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TECHNICAL NOTE

Updated Recommendations for Handling STR PCR Amplification Kits and Allelic Ladder Decontamination

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Introduction

With more sensitive DNA testing methods, rigorous procedures must be implemented to prevent inadvertent contamination in human identification laboratories. The allelic ladder of STR kits contains amplified product and is a contamination risk to forensic operations at numerous points in the product receipt pathway and analysis process. While using cleaning reagents is common practice to reduce the risk of ladder and sample-to-sample contamination, carefully considering decontamination protocols is recommended. Cleaning reagents can inhibit PCR amplification and impact the integrity of PCR components; therefore, it is critical to balance decontamination practices and the risk of impacting DNA profile quality.

This document provides insight into the amplification kit manufacturing and assembly process designed to minimize allelic ladder transfer. Based on the results of internal studies demonstrating the impact of common cleaning reagents, recommendations are also provided to assist laboratories in reducing the risk of unintentional contamination and PCR inhibition during the receipt, disassembly, and handling of STR kits during routine operations.

Precautions Taken During Kit Manufacturing (not exhaustive)

During the manufacture of STR kits, great care is taken to prevent the introduction of allelic ladder to non-allelic ladder kit components and packaging. Additionally, the manufacturing and assembly process is under continuous evaluation to identify areas of improvement.

Manufacturing Facility Precautions

- Two manufacturing sites several miles apart independently house PCR reagent or allelic ladder manufacturing.
- Ladder and PCR reagent tubes or bottles are filled on automated filling systems and then fed into a separate room for packaging before being manually handled.
    - A mandatory one-way flow ensures operators in the allelic ladder filling area cannot enter the allelic ladder packaging area or PCR reagent manufacturing site on the same day.
    - The ladder tube placement in the light-protective brown bottle (overtube) is automated in the packaging room before the overtube is handled.
    - A by-product of the automated filling process is that the allelic ladder may become aerosolized and leave trace amounts of allelic ladder on the exterior surface of the tube.
- Regular cleaning and environmental monitoring occur at all areas of the PCR component and allelic ladder manufacturing sites.
    - Routine swabbings from packaging and critical areas, including operator touch points, are monitored for contamination by amplifying swab elutes with the Applied Biosystems™ GlobalFiler™ PCR Amplification Kit 31-cycle protocol.
    - Data is analyzed on the Applied Biosystems™ 3500xL Genetic Analyzer, with instrument-specific minimum thresholds to detect trace amounts of allelic ladder.

- A swab is considered positive for allelic ladder contamination when at least one locus has more than 5 alleles in succession (Figure 1).
- The detection of allelic ladder within the manufacturing areas triggers a reassessment of the contaminated location and other materials manufactured at that time.

