Developmental Validation of Amplicon Rx, a post-PCR Purification System

Introduction

Allelic drop out and allele peaks below analytical threshold are common observation in forensic DNA analysis. These issues can be due to low amount of template, degraded DNA or inhibited amplification, or some combination of these problems. These are not unusual observation when biological material from crime scenes are processed and analyzed. These issues can also be addressed by increasing the number of PCR cycles during amplification (though 'drop-in' of extraneous alleles can often result), the use of inhibitor resistant PCR buffers, the addition of excess TAQ enzymes, the use of mini-STR primers sets or some combination of these options. These solutions can be expensive, time consuming, or controversial (*e.g.,* additional PCR cycles), or impossible due to limiting amounts of forensic evidence. Another approach, attempting to recover all of the amplicons in the PCR reaction is not only logical, but the most conservative and cost effective solution. Post-PCR clean up is designed to improve the efficiency electrokinetic injection of the amplicons produced in the PCR reaction. In addition Amplicon Rx™ will concentrate the remaining 90% of the PCR reaction that had been previously sampled. It is an obvious first solution to dealing with forensic samples that produce weak (*i.e.,* below laboratory threshold) DNA profiles.

The standard protocol for post-PCR prep for capillary electrophoresis (*i.e.,* the addition of an aliquot of the PCR reaction to formamide and dye-labeled size standards) only samples between 4% and 8% of the actual multiplex PCR reaction. The remaining 92-96% of the amplicons are never used – no other analytical technique discards 90% of the reaction product. Amplicon Rx™ selectively retains the amplified products of the PCR reaction and discards the bulk of the unincorporated primers, dNTPs, and salts that compete with the PCR amplicons for electrokinetic injection into the capillary. This selectivity provides the RFU 'boost' seen with technique.

Here we describe the developmental validation of a post-PCR purification system, Amplicon Rx™, that effectively 'cleans-up' the PCR reaction by (i) removing unincorporated primers, dNTPs, and salts, and (ii) concentrates the 90% of the amplicons that have been so expensively (in both time and reagents costs) produced. By eluting the amplicons in formamide/ROX/LIZ, the entire PCR reaction is now prepared for capillary electrophoresis.

The proprietary technology uses a non chaotropic low salt buffer, which, in a single step, binds PCR amplicons onto the spin filter column while washing away the bulk of the unincorporated primers, dNTPs and salts of the PCR reaction. Additional wash steps are not required and are not recommended. PCR amplicons are eluted with a Formamide/Size Standards mix, which conveniently prepares the entire elution for capillary electrophoresis without no further manipulation. The procedure takes 10 minutes and is performed in three easy steps (1)mix the PCR reaction with binding buffer at the indicated ratio and load the binding buffer/PCR reaction mixture onto the column, (2) centrifuge and discard the wash through, and (3) elute the purified amplicons with Formamide/Size Standards mix – see Figure 1.

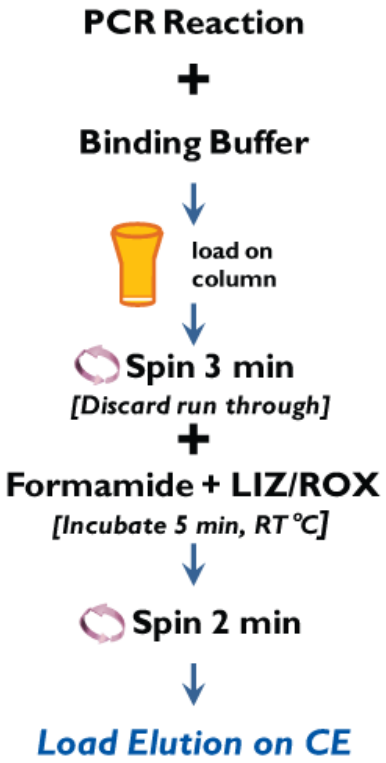

Figure. 1 Schematic of Amplicon Rx™ procedure for post-PCR purification

Experiment: Post-PCR recovery of Low Template Amount 9947A Control Sample

9947A Control DNA from an Identifiler kit at a concentration 100 pg/µL, was diluted 1:2 in x0.1 TE buffer to obtain 50 pg/µL working stock. From this working stock, 1 µL was taken for amplification with Identifiler in 12.5 µL PCR volume under standard cycling conditions. Figure 2 demonstrates analyzed data obtained from 50 pg 9947A sample using standard CE analysis protocol (Figure 2A)and after treatment with Amplicon Rx (Figure 2B). Table 1 shows signal boost that was observed as the result of Amplicon Rx™ post-PCR treatment.

