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TECHNICAL NOTE

CRIMINALISTICS

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The Effect of Cleaning Agents on the Ability to Obtain DNA Profiles Using the Identifiler™ and PowerPlex® Y Multiplex Kits

ABSTRACT: A year after the introduction of Identifiler™ into the forensic DNA laboratories of the Institute of Environmental Science and Research Limited (ESR), increasing occurrences of dropout of the three loci, D7S820, D18S51, and FGA, were observed in samples where the DNA was not degraded and sufficient DNA was present that full DNA profiles were to be expected. The dropout was either partial or complete at these loci. Full profiles could sometimes be obtained by reamplification of samples using the same input amount of DNA. After a thorough investigation of the methods and procedures used in the laboratory, the cause of this inhibition was identified as the cleaning agent TriGene™ ADVANCE. This was determined after the deliberate addition of varying amounts of different cleaning reagents into the DNA amplification reactions. At concentrations of 0.004% TriGene™ ADVANCE caused inhibition resulting in tri-loci dropout. At concentrations of 0.04% and higher, complete inhibition was observed. An effect was also seen on the amplification of samples using the Y STR profiling system PowerPlex®Y. This work highlights the importance of checking all reagents and chemicals prior to use, even those with no apparent direct influence on the DNA profiling process.

KEYWORDS: forensic science, DNA typing, Identifiler™, PowerPlex®Y, inhibition, allelic dropout, TriGene™

A year after the introduction of Identifiler™ (Applied Biosystems, Foster City, CA; [1]) into our laboratory, we observed a gradual increase in the prevalence of dropout of the loci D7S820, D18S51, and FGA. By dropout, we mean the partial or complete absence of amplified products at these loci. This dropout was observed in amplified extracts of reference, casework, and external proficiency test samples with otherwise sufficient measured quantities of genomic DNA as determined by Quantifiler™ (Applied Biosystems). In the affected samples, the amplification of the remaining loci was as expected with allelic peaks typically >1000 RFU when 1.5 ng of DNA was amplified. The dropout of the affected loci ranged from significantly reduced peak heights at the affected loci, to imbalance of heterozygote peaks greater than the laboratory's 60% imbalance threshold, to complete dropout of all alleles at all three loci. These alleles would often be recovered by reamplification of the same sample extracts under identical conditions with identical amounts of extracted DNA added. The samples did not appear to be affected by degradation.

Of primary concern was that, if unrecognized, such dropout, especially that causing severe allele imbalance, could cause a mismatch when reference samples were compared with casework samples, as a heterozygous locus may be designated as a homozygote at the affected loci, leading to a false exclusion.

After ruling out other factors, such as a problem with the Identifiler™ multiplex kit components, we determined that the most likely cause was inhibition of the Identifiler™ reaction by as yet unknown mechanism. It has previously been shown that some Identifiler™ loci are more adversely affected by small changes in the amplification reaction, such as varying concentrations of $MgCl_2$, than other loci (1). We have observed that the loci D7S820, D18S51, and FGA are often affected by inhibition. We were faced with two alternatives:

- Determine the cause of inhibition and remove it from the laboratory; or
- Accept inhibition may be present and overcome it by altering the amplification reaction.

We undertook a stepwise analysis of all procedural steps in an attempt to determine the cause of the allelic dropout that was occurring to determine whether removal or remediation of the inhibition was the best alternative.

Materials and Methods

The standard methods used in our laboratory can be summarized as follows:

Reference samples from individuals were extracted using Chelex® (Bio-Rad, Hercules, CA; [2]) in a semiautomated manner on a Xiril 150-2-4 liquid handling robot (Xiril AG, Zurich, Switzerland).

Casework samples (typically blood, semen, saliva, and trace samples) were extracted using DNA IQ™ (Promega Corp., Madison, WI; [3–6]) according to the manufacturer's instructions using either a manual approach or on a Microlab® Hamilton (Bonaduz, Switzerland) STARlet liquid handling robot. Differential extraction of semen stained samples was carried out with Differex™ (Promega Corp.; [7]) and DNA IQ™ or, prior to the implementation of Differex™, with a differential lysis procedure and organic extraction method (8–10).

Quantitation of all samples was carried out with Quantifiler™ on 7500 instruments according to the manufacturer's instructions (11).

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Amplification was with Identifiler™ according to the manufacturer's instructions in a 9700 thermal cycler with silver block. The optimum amount of template DNA added to these reactions was 1.5 ng. Alternatively, amplification with PowerPlex®Y (Promega Corp.; [12]) was carried out according to the manufacturer's instructions using 0.5 ng template DNA.