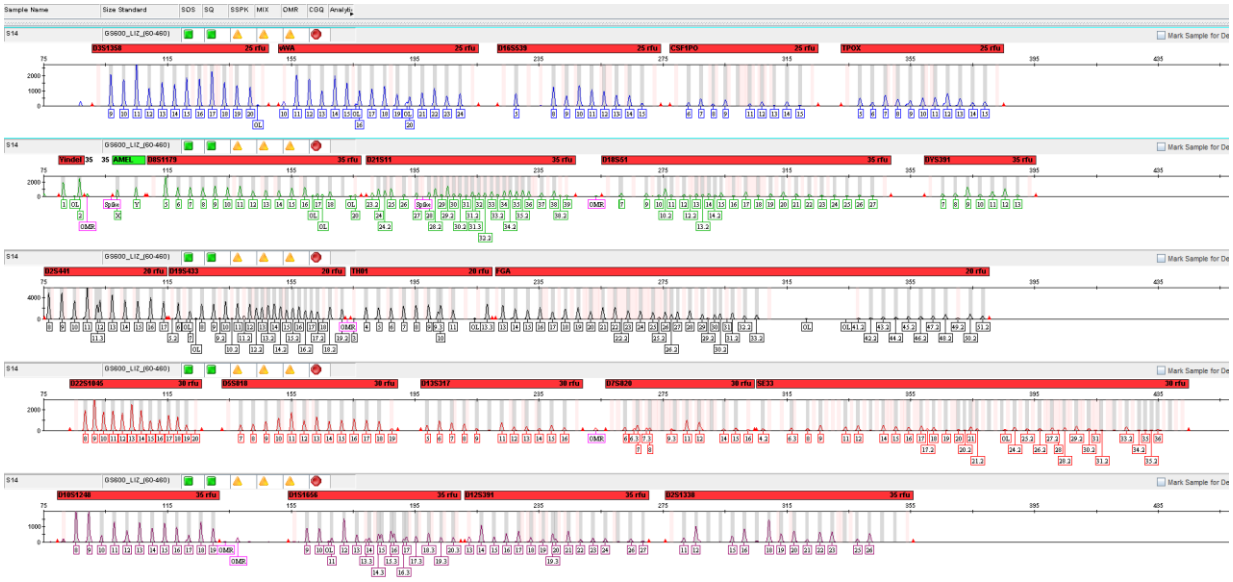

Figure 1. An electropherogram from the environmental monitoring of the brown overtube for the allelic ladder in the Globalfiler STR kit. Multiple loci have more than 5 alleles in succession, confirming the presence of allelic ladder.

Packaging Precautions

- Allelic ladder primary packaging: The allelic ladder tube is confined within a brown overtube.
- Allelic ladder secondary packaging: The overtube is placed within a cardboard insert inside an outer cardboard box, which is then shrink-wrapped.
- PCR reagents are placed within a cardboard insert inside another outer cardboard box, which is then shrink-wrapped.
- The shrink-wrapped ladder and PCR reagent boxes are placed in a PCR amplification kit product sleeve.

Figure 2. Allelic ladder packaging

Although we have implemented extensive steps at our manufacturing site to minimize the incidence of allelic ladder on external packaging, we recommend treating all STR kit components as if trace amounts of material may be present.

Recommendations for STR Kit Handling

We recommend the following STR kit handling processes to minimize the risk of introducing allelic ladder into pre-amplification areas. These recommendations have been simplified as we have evolved our manufacturing processes. Even with these recommendations, laboratories should consider ladder contamination risk and carefully handle allelic ladder packaging.

Note: The following are considerations for decontamination protocols. Laboratories should determine their own procedures after testing and following local quality and environmental policies for chemical handling.

Kit Receipt & Disassembly (designated disassembly location)

- Wear disposable gloves and change frequently during the kit disassembly process.
- Identify a location within the laboratory suitable for kit disassembly. Ideally, this location should NOT be in the pre or post-PCR sections of the lab.
- Remove the outermost shrink-wrap layer from the kit sleeve and discard.
- Immediately transfer the allelic ladder box (with the shrink-wrap layer still intact) to the post-PCR room.
- Discard or wipe down the shrink-wrap or exterior of the PCR reagent box before transferring the PCR reagents to a location that meets storage guidelines for the applicable kit.
- Clean the areas where the kit disassembly occurred.
- Remove traces of cleaning reagent.

Allelic Ladder Handling (in the post-PCR room)

- Wear disposable gloves and change frequently when handling the allelic ladder packaging.
- Wipe down and then remove the shrink-wrap.
- Wipe down the allelic ladder box.
- Remove and wipe down the overtube from the allelic ladder box before removing and wiping down the ladder tube.
- Remove traces of cleaning reagent.
- Never take materials from the post-PCR lab to the pre-PCR lab.

Note: The Thermo Fisher Scientific Research and Development team uses bleach at a 0.8% sodium hypochlorite concentration and 70% isopropanol solutions for cleaning. Squeeze bottles are used instead of spray bottles to reduce the risk of aerosolized products adhering to surfaces. If spray bottles are used, the cleaning solution is sprayed onto a wipe first, then the lab surface is wiped down. After the cleaning process, water is then used to remove any remaining cleaning reagents.

If not handled properly, allelic ladder can transfer through secondary and tertiary contact. Maintain a strict separation between pre and post-PCR environments.