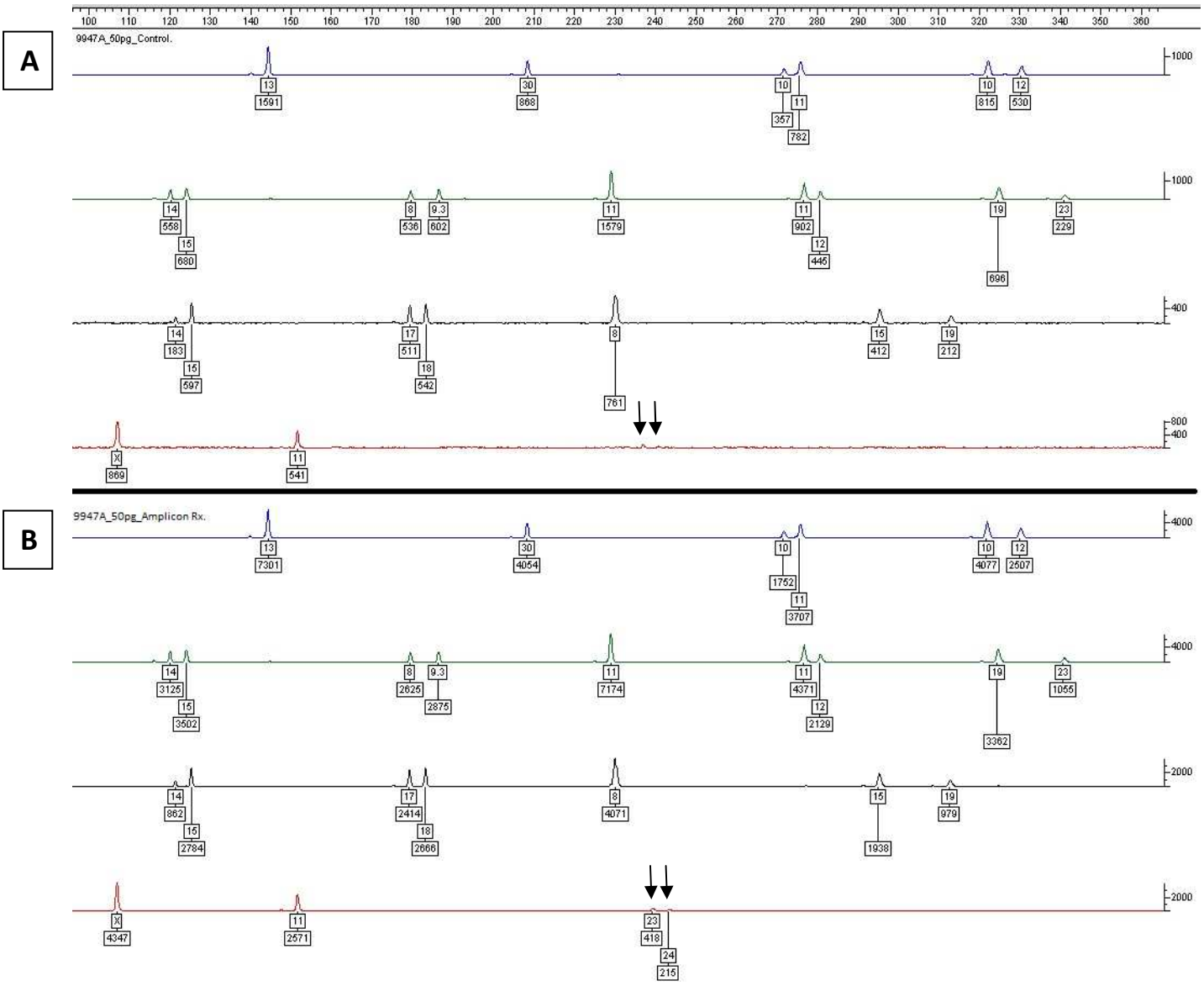

Figure 2. CE analysis of untreated (A) and Amplicon Rx™ treated (B) PCR reaction.

50 pg of 9947A amplified with Identifiler in 12.5 µL PCR volume. Arrows identify FGA alleles 23 and 24 which are below 150 RFU in the untreated sample (A) and that were recovered after Amplicon Rx™ treatment to 418 RFU and 215 RFU, respectively (B). Note the different RFU scales for sections (A) and (B).

Table 1. Signal boost after Amplicon Rx post-PCR treatment of 50 pg 9947A amplified with Identifiler

Marker	Alleles	Untreated sample RFUs	Amplicon Rx sample RFUs	Signal Boost (times)
D8S1179	13	1591	7301	4.6
D21S11	30	868	4054	4.7
D7S820	10	357	1752	4.9
D7S820	11	782	3707	4.7
CSF1PO	10	815	4077	5.0
CSF1PO	12	530	2507	4.7
D3S1358	14	558	3125	5.6
D3S1358	15	680	3502	5.2
TH01	8	536	2625	4.9
TH01	9.3	602	2875	4.8
D13S317	11	1579	7174	4.5
D16S539	11	902	4371	4.8
D16S539	12	445	2129	4.8
D2S1338	19	696	3362	4.8
D2S1338	23	229	1055	4.6
D19S433	14	183	862	4.7
D19S433	15	597	2784	4.7
vWA	17	511	2414	4.7
vWA	18	542	2666	4.9
TPOX	8	761	4071	5.3
D18S51	15	412	1938	4.7
D18S51	19	212	979	4.6
D5S818	11	541	2571	4.8
FGA	23	97	418	4.3
FGA	24	67	215	3.2
Amelogenin	X	869	4347	5.0

Conclusion: Amplicon Rx™ Post-PCR treatment of 50 pg 9947A helped recover two alleles to above 150 RFU threshold and provided an average RFU boost of 4.7 fold. Amplicon Rx™ Post-PCR treatment did not introduce any new alleles that were not present in the original sample.

9947A Control DNA from an Identifiler kit at a concentration 100 pg/μL, was diluted 1:4 in x0.1 TE buffer to obtain 25 pg/μL working stock. From this working stock, 1 μL was taken for amplification with Identifiler in 12.5 μL PCR volume under standard cycling conditions. Figure 3 shows analyzed data obtained from 25 pg 9947A sample using standard CE analysis protocol (Figure 3A)and after treatment with Amplicon Rx™ (Figure 3B). Table 2 shows signal boost that was observed as the result of Amplicon Rx post-PCR treatment.