Amplified products were separated on 3130*xl* Genetic Analysers (Applied Biosystems), and duplicate analysis of DNA profiles was accomplished with the GeneMapper™ *ID* version 3.2 (Applied Biosystems) and FaSTR DNA software systems (13).

Addition of Cleaning Compounds

TriGene™ ADVANCE (MediChem International Ltd., Kent, U.K.) and Virkon® (DuPont, Wilmington, DE) were prepared according to the manufacturer's instructions and added at various concentrations (ranging from 0.04% to 0.0004%) to amplification reactions as described in the results. Amplifications performed as part of the investigative process were using the positive control supplied with the Identifiler™ amplification kit, 9947A, an internal positive control extracted from liquid whole blood using DNA IQ™, or the PowerPlex®Y male positive control supplied with the kit.

Results and Discussion

An Identifiler™ electropherogram showing the characteristic tri-loci dropout can be seen in Fig. 1 and a "recovered" electropherogram of the same sample in Fig. 2. By recovered, we mean reamplification of the sample extract using the same amount of template DNA resulting in a "full" Identifiler™ profile.

We considered it unlikely that the sample type had an effect because reference samples (predominantly saliva samples on FTA card) and casework samples of all types were similarly affected. Likewise, because several different DNA extraction methods had been used, we considered it unlikely that the extraction methods themselves were the cause. No correlation was found between the dropout and particular sample types, extraction methodology, or substrate.

The results obtained from Quantifiler™ were also reviewed. There was no correlation between the quantitation results for the affected samples and the dropout observed following amplification. In particular, there was no apparent relationship between inhibition detected in DNA profiles and the results of the internal polymerase chain reaction (PCR) control in affected samples. Re-running of affected samples on the 3130*xl* Genetic Analysers did not recover alleles demonstrating that the postamplification step was not responsible, in particular there was no 3130 reagent or hardware effect. This was confirmed by re-running samples at an offsite laboratory where the same profiles, with the tri-loci dropout were obtained.

As part of the stepwise investigative process, the amplification reaction itself was investigated. The methodology, setup, and temperature verification of the 9700 thermal cyclers was checked and found to be in order. There was no well position effect. The work performance of each technician that carried out manual and