Decontamination Studies and the Impact of Cleaning Reagents on PCR Amplification

While labs must be diligent in cleaning to minimize contamination, care should be taken to remove residual cleaning reagents left on lab surfaces, small equipment, consumables, and other items. The following sections describe studies performed by the Thermo Fisher Scientific Human Identification Research and Development team to understand the impact of typical lab cleaning agents on downstream STR data. The cleaning agents tested were 8.25% concentrated bleach, 70% ethanol, 70% isopropanol, Thermo Scientific™ DNA AWAY™ Surface Decontaminant, and Invitrogen™ DNAZap™ PCR DNA Degradation Solutions.

Bleach Studies

A stock solution of 8.25% Clorox Germicidal bleach was diluted to 1000 ppm (0.1% sodium hypochlorite), 200 ppm (0.02% sodium hypochlorite), and 10 ppm (0.001% sodium hypochlorite) and then spiked into Applied Biosystems™ GlobalFiler™ IQC master mix or primer mix (Table 1). 1ng Control DNA 007 was amplified with spiked PCR reagents on Days 0, 7, and 30.

Table 1. PCR setup volumes with bleach dilutions run at 29 cycles on a GeneAmp™ PCR System 9700 thermal cycler.

Reagent	Standard GFIQC Reaction	Master Mix with Bleach	Primer Mix with Bleach
DNA suspension buffer (uL)	5	2.5	2.5
GFIQC Master mix (uL)	7.5	10	7.5
GFIQC Primer Set (uL)	2.5	2.5	5

Primer Mix Spike Results: Dye Degradation

On Day 0, the sample quality was minimally impacted in the 0.001% bleach primer mix, and amplification was performed the same day (Figure 3a). All expected peaks were present, with artifacts only seen at the Amelogenin locus. The dyes in the primer mix have started to degrade in the presence of 0.02% bleach, which is evident from the artifacts in the VIC™ and NED™ dye channels (Figure 3b), as well as a drop in peak heights compared to the 0.001% bleach sample. These artifacts appear similar to spectral pull-up peaks, falling in another dye channel and at a base pair size aligned with the parent peaks. However, these artifacts are due to dye degradation, which has affected the typical fluorescent properties of the dyes. As bleach concentrations increase to 0.1%, the dyes degrade entirely, and no data is generated (Figure 4).