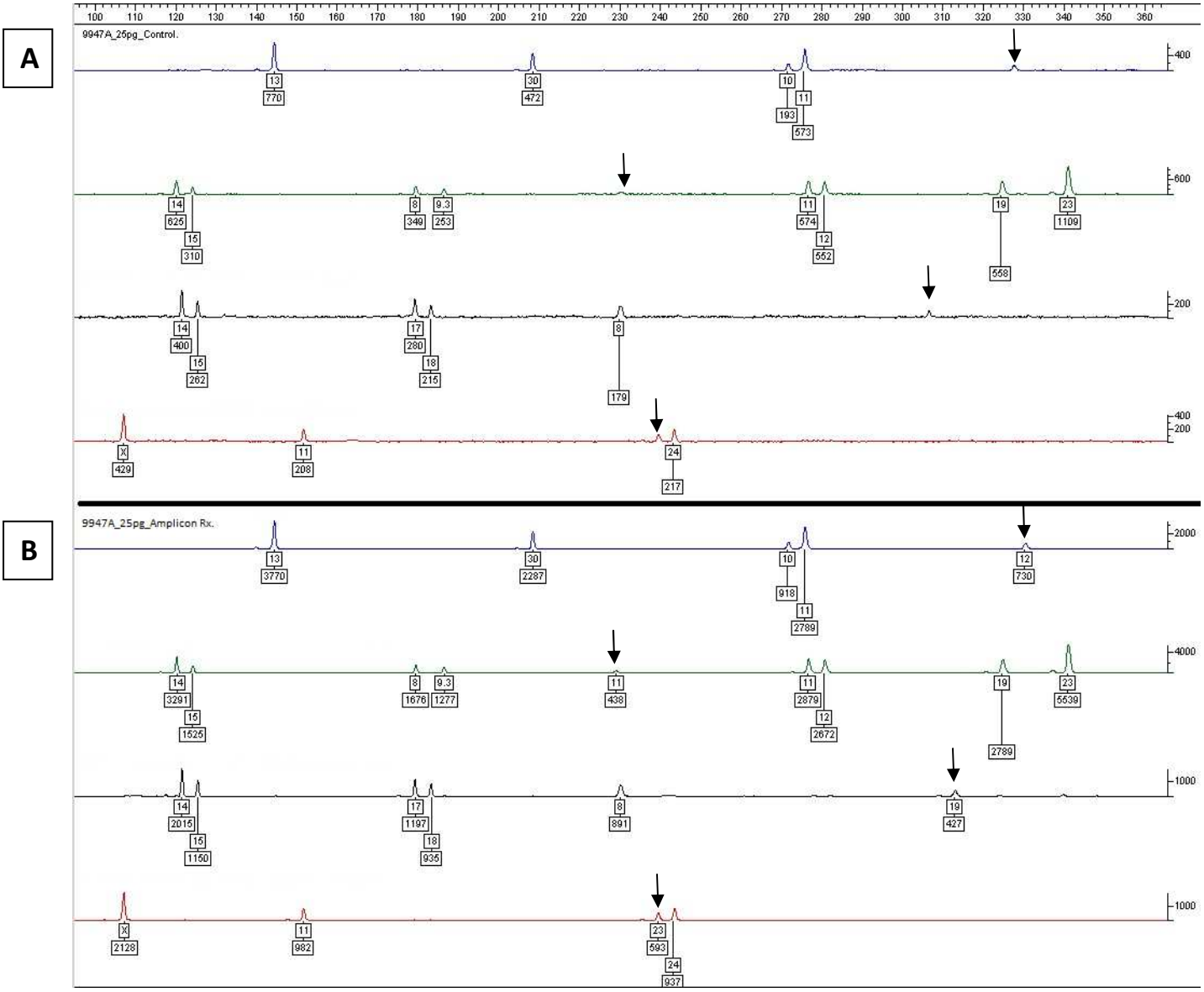

Figure 3. CE analysis of untreated (A) and Amplicon Rx™ treated (B) PCR reaction.
25 pg of 9947A amplified with Identifiler in a 12.5 μL PCR reaction volume. Arrows identify CSF1PO allele 12, D13S317 allele 11, D18S51 allele 19, and FGA allele 23, which were below 150 RFU in the untreated sample (A), and were recovered after Amplicon Rx™ treatment to 730 RFU, 438 RFU, 427 RFU and 593 RFU, respectively (B). Note RFU different scales in (A) and (B).

Table 2. Signal boost after Amplicon Rx post-PCR treatment of 25 pg 9947A amplified with Identifiler

Marker	Alleles	Untreated	Amplicon Rx	Signal Boost (times)
D8S1179	13	770	3770	4.9
D21S11	30	472	2287	4.8
D7S820	10	193	918	4.8
D7S821	11	573	2789	4.9
CSF1PO	10	drop out	drop out	
CSF1PO	12	138	730	5.3
D3S1358	14	625	3291	5.3
D3S1358	15	310	1525	4.9
TH01	8	349	1676	4.8
TH01	9.3	253	1277	5.0
D13S317	11	109	438	4.0
D16S539	11	574	2879	5.0
D16S539	12	552	2672	4.8
D2S1338	19	558	2789	5.0
D2S1338	23	1109	5539	5.0
D19S433	14	400	2015	5.0
D19S433	15	262	1150	4.4
vWA	17	280	1197	4.3
vWA	18	215	935	4.3
TPOX	8	179	891	5.0
D18S51	15	drop out	drop out	
D18S51	19	110	427	3.9
D5S818	11	208	982	4.7
FGA	23	135	593	4.4
FGA	24	270	1186	4.4
Amelogenin	X	429	2128	5.0

Conclusion: Amplicon Rx™ Post-PCR treatment of 25 pg 9947A sample recovered four alleles to above 150 RFU threshold and provided an average RFU boost of 4.7 fold. Amplicon Rx™ Post-PCR treatment did not introduce any new alleles that were not present in the original sample.

