(4), 810–821. Journal of Forensic Sciences, 55(1), 265–268.

Gill, P., et al. (2012). DNA Commission of the International Society of Forensic Genetics: Recommendations on the evaluation of STR typing results that may include drop-out and/or drop-in using probabilistic methods. *Forensic Science International: Genetics, 6*(6), 679–688.

Gill, P., & Haned, H. (2013). A new methodological framework to interpret complex DNA profiles using likelihood ratios. *Forensic Science International: Genetics, 7*, 251–263.

Kelly, H., et al. (2014). A comparison of statistical models for the analysis of complex forensic DNA profiles. *Science & Justice, 54*, 66–70.

Perlin, M. W., et al. (2011). Validating TrueAllele DNA mixture interpretation. *Journal of Forensic Sciences, 56*(6), 1430–1447.

Perlin, M. W. (2012). Easy reporting of hard DNA: computer comfort in the courtroom. *Forensic Magazine, 9*(4), 32–37. Available at http://www.cybgen.com/information/publication/page.shtml. Accessed March 24, 2014.

High-Density SNP Arrays?

Braun, R., et al. (2009). Needles in the haystack: identifying individuals present in pooled genomic data. *PLoS Genetics, 5*(10), 1–8.

The Center for Advanced Forensic DNA Analysis™. Available at http://advancedforensicdna.com/. Accessed March 24, 2014.

Clayton, D. (2010). On inferring presence of an individual in a mixture: a Bayesian approach. *Biostatistics, 11*(4), 661–673.

Egeland, T., et al. (2012). Complex mixtures: a critical examination of a paper by Homer, et al. *Forensic Science International: Genetics, 6*, 64–69.

Homer, N., et al. (2008). Resolving individuals contributing trace amounts of DNA to highly complex mixtures using high-density SNP genotyping microarrays. *PLoS Genetics, 4*(8), 1–9.

Jacobs, K. B., et al. (2009). A new statistic and its power to infer membership in a genome-wide association study using genotype frequencies. *Nature Genetics, 41*(11), 1253–1257.

McElfresh, K. C., et al. (2009). Utilizing 300,000 or more single nucleotide polymorphisms for the identification of individuals within a forensic mixture. In *Proceedings of the 20th International Symposium on Human Identification*. Available at http://www.promega.com/~/media/files/resources/conference%20proceedings/ishi%2020/oral%20presentations/mcelfresh.pdf?la=en. Accessed March 24, 2014.

McElfresh, K. C., et al. (2011). Utilizing ultra-high-density SNP arrays to analyze forensic mixtures: an operational assessment. In *Proceedings of the 22th International Symposium on Human Identification*. Available at http://www.promega.com/~/media/files/resources/conference%20proceedings/ishi%2021/poster%20abstracts/poster_24.pdf?la=en. Accessed March 24, 2014.

Sampson, J., & Zhao, H. (2009). Identifying individuals in a complex mixture of DNA with unknown ancestry. *Statistical Applications in Genetics and Molecular Biology, 8*(1), 37.

Visscher, P. M., & Hill, W. G. (2009). The limits of individual identification from sample allele frequencies: theory and statistical analysis. *PLoS Genetics, 5*(10), 1–6.

Voskoboinik, L., & Darvasi, A. (2011). Forensic identification of an individual in complex DNA mixtures. *Forensic Science International: Genetics, 5*, 428–435.

Poor-Quality Samples Being Submitted

Bitner, S. E., et al. (2014). A touch-ing retrospective: a review of five years of touch DNA samples. *Proceedings of the American Academy of Forensic Sciences 66th Annual Scientific Meeting, 20*, 98–99.

Dieltjes, P., et al. (2011). A sensitive method to extract DNA from biological traces present on ammunition for the purpose of genetic profiling. *International Journal of Legal Medicine, 125*, 597–602.

Dufva, C., & Nilsson, A. (2011). Success rate of LT DNA analyses in casework. *Forensic Science International: Genetics Supplement Series, 3*, e271–e272.

Hunt, T. R. (2014). It's rapid, but is it relevant? Balancing speed and evidentiary significance in the coming age of real-time DNA analysis. *Proceedings of the American Academy of Forensic Sciences 66th Annual Scientific Meeting, 20*, 265–266.

Irwin, J. A., et al. (2007). Application of low copy number STR typing to the identification of aged, degraded skeletal remains. *Journal of Forensic Sciences, 52*, 1322–1327.

Pizzamiglio, M., et al. (2004). Forensic identification of a murderer by LCN DNA collected from the inside of the victim's car. *Progress in Forensic Genetics, 10*(ICS 1261), 437–439.

Prinz, M., et al. (2006). Maximization of STR DNA typing success for touched objects. *Progress in Forensic Genetics, 11*(ICS 1288), 651–653.

Raymond, J. J., et al. (2008). Trace DNA analysis: do you know what your neighbour is doing? a multi-jurisdictional survey. *Forensic Science International: Genetics, 2*, 19–28.

Raymond, J. J., et al. (2009). Trace DNA success rates relating to volume crime offences. *Forensic Science International: Genetics Supplement Series, 2*, 136–137.

Raymond, J. J., et al. (2009). Trace evidence characteristics of DNA: a preliminary investigation of the persistence of DNA at crime scenes. *Forensic Science International: Genetics, 4*, 26–33.

Roman, J. K., et al. (2008). *The DNA field experiment: cost-effectiveness analysis of the use of DNA in the investigation of high-volume crimes*. Washington, DC: Urban Institute Justice Policy Center. Available at https://www.ncjrs.gov/pdffiles1/nij/grants/222318.pdf. Accessed March 24, 2014.

Samples, M., Vasquez, T. L., & Umback, N. J. (2014). What's in a name? That which we call 'touch DNA' perhaps should be split in twain. *Proceedings of the American Academy of Forensic Sciences 66th Annual Scientific Meeting, 20*, 96.

Complexity Threshold

Geddes, L. (2010). Fallible DNA evidence can mean prison or freedom. *New Scientist*, (issue 2773). Available at http://www.newscientist.com/article/mg20727733.500-fallible-dna-evidence-can-mean-prison-or-freedom.html. Accessed March 24, 2014.

Gordon, J. S. (2012). Characterization of error tradeoffs in human identity comparisons: determining a complexity threshold for DNA mixture interpretation. *Unpublished master's thesis for Boston University School of Medicine Forensic Science Program*.

Grgicak, C. M. (2012). Complexity thresholds and exclusion criteria. In *Presentation at ISHI 2012 Mixture Interpretation Workshop*. Available at http://www.cstl.nist.gov/strbase/training/ISHI2012-MixtureWorkshop-ComplexityThreshold.pdf. Accessed March 24, 2014.

Pascali, V., & Prinz, M. (2012). Highlights of the conference "The hidden side of DNA profiles: Artifacts, errors and uncertain evidence." *Forensic Science International: Genetics*, 6, 775–777.

Rome meeting (2012). Video of conference entitled "The hidden side of DNA profiles: artifacts, errors and uncertain evidence" kindly provided by Vince Pascali to Author following the April 27–28, 2012 meeting.

Rudin, N., & Inman, K. (2012). The discomfort of thought – a discussion with John Butler. *The CACNews. 1st Quarter, 2012*, 8–11.