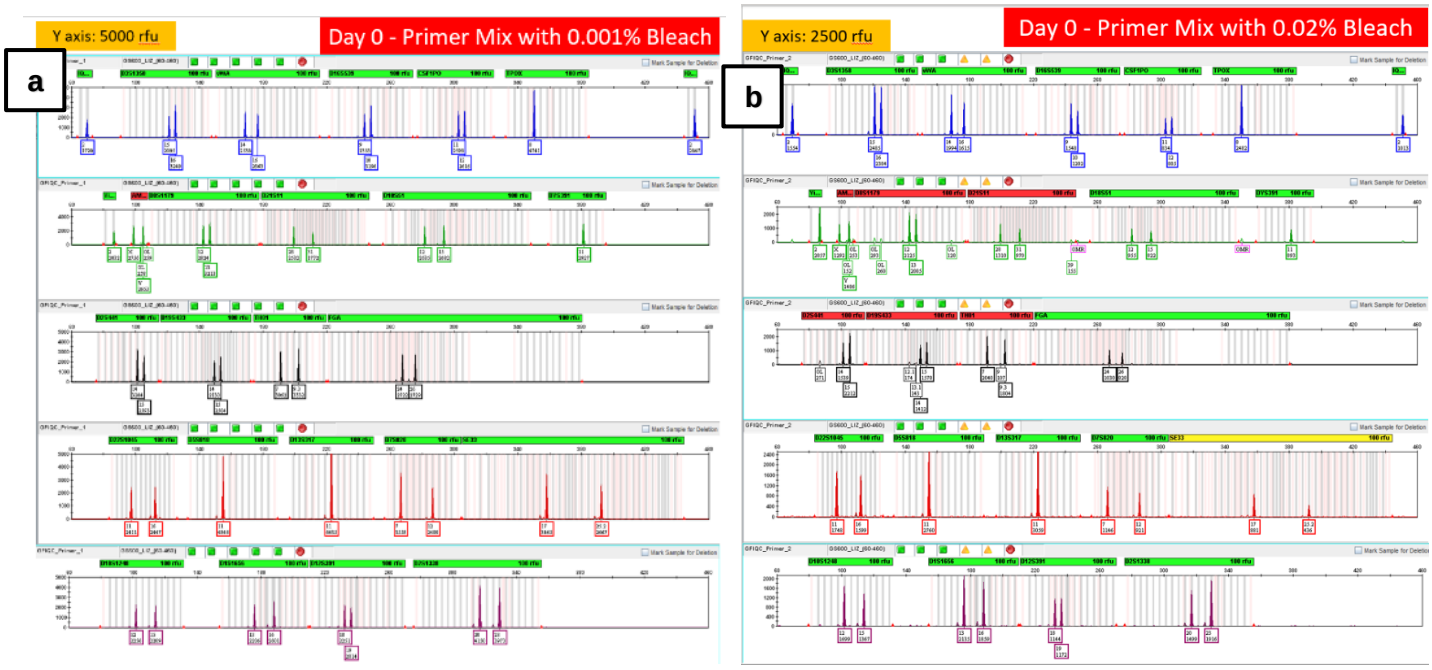

Figure 3. Electropherograms of the 0.001% (a) and 0.02% (b) bleach-spiked GlobalFiler IQC primer mix. Samples were amplified and run on a 3500 Genetic Analyzer on Day 0. Data was analyzed with an analytical threshold of 100 RFU in GeneMapper™ ID-X v1.7. The known Control 007 DNA alleles are highlighted.

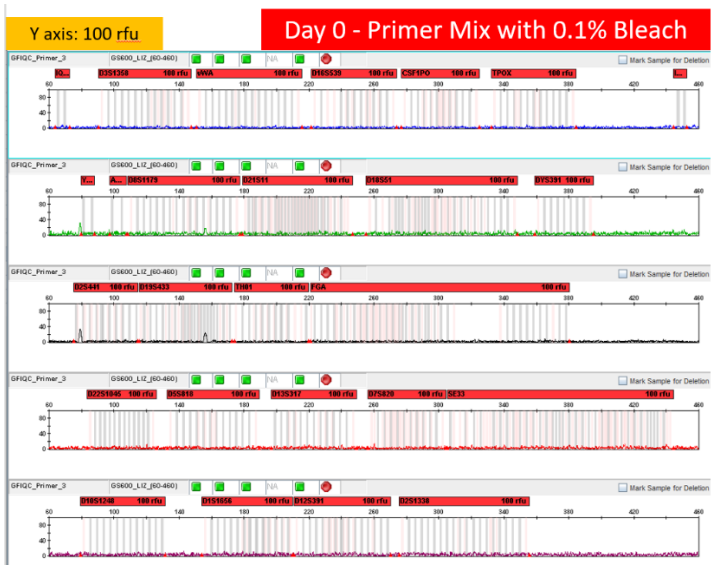

Figure 4. Electropherogram of the 0.1% bleach-spiked GlobalFiler IQC primer mix. The sample was amplified and run on a 3500 Genetic Analyzer on Day 0. Data was analyzed with an analytical threshold of 100 RFU in GeneMapper ID-X v1.7.

For the 0.001% and 0.02% bleach-spiked primer mix, the impact on data quality worsens on Day 7 and Day 30, showing more artifactual data, a decrease in peak height, and an inter-color peak height imbalance in multiple dye channels. The 0.02% bleach samples also genotype incorrectly, with alleles falling out-of-bin in both Day 7 and 30 runs (Figure 5a and 5b). By Day 30, only 4 of the expected 24 alleles were genotyped correctly in the NED, SID™, and TAZ™ dye channels.