Experiment: Post-PCR Purification of Touch DNA Samples

A Touch DNA sample was collected by swabbing the surface of screwdriver handle. The entire DNA extract was PCR amplified with Identifiler in 6.25 µL PCR volume under standard cycling conditions. Figure 4 shows analyzed data obtained from the screwdriver sample using standard CE analysis protocol (Figure 4A) and after treatment with Amplicon Rx™ (Figure 4B).

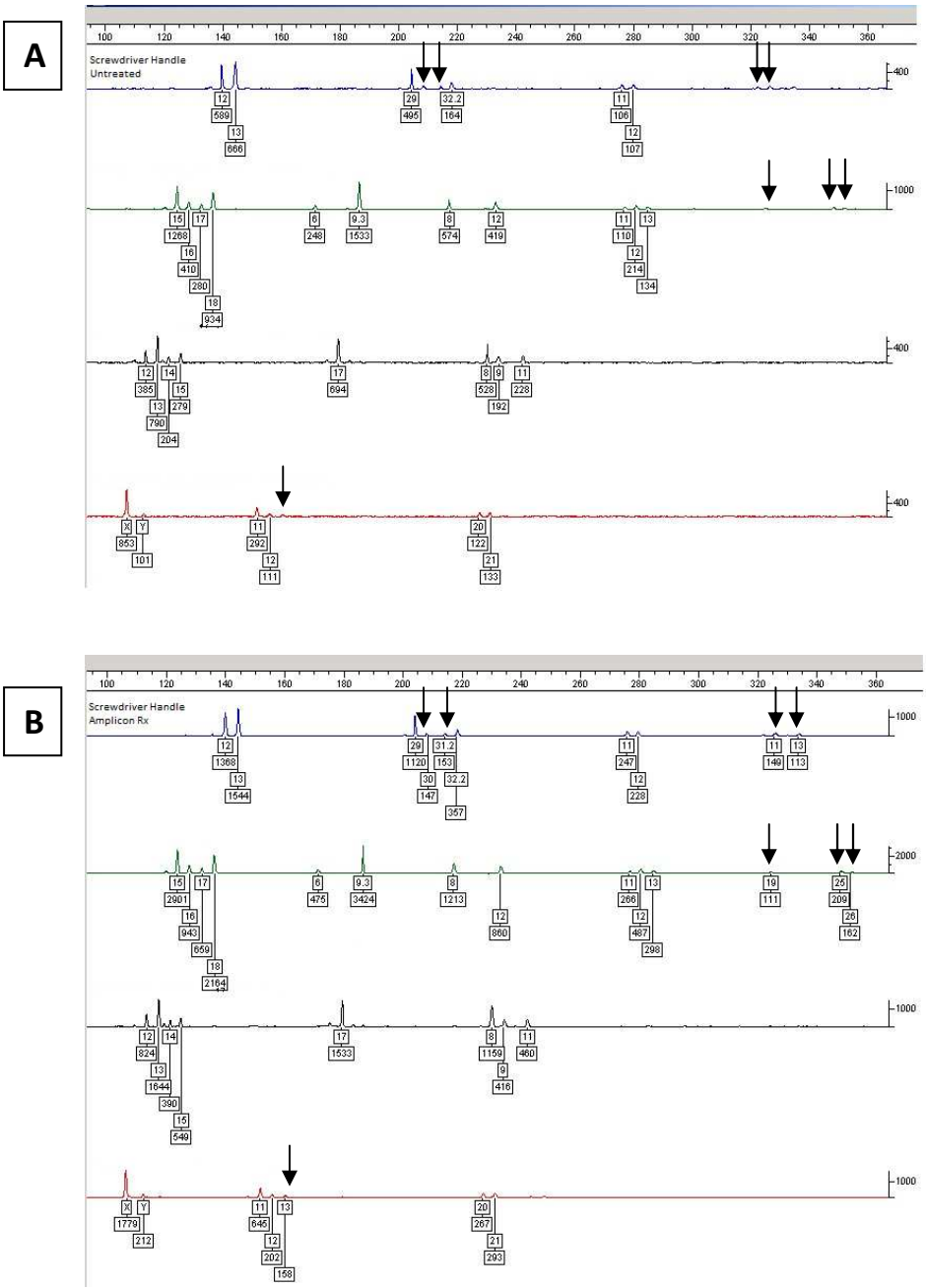

Figure 4. CE analysis of untreated (A) and Amplicon Rx™ treated (B) PCR reaction.
Touch DNA sample (screwdriver handle) was amplified with Identifiler in a 6.25 µL PCR reaction volume. Arrows identify D21S11 alleles 30 and 31.2, CSF1PO alleles 11 and 13, D2S1338 alleles 19, 25 and 26, and D5S818 allele13, which were below 100 RFU in the untreated sample (A), and were recovered after Amplicon Rx™ treatment to 147, 153, 149, 113, 111, 209, 162 and 158 RFU, respectively (B). Note different RFU scales in (A) and (B).

Table 3 summarizes data from an analysis of a mixed sample processed by Amplicon Rx™ post-PCR treatment. Allelic peak heights in RFU and peak height ratios for each marker are shown. For example, at D8S1179, the peak height ratio of alleles 12 and 13 is 0.88 (589/666) in the untreated sample, and 0.89 (1368/1544) in the Amplicon Rx™ treated sample. At D3S1358, peak height ratios in the untreated sample are: 3.09 for alleles 15 and 16 (1268/410), 1.46 for alleles 16 and 17 (410/280) and 0.30 for alleles 17 and 18 (280/934). In the Amplicon Rx™ treated samples the peak height ratios are: 3.08 for alleles 15 and 16 (2901/943), 1.43 for alleles 16 and 17 (943/659) and 0.30 for alleles 17 and 18 (659/2164). The last column in the table shows the difference between peak height ratios in the untreated sample and peak height ratios in the sample treated with Amplicon Rx™ for each allelic pair examined, by dividing the "Untreated Ratio" by the "Amplicon Rx™ Ratio".