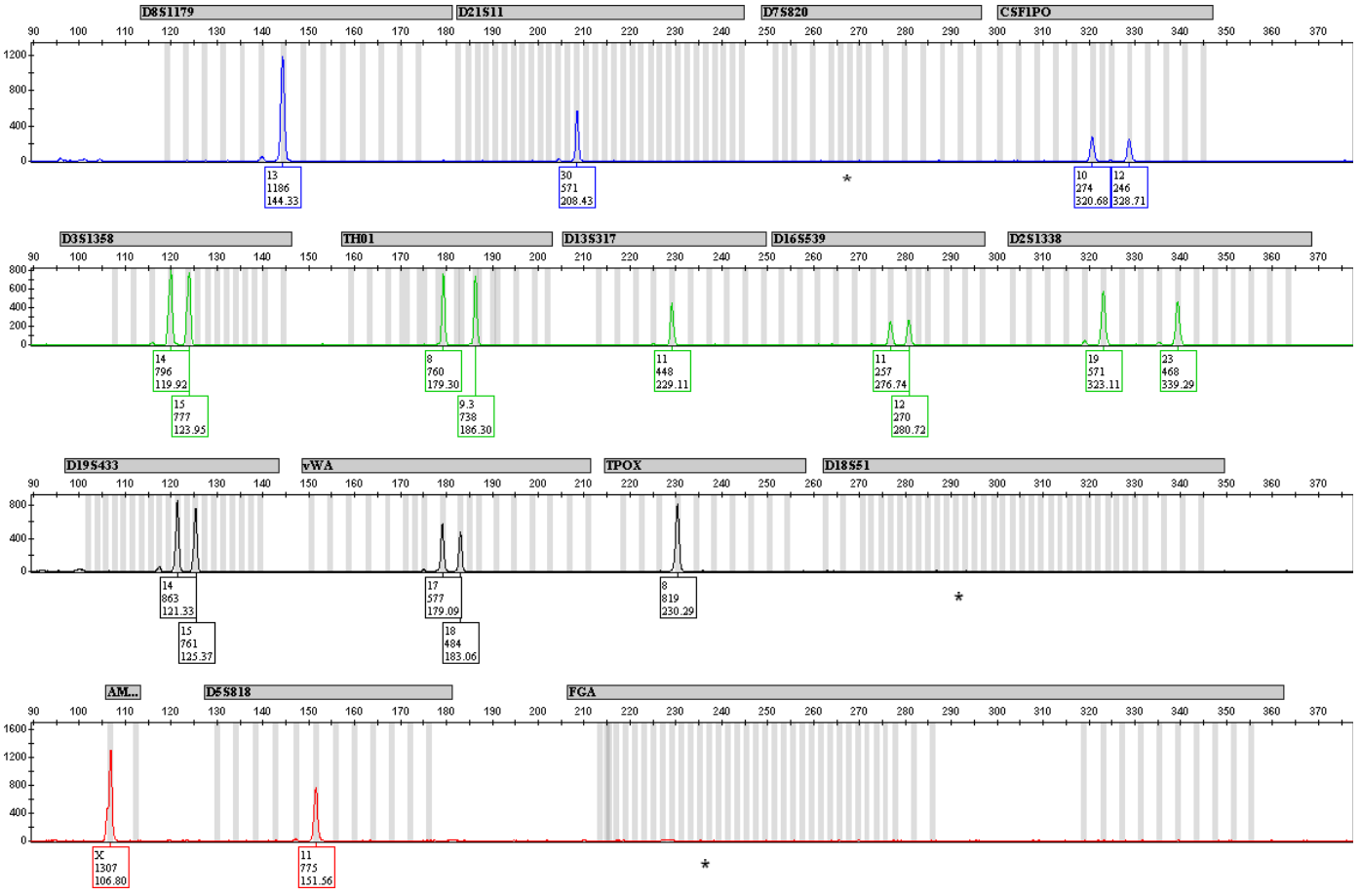

FIG. 1—*An Identifiler™ electropherogram showing the characteristic tri-loci dropout at the loci D7S820, D18S51, and FGA, as shown by the locus markers above the profile.*