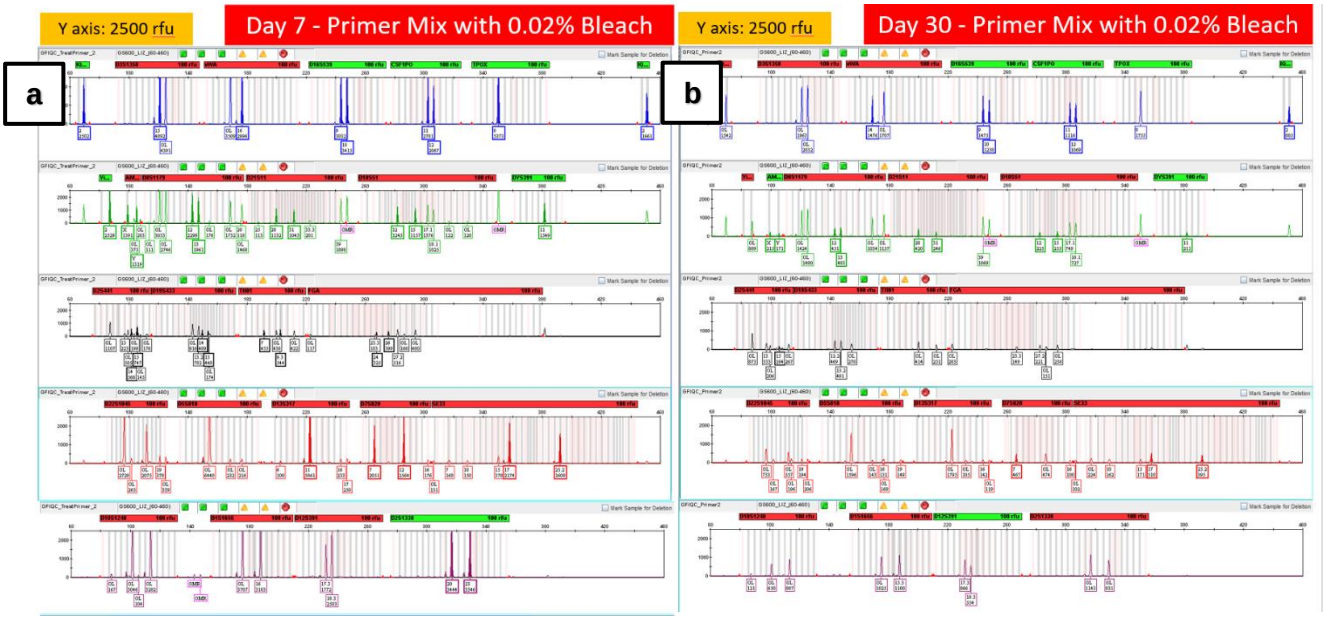

Figure 5. Electropherograms of 0.02% of bleach-spiked GlobalFiler IQC primer mix. Samples were amplified and run on a 3500 Genetic Analyzer on Day 7 (a) and Day 30 (b). Data was analyzed with an analytical threshold of 100 RFU in GeneMapper ID-X v1.7. The known Control 007 DNA alleles are highlighted.

Master Mix Spike Results: PCR Inhibition

On day 0, when bleach was spiked into the master mix at 0.001%, data quality was not impacted, and a complete profile was generated (Figure 6a). At 0.02% bleach, PCR inhibition is evident in the profile with dropout of all but some smaller markers (Figure 6b). The large internal quality control peak (IQCL) is also dropped out. At 0.1% bleach, there is complete profile dropout (Figure 6c).

Days 7 and 30 showed similar evidence of inhibition in the 0.02% and 0.1% bleach amplifications. The 0.1% bleach amplifications continued to have no impact on data quality. These results indicate that the effect of bleach contamination in the master mix is immediate on Day 0 rather than worsening over time.