Table 3. Allelic peak height ratios before and after treatment with Amplicon Rx™

(Touch DNA sample from screwdriver handle)

Marker	Alleles	Untreated RFU	Untreated Ratio	Amplicon Rx™ RFU	Amplicon Rx™ Ratio	Untreated Ratio / AmpliconRx™ Ratio
D8S1179	12	589	0.88	1368	0.89	0.998
D8S1179	13	666		1544		
D21S11	29	495	3.02	1120	3.14	0.962
D21S11	32.2	164		357		
D7S820	11	106	0.99	247	1.08	0.914
D7S820	12	107		228		
D3S1358	15	1268	3.09	2901	3.08	1.005
D3S1358	16	410	1.46	943	1.43	1.023
D3S1358	17	280	0.30	659	0.30	0.984
D3S1358	18	934		2164		
TH01	6	248	0.16	475	0.14	1.166
TH01	9.3	1533		3424		
D13S317	8	574	1.37	1213	1.41	0.971
D13S317	12	419		860		
D16S539	11	110	0.51	266	0.55	0.941
D16S539	12	214	1.60	487	1.63	0.977
D16S539	13	134		298		
D19S433	12	385	0.49	824	0.50	0.972
D19S433	13	790	3.87	1644	4.22	0.919
D19S433	14	204	0.73	390	0.71	1.029
D19S433	15	279		549		
TPOX	8	528	2.75	1159	2.79	0.987
TPOX	9	192	0.84	416	0.90	0.931
TPOX	11	228		460		
D5S818	11	292	2.63	645	3.19	0.824
D5S818	12	111		202		
FGA	20	122	0.92	267	0.91	1.007
FGA	21	133		293		

Conclusion: Amplicon Rx™ Post-PCR treatment of Touch DNA sample from screwdriver handle recovered eight alleles to above 100 RFU threshold and preserved the allelic peak height ratios. Amplicon Rx™ Post-PCR treatment did not introduce any new alleles that were not present in the original sample.

Another Touch DNA sample was collected by swabbing the surface of a piece of paper. The entire DNA extract was PCR amplified with Identifiler in 6.25 µL PCR reaction under standard cycling conditions. Figure 5 shows analyzed data obtained from the swabbed paper sample using standard CE analysis protocol (Figure 5A) and after treatment with Amplicon Rx™ (Figure 5B).