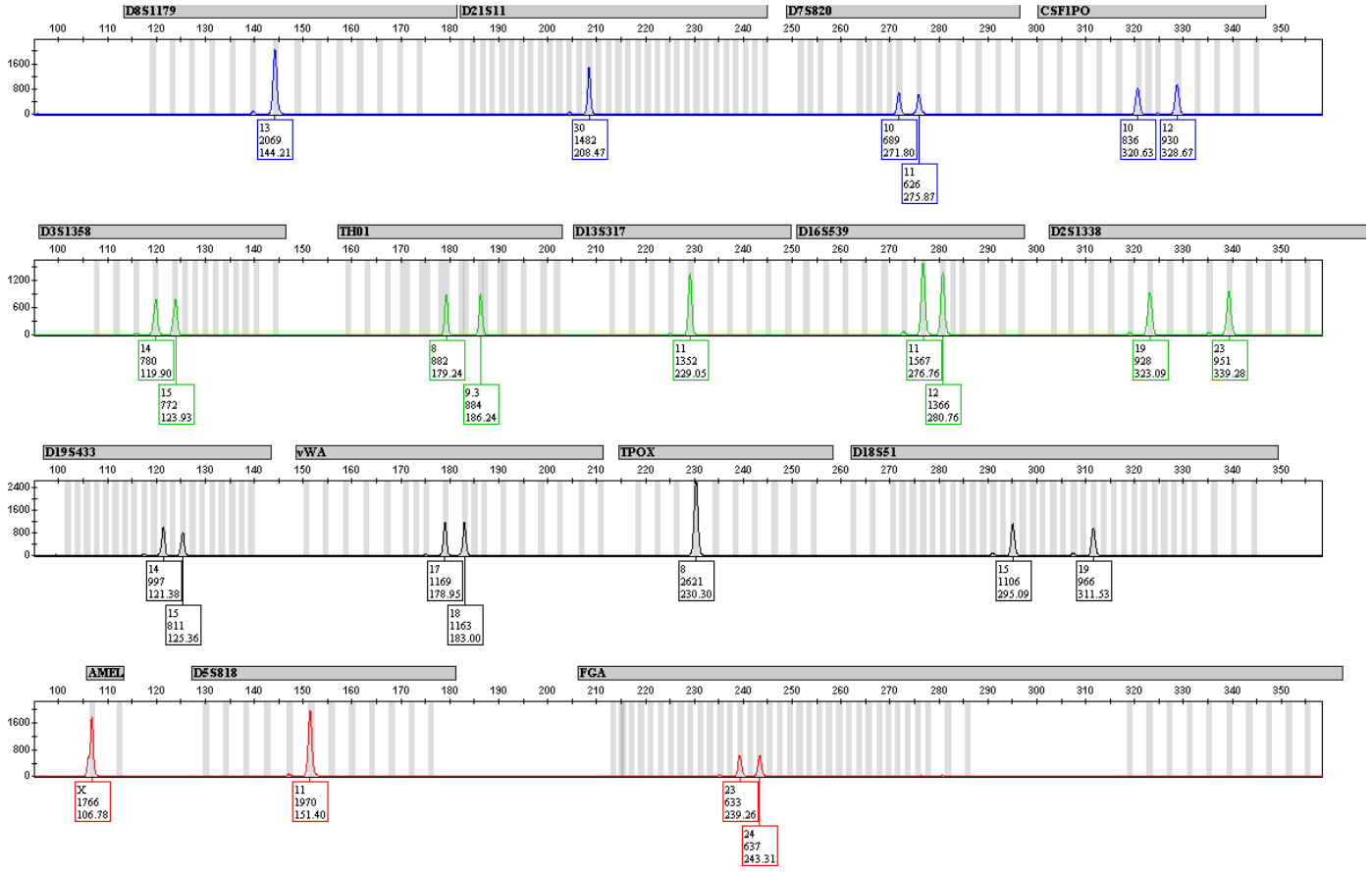

FIG. 2—*An Identifiler™ electropherogram showing the recovery of the loci D7S820, D18S51, and FGA as shown by the locus markers above the profile.*

automated amplification steps was assessed and no correlations were found. Amplifications in tube and plate formats were similarly affected, demonstrating that amplification format was not responsible for the dropout.

As Identifiler™ was generally known to be susceptible to inhibition and small variations in temperature during amplification, presumably as a result of the balancing of 16 primers, we formed the view that inhibition of the amplification reaction was the most likely cause of the dropout.

The lot numbers of the Identifiler™ multiplex kits used and the quality control testing of the kits at the Institute of Environmental Science and Research Limited (ESR) prior to their use were reviewed, and no correlation was identified.

We investigated in detail reagents other than the amplification kit that were common to both the reference sample and casework workstreams. One such reagent was TE buffer (10 mM Tris HCl, 0.1 mM EDTA) in which samples are diluted prior to PCR as recommended by Applied Biosystems. Historically, we used buffer that had been made within our laboratory, and quality-control tested before use. As part of this investigation, we purchased ready-made TE buffer (Invitrogen, Carlsbad, CA) and although use of this commercial TE buffer did appear to overcome the problem in the short term, within 3 weeks dropout was again observed in the data generated.

Adding an additional 1 μL of AmpliTaq Gold® DNA polymerase (Applied Biosystems) to the Identifiler™ reaction of affected samples was tested as this had previously been suggested as a mechanism to overcoming inhibition (14). This had no apparent effect on alleviating dropout. Dilution of samples prior to amplification had previously been established as an effective way to recover DNA profiles from samples exhibiting inhibition at quantitation (15). Again, this strategy did not recover the missing loci.

We have examined the DNA sequences of the short tandem repeats that are part of the Identifiler™ multiplex. There are no significant differences in the repeat sequence nor the repeat structure between the three affected loci and the remaining Identifiler™ loci. As primer sequences are not available to us for the Identifiler™ multiplex, we are unable to comment on whether there are differences in the guanine-cytosine content or other factors that may have affected the DNA profiling success of these particular loci.

During the investigation described earlier, it became clear that Identifiler™ amplifications setup on the Microlab® Hamilton STARlet and Xiril liquid handling robots were particularly prone to the partial or complete dropout of the affected loci compared with manual amplifications. Both robot types were used for setting up Quantifiler™ reactions, and no issues were apparent with this method. A consideration was that inadequate mixing of the amplification master mix reagents prior to amplification setup may have been to blame. The hypothesis was that this might explain the variability observed in results and the ability to recover affected loci by reamplification on occasion. Each robot sequence was checked in detail including the mixing and aliquoting steps, and no obvious issues were identified.

It was noted that the plastic troughs into which the various amplification reagents were placed for liquid handling robot methods absorbed the green color from the cleaning agent TriGene™ ADVANCE used in their cleaning process. This was not completely removed after vigorous manual washing and a normal washing cycle through a dishwasher. It was considered that this

could be a potential pathway for trace amounts of cleaning compound to be introduced to our amplification setups. While this was thought to be a potential cause of the dropout observed, this did not completely explain the results as manually amplified samples were similarly affected, although at lower frequencies.