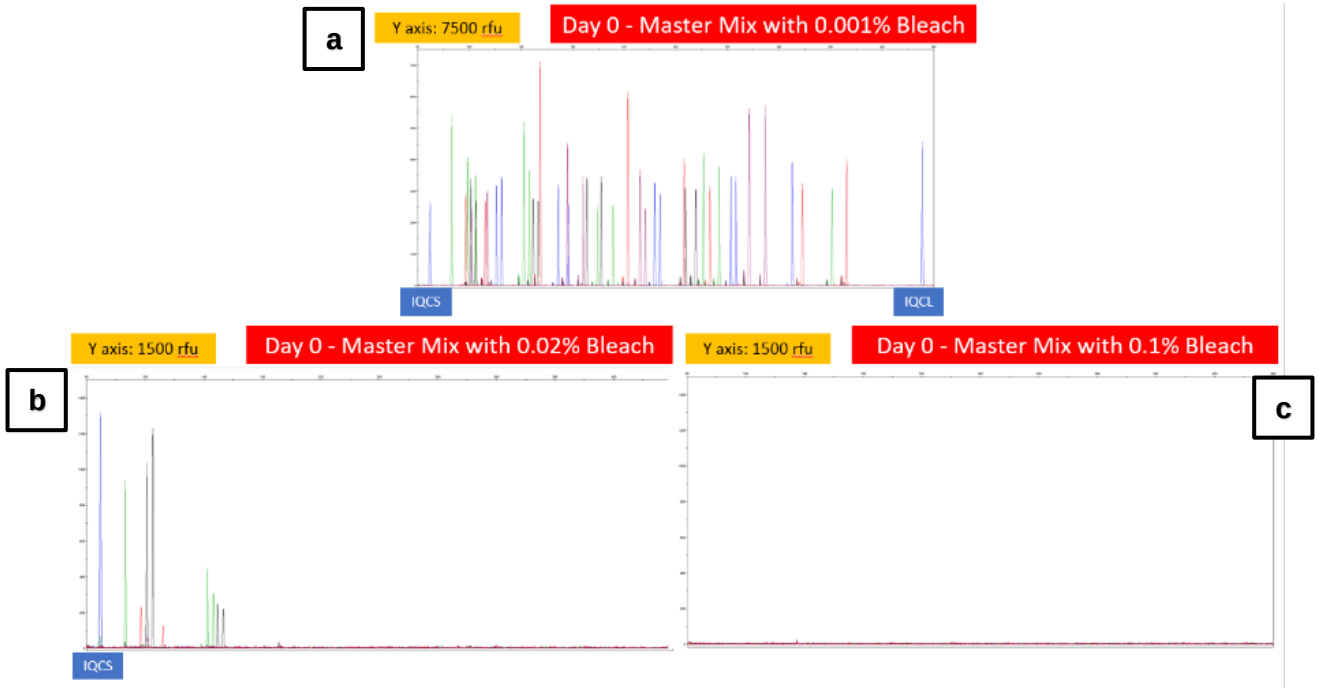

Figure 6. Sample plots with the dye channels overlaid when 0.001% (a), 0.02% (b), or 0.1% (c) bleach is spiked into the GlobalFiler IQC master mix. The samples were amplified and run on a 3500 Genetic Analyzer on Day 0. Data was analyzed with an analytical threshold of 100 RFU in GeneMapper ID-X v1.7.

Alcohol Studies

70% ethanol (EtOH) or 70% isopropanol (IPA) was added directly to 10 µl of Control DNA 007 in varying amounts (0-3 µl, in duplicate) (Table 2) before being added to the final GlobalFiler IQC PCR reaction (1 ng final DNA input). The first replicates were amplified on an Applied Biosystems™ VeritiPro™ thermal cycler and run on the 3500xL Genetic Analyzer on Day 0. The second replicates were stored at 4°C, then amplified and run on Day 3.

Table 2. Sample plus alcohol volumes before being added to the final PCR reaction. Samples were run at 29 cycles on a VeritiPro thermal cycler.

	Control	0.5	1	1.5	2	2.5	3
0.1 ng/µl DNA (µl)	10	10	10	10	10	10	10
70% EtOH/ IPA (µl)	0	0.5	1	1.5	2	2.5	3

Results: PCR Inhibition

Day 0 results show that as alcohol volume increases, the peak heights of the alleles decrease (Figure 7, Table 3). Another observation is that higher peak heights are maintained in the NED dye channel. An inhibition pattern seen in the GlobalFiler amplification kit family is “High NED” (Figure 8). A higher concentration of NED-labeled primers is needed to maintain inter-color balance. Therefore, when an inhibitor is present in the PCR reaction, peaks in the NED dye channel may persist over others, as seen in this study.