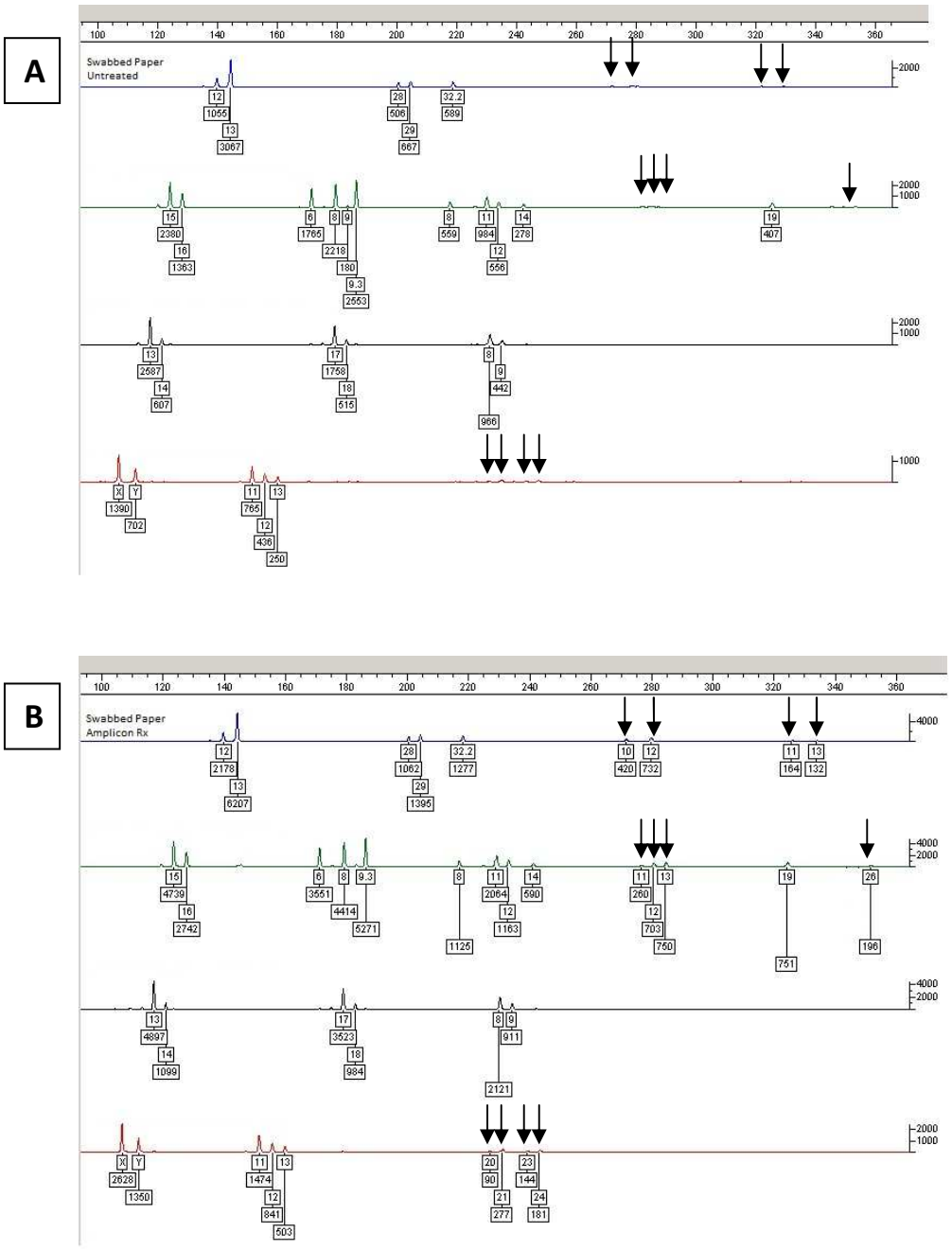

Figure 5. CE analysis of untreated (A) and Amplicon Rx™ treated (B) PCR reaction.
Touch DNA sample (swabbed paper) was amplified with Identifiler in a 6.25 µL PCR reaction. Arrows identify D7S820 alleles 10 and 12, CSF1PO alleles 11 and 13, D16S539 alleles 11, 12 and 13, D2S1338 allele 26, and FGA alleles 20, 21, 23 and 24 which were below 100 RFU in the untreated sample (A), and were recovered after Amplicon Rx™ treatment to 420, 732, 164, 132, 260, 703, 750, 196, 90, 277, 144 and 181 RFU, respectively (B). Note different RFU scales in (A) and (B).

Table 4 summarizes data collected from a mixed sample analyzed before and after Amplicon Rx™ post-PCR treatment. Allelic peak heights in RFU and peak height ratios for each marker are shown. For example, at D8S1179, peak height ratio of alleles 12 and 13 is 0.34 (1055/3067) in the untreated sample, and 0.35 (2178/6207) in the Amplicon Rx™ treated sample. At D13S317, peak height ratios in the untreated sample are 0.57 for alleles 8 and 11 (559/984), 1.77 for alleles 11 and 12 (984/556) and 2.00 for alleles 12 and 14 (556/278). In the Amplicon Rx™ treated sample, the peak height ratios are: 0.55 for alleles 8 and 11 (1125/2064), 1.77 for alleles 11 and 12 (2064/1163) and 1.97 for alleles 12 and 14 (1163/590). The last column in the table shows the difference between peak height ratios in the untreated sample and peak height ratios in the sample treated with Amplicon Rx™ for each allelic pair examined, by dividing the "Untreated Ratio" by the "Amplicon Rx™ Ratio".

Table 4. Allelic peak height ratios before and after treatment with Amplicon Rx™
(Touch DNA sample from swabbed paper)

Marker	Alleles	Untreated RFU	Untreated Ratio	AmpliconRx™ RFU	AmpliconRx™ Ratio	Untreated Ratio / AmpliconRx™ Ratio
D8S1179	12	1055	0.34	2178	0.35	0.980
D8S1179	13	3067		6207		
D21S11	28	506	0.76	1062	0.76	0.996
D21S11	29	667	1.13	1395	1.09	1.037
D21S11	32.2	589		1277		
D3S1358	15	2380	1.75	4739	1.73	1.010
D3S1358	16	1363		2742		
TH01	6	1765	0.80	3551	0.80	0.989
TH01	8	2218	0.87	4414	0.84	1.037
TH01	9.3	2553		5271		
D13S317	8	559	0.57	1125	0.55	1.042
D13S317	11	984	1.77	2064	1.77	0.997
D13S317	12	556	2.00	1163	1.97	1.015
D13S317	14	278		590		
D19S433	13	2587	4.26	4897	4.46	0.956
D19S433	14	607		1099		
vWA	17	1758	3.41	3523	3.58	0.953
vWA	18	515		984		
TPOX	8	966	2.19	2121	2.33	0.939
TPOX	9	442		911		
D5S818	11	765	1.75	1474	1.75	1.001
D5S818	12	436	1.74	841	1.67	1.043
D5S818	13	250		503		

Conclusion: Amplicon Rx™ Post-PCR treatment of Touch DNA sample from swabbed paper recovered eleven alleles above a 100 RFU threshold and preserved allelic peak height ratios. Amplicon Rx™ Post-PCR treatment did not introduce any new alleles that were not present in the original sample.

Experiment: Post-PCR Purification of DNA Samples from Fingerprints on Glass Slides

Left and right index fingerprints were deposited on glass slides and a touch/fingerprint DNA samples was collected by swabbing these surfaces. The entire DNA extract was PCR amplified with Identifiler in a 25 µL PCR volume under standard cycling conditions. Post-PCR purification with Amplicon Rx™ resulted in oversaturating signal. In order to accurately calculate the signal boost, Amplicon Rx™ treated and untreated control samples from left and right index fingerprints were diluted 1:10 and 1:5, respectively, in Formamide/LIZ and re-injected. Figures 6 shows raw data of untreated controls (Figure 6 A and B) and Amplicon Rx™ treated (Figure 6 C and D) samples.