For 2 years, since the introduction of low copy number techniques at ESR, we have been using the cleaning agent TriGene™ and TriGene™ ADVANCE for all aspects of laboratory cleaning from the floors, surfaces, and walls to the reagent bottles, racks, benches, and laboratory equipment. As with all cleaning agents, it is advisable to rinse and wipe all areas of materials that are in repeated contact with TriGene™ to avoid the buildup of the chemicals. Benches and equipment, such as tweezers and scissors, in our laboratory are also wiped with 70% ethanol prior to and during use to remove such buildup.

TriGene™ ADVANCE was chosen as the preferred laboratory cleaning agent for its performance in studies in our laboratory and its relative safety of use. It is a nonalcohol-based, nonoxidizing solution of pH 5.5. The formulation also contains detergent, color, and various fragrances and is delivered by a microemulsion of nanoparticles, which carry the active ingredients through bacterial cell walls and can penetrate very small crevices on surfaces, allowing a lower and safer amount to be used. The active ingredients comprise halogenated tertiary amines (polymeric biguanide hydrochloride, alkyl dimethyl benzyl ammonium chloride, and didecyl dimethyl ammonium chloride). Because similar compounds have been shown to cause bacterial apoptosis (cell death) by crossing the cell membrane, causing transcriptional changes in the genes associated with cell death and inflammatory response and interfering with the helical structure of the DNA rendering it unable to replicate (16), we hypothesize that this is a possible mode of action of TriGene™ ADVANCE.

Previously, and without incident, we had used the detergent Virkon® followed by a 70% ethanol rinse for surfaces and equipment as our laboratory cleaning agent. Virkon® is the trade name of a disinfectant active against viruses, bacteria, and fungi pathogenic to animals and poultry. It was used at a concentration of 1% as recommended. Virkon® is composed of peroxygen compounds, organic acids, surfactant, and buffer. The active ingredients are potassium peroxymonosulphate (20.4%) and sodium chloride (1.5%) equivalent to 9.75% of available chlorine. Virkon® acts by general oxidative disruption of key structures and compounds including DNA. TriGene™ replaced Virkon® as our cleaning agent of choice because of its noncorrosive properties and its demonstrated improved performance.

As suspicion fell on the use of TriGene™ ADVANCE for cleaning purposes, we undertook a series of amplifications adding increasing concentrations of Virkon®, TriGene™ ADVANCE, and ethanol to Identifiler™ and PowerPlex® Y amplifications and observing the effect, if any, on the DNA profiles produced.

We determined that when up to 0.04% concentrations of TriGene™ ADVANCE was added to an Identifiler™ amplification reaction, no DNA profiles were produced indicating that complete inhibition of the Identifiler™ reaction was occurring. As the amount of TriGene™ ADVANCE decreased to 0.004%, the DNA profiles were recovered except for the loci D7S820, D18S51, and FGA (referred to as tri-loci dropout). The same samples amplified concurrently without the addition of TriGene™ ADVANCE gave results as expected showing no signs of inhibition. The addition of TriGene™ ADVANCE at <0.0004% concentrations had no apparent effect on the DNA profiles (data not shown). A concentration of 0.04% TriGene™ ADVANCE in an amplification product would entail the addition of 1 μL of 1% TriGene™ ADVANCE (the concentration used for cleaning), which is thought to be an unlikely scenario.

In contrast, the addition of comparable amounts of Virkon®, ethanol, or the powdered dishwashing detergent used in our laboratory (Finish®; Reckitt Benckiser, Slough, U.K.) did not affect the DNA profiles, and full profiles were obtained; specifically, the performance of D7S820, D18S51, and FGA was not affected. A summary of DNA profiling results for control DNA 9947A and ESR's internal positive control DNA amplified with Identifiler™ is in Table 1.

The Identifiler™ results for all samples (ESR's internal positive control and 9947A) contaminated with 0.004% TriGene™ ADVANCE resulted in complete dropout of the loci D7S820, D18S51, and FGA. All positive controls amplified concurrently with no TriGene™ ADVANCE added resulted in full profiles of the expected DNA genotype.

A trial was also undertaken comparing the effectiveness of reagent troughs versus tubes for use on the robots for the mastermix solution. Using the troughs (stained green), dropout of the affected loci was observed; however, when new disposable tubes were used, no dropout was observed.