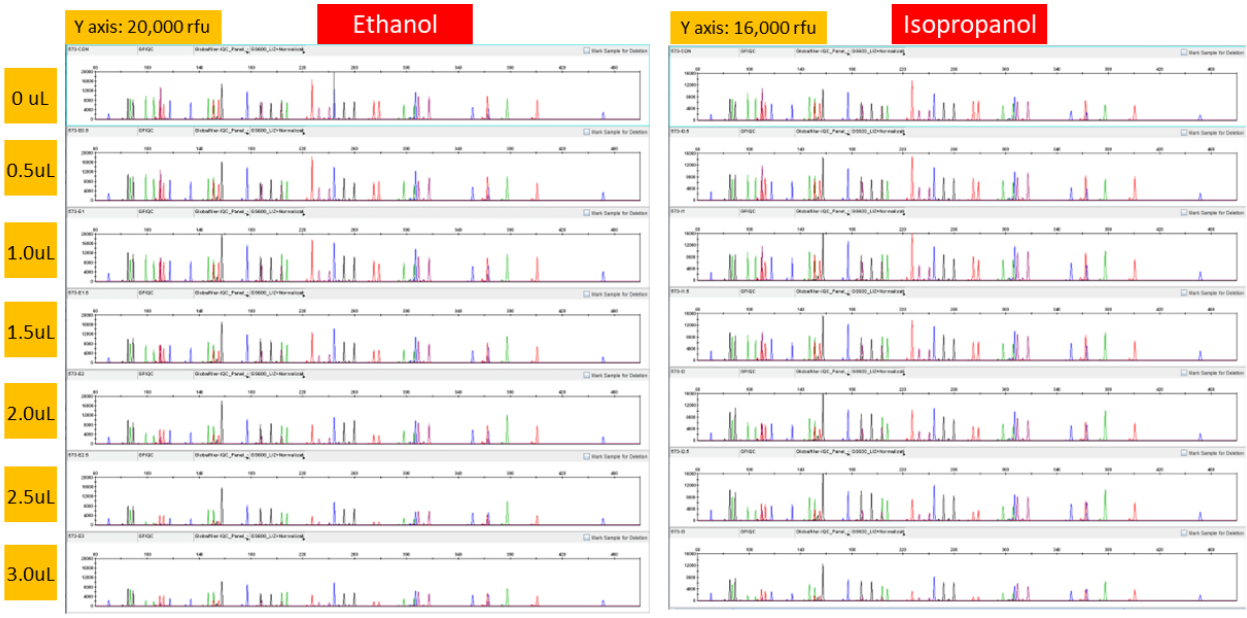

Figure 7. Sample plots with the dye channels overlaid for the ethanol and isopropanol studies. Samples were amplified with the GlobalFiler IQC kit and run on a 3500xL Genetic Analyzer on Day 0. Data was analyzed with an analytical threshold of 175 RFU in GeneMapper ID-X v1.7.