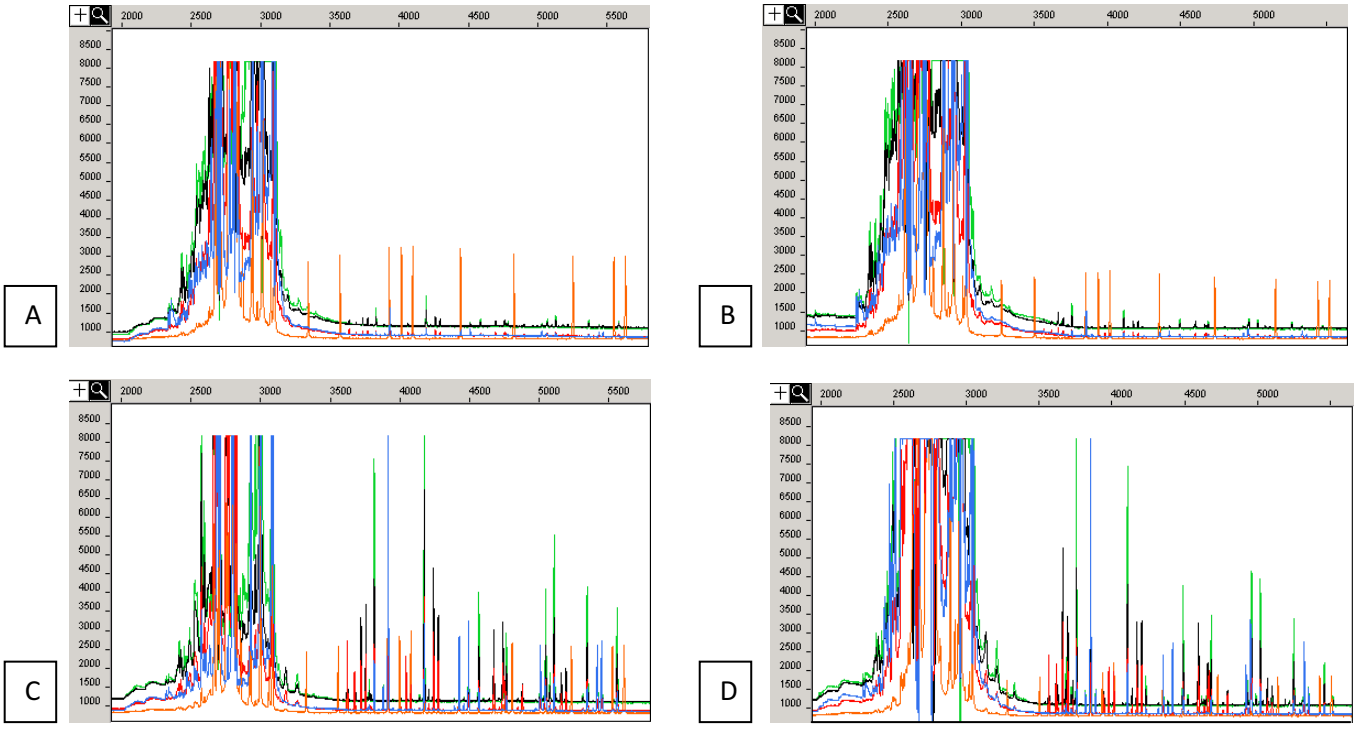

Figure 6. Signal boost after Amplicon Rx™ post-PCR treatment of left and right index fingerprint DNA samples amplified with Identifiler

A. Untreated control sample from left index fingerprint diluted 1:10;
B. Untreated control sample from right index fingerprint diluted 1:5;
C. Amplicon Rx™ treated sample from left index fingerprint diluted 1:10;
D. Amplicon Rx™ treated sample from right index fingerprint diluted 1:5.

Table 5 summarizes the RFU boost in allelic peak signal. Amplicon Rx™ Post-PCR treatment did not introduce any new alleles that were not present in the original sample.

Table 5. Signal boost after Amplicon Rx™ post-PCR treatment of left and right index fingerprint DNA samples amplified with Identifiler

Marker/Allele	Left Index Fingerprint Signal Boost (times)	Right Index Fingerprint Signal Boost (times)	Average Signal Boost (x)
D8S1179/Homozygote	9.6	11.1	10.3
D21S11/Allele 1	13.3	12.9	13.1
D21S11/Allele 2	13.2	12.8	13.0
D7S820/Allele 1	13.6	13.0	13.3
D7S820/Allele 2	12.6	12.6	12.6
CSF1PO/Allele 1	12.7	13.1	12.9
CSF1PO/Allele 2	12.7	10.4	11.6
D3S1358/Homozygote	13.4	11.1	12.3
TH01/Homozygote	7.5	12.2	9.8
D13S317/Allele 1	12.0	12.0	12.0
D13S317/Allele 2	11.0	11.3	11.2
D16S539/Allele 1	12.6	12.7	12.7
D16S539/Allele 2	13.8	12.5	13.2
D2S1338/Allele 1	13.4	11.9	12.6
D2S1338/Allele 2	11.8	10.5	11.2
D19S433/Allele 1	10.9	11.0	11.0
D19S433/Allele 2	11.8	11.0	11.4
vWA /Allele 1	12.7	11.6	12.2
vWA /Allele 2	10.9	11.6	11.3
TPOX/Allele 1	12.4	11.8	12.1
TPOX/Allele 2	10.9	9.3	10.1
D18S51/Allele 1	7.7	7.2	7.4
D18S51/Allele 2	9.5	8.9	9.2
D5S818/Allele 1	12.3	11.2	11.7
D5S818/Allele 2	11.0	10.2	10.6
FGA/Allele 1	11.1	9.5	10.3
FGA/Allele 2	12.7	8.5	10.6
Amelogenin/X	12.2	10.0	11.1
Amelogenin/Y	11.2	10.0	10.6

Conclusion: Amplicon Rx™ Post-PCR treatment of fingerprint samples amplified with Identifiler in a 25 μL PCR reaction provided an average RFU boost of 7.4 - 13.3 fold. Amplicon Rx™ Post-PCR treatment did not introduce any new alleles that were not present in the original sample.