In addition, it was occasionally observed that previously unaffected samples injected onto the 3130*xl* following samples where TriGene™ ADVANCE had been deliberately added subsequently failed to analyze because of the poor quality of the internal size standards preventing analysis of the sample profile. This was confirmed by the presence of inhibition in samples containing only HiDi Formamide™ (Applied Biosystems) and GS-500 LIZ™ (Applied Biosystems) run after a series of TriGene™ contaminated samples (data not shown). In subsequent experiments, the 3130*xl* capillaries were flushed with at least a single run of HiDi Formamide™ to remove contaminants from the array prior to running further samples.

Y STR DNA analysis is also carried out at ESR using the PowerPlex® Y multiplex kit. Because this multiplex kit is carried out at a higher number of PCR cycles, it is more sensitive than Identifiler™ and is routinely used on samples with very small amounts of male DNA sometimes mixed with much larger amounts of female DNA and partial profiles comprising only a few alleles are often produced. This means that it is difficult to assess from casework sample analysis whether or not any effect of cleaning reagents was also observed with this analysis system. To assess

TABLE 1—*A summary of DNA profiling results for control DNA 9947A and Environmental Science and Research Limited's internal positive control DNA amplified with Identifiler™ and treated with different cleaning agents.*

Dilution of cleaning agent (%)	Cleaning Agents Used in the Study			
	Ethanol	Dishwasher Powder	TriGene™ ADVANCE	Virkon®
	Identifiler™ DNA Typing Results (N = 5 samples)			
0.04	Full profiles	Full profiles	No results	Full profiles
0.004	Full profiles	Full profiles	Tri-loci drop all samples	Full profiles
0.0004	Full profiles	Full profiles	Full profiles	Full profiles

TABLE 2—*PowerPlex® Y amplification results using 0.5 ng of the PowerPlex® Y kit control male DNA.*

Dilution of Cleaning Agent (%)	Cleaning Agents Used in the Study	
	TriGene™ ADVANCE	Virkon®
	Identifiler™ DNA Typing Results (N = 5 samples)	
0.04	No results	Full profiles
0.004	Partial profiles all samples	Full profiles
0.0004	Full profiles	Full profiles

this, we carried out a similar experiment adding increasing amounts of TriGene™ ADVANCE and Virkon® to duplicate amplifications containing 0.5 ng male DNA (Table 2).

DNA profiles obtained after the addition of 0.004% TriGene™ ADVANCE to both the PowerPlex® Y kit control male DNA and ESR's internal positive control DNA were partial. The number of alleles typed varied for each sample from two to 11 of a possible 12 alleles. There was no pattern of dropout as for Identifiler™ samples.

We found that full PowerPlex® Y DNA profiles were generated after the addition of Virkon® at all amounts tested, from a 0.04% solution to a 0.0004% solution. Virkon® is typically used as a 1% solution for cleaning benches and equipment and is rinsed off prior to the item being used. The addition of comparable amounts of TriGene™ ADVANCE (0.04% solution) gave no PowerPlex® Y DNA profiles.

After changing back to a cleaning and washing regime using Virkon® and 70% ethanol exclusively, the inhibition of amplification of D7S820, D18S51, and FGA has been prevented and is no longer being observed in our laboratory.

Conclusion

It would seem that the choice of cleaning compounds can adversely affect the performance of the highly sensitive and complex STR multiplexes used in many forensic laboratories. Our conclusion is that TriGene™ ADVANCE, carefully developed to deliver a nonoxidative disinfectant in a nano-emulsion, causes inhibition if introduced into the amplification reaction. This may occur if it is not completely removed after immersion of the items or wiping of surfaces. This is made much more difficult by the nano-emulsion properties, which may cause tiny amounts of detergent to persist on glassware, plastic items, or benches.

It is hypothesized that residual TriGene™ ADVANCE was being introduced into the Identifiler™ amplification reactions from "contaminated" reagent troughs used on the liquid handling robots. Less frequently, trace amounts of TriGene™ ADVANCE from pipettes, reagent bottles (such as those containing TE buffer), racks, and bench surfaces were contaminating amplifications setup manually. These minute amounts of TriGene™ ADVANCE were having the effect of inhibiting the Identifiler™ reactions resulting in dropout of the loci D7S820, D18S51, and FGA.

By a careful process of deduction, we have demonstrated that inhibition of the PCR can occur if a seemingly sensible but incorrect choice is made.

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