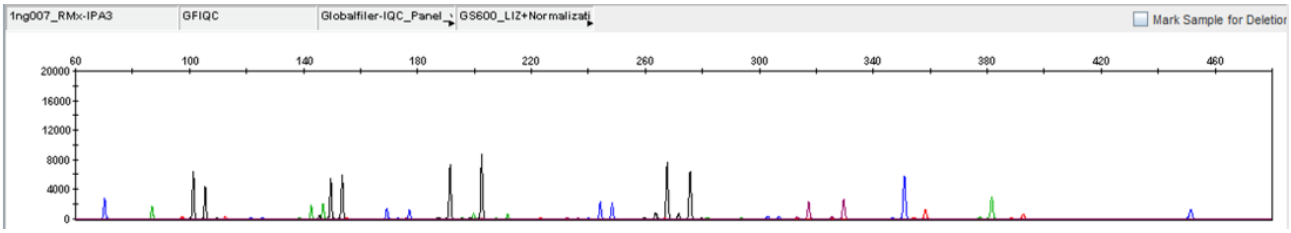

Figure 8. Sample plots with the dye channels overlaid for a 007 DNA:3 µl IPA sample showing the "High NED" inhibition pattern. The peaks in the NED channel have average peak heights above 4000 RFU, while the other dye channels show average peak heights of less than 2000 RFU. Data was analyzed with an analytical threshold of 175 RFU in GeneMapper ID-X v1.7.

The average peak heights for the Day 3 samples increased relative to Day 0, as shown in Table 3. One theory that these results support is the evaporation of the alcohol over time, which results in less inhibition of the PCR reaction. However, it should also be considered that there is run-to-run variation inherent in capillary electrophoresis (CE) workflows. Additional studies would need to be performed to determine the impact of either possibility.

Table 3: Comparison of the mean heterozygote peak heights and percent change for the samples at each ethanol and isopropanol volume on Day 0 and Day 3.

	Sample Name	Mean Peak Height (RFU)		Mean Peak Height (RFU)
		Day 0	Day 30	
No EtOH or IPA	CONTROL	7198	7802	8%
Ethanol	EtOH 0.5	7410	10314	39%
	EtOH 1.0	7589	9107	20%
	EtOH 1.5	6833	9632	41%
	EtOH 2.0	5368	8047	50%
	EtOH 2.5	4482	6675	49%
	EtOH 3.0	3869	4886	26%
Isopropanol	IPA 0.5	7404	8413	14%
	IPA 1.0	7886	8248	5%
	IPA 1.5	7886	8816	12%
	IPA 2.0	6501	8100	25%
	IPA 2.5	6557	7968	22%
	IPA 3.0	4804	7587	58%

DNAZap and DNA AWAY Studies

DNAZap or DNA AWAY studies were set up similarly to the alcohol studies; however, they were only amplified and run on Day 0. The amount of surface decontaminate added to the Control DNA 007 is shown in Figure 9 (DNAZAP 0-4 µl, DNA AWAY 0-3.5 µl).

Results: Inhibition

In Figure 9, the results of DNAZap and DNA AWAY are shown where increasing amounts of the reagent increase the inhibitory impact (ski-slope, decreased peak heights, dropout). Further, “High NED” can be seen in the DNA Away samples as the inhibitor volume increases.

Figure 9. Sample plots with the dye channels overlaid for the DNAZap and DNAAway studies. The samples were amplified with the GlobalFiler IQC kit and run on a 3500xL Genetic Analyzer on Day 0. Data was analyzed with an analytical threshold of 175 RFU in GeneMapper ID-X v1.7.

Conclusions

Thermo Fisher Scientific has rigorous processes in place to prevent the presence of trace levels of allelic ladder on STR kit packaging material. While we continue to investigate areas of continuous improvement for the automated filling and packaging processes, including methods for reducing aerosols, no process is infallible. The inadvertent transfer of allelic ladder can be mitigated by good handling practices during kit receipt, disassembly, and storage in the laboratory.

Laboratories should consider their cleaning and decontamination strategies and how they may affect their downstream STR profiling results. Studies performed by Thermo Fisher Scientific show that increasing amounts of common cleaning reagents into the sample extract, master mix, or primer mix can negatively impact profile quality due to dye degradation or inhibition. Minimizing aerosols and additional cleaning with water can help remove residual cleaning reagents that may be transferred to the PCR reaction.

Revision History

Revision	Date	Description
A00	09-Aug-17	Initial publication.
A01	13-Aug-19	Added more detail and pictures regarding the allelic ladder filling and packaging process. Revised kit disassembly recommendations. Minor other edits.
A02	25-Mar-24	Updated revision numbers from A, B, C to A00, A01, A02. Additional detail on the manufacturing of allelic ladder. Added studies showing downstream DNA profile impact when using common lab cleaning reagents.