Experiment: Amplicon Rx™ Post-PCR Treatment of Samples Amplified with PowerPlex 16 and Y-filer

In order to verify that the RFU bust with Amplicon Rx™ treated samples is not limited to Identifiler PCR amplification kits, positive control samples amplified with PowerPlex 16 and Y-filer were analyzed before and after Amplicon Rx™ treatment.

Positive Control 2800M was PCR amplified with PowerPlex 16 kit and mixed 1:20 with the PowerPlex 16 amplified Negative Control (blank that contained PCR primer mix, buffer and enzyme, but no DNA template) as follows: 1.25 µL of Positive Control 2800M was mixed with 23.75 µL of Negative Control. 1 µL of this sample was used for capillary electrophoresis as per standard protocol (Figure 7A), while the rest of the volume was treated with Amplicon Rx™ before electrophoresis (Figure 7B).

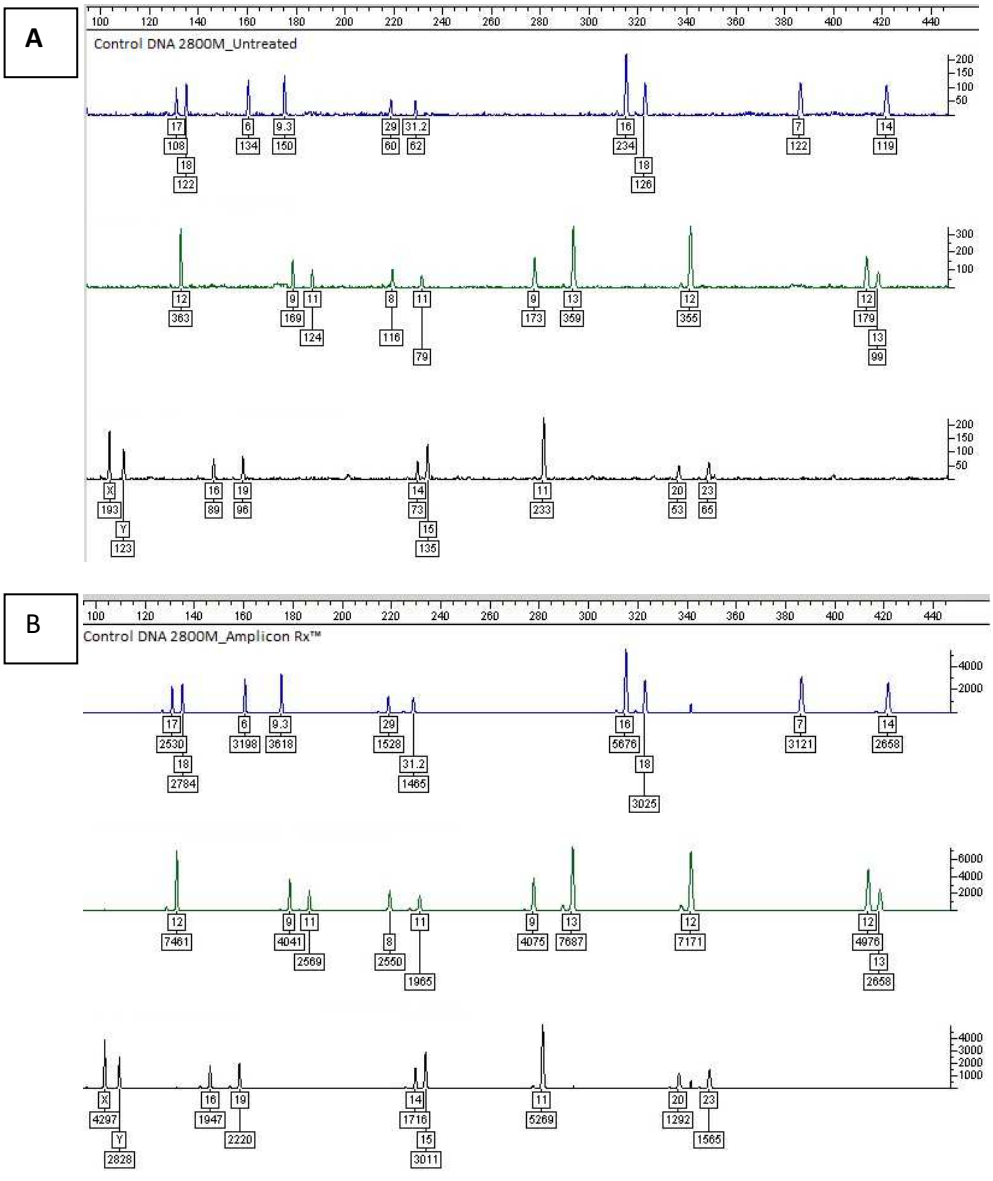

Figure 7. RFU boost after Amplicon Rx™ treatment of PowerPlex 16 amplified Positive Control 2800M sample
A - Untreated Control sample; B - Amplicon Rx™ treated sample. Note different RFU scales in (A) and (B).

Table 6 summarizes the RFU signal boost observed as the result of Amplicon Rx™ Post-PCR treatment.

Table 6. RFU boost after Amplicon Rx™ treatment of PowerPlex 16 amplified Positive Control 2800M sample

Marker	Alleles	Unterated sample RFUs	Amplicon Rx sample RFUs	Signal Boost (x)
D3S1358	17	108	2530	23.4
D3S1358	18	122	2784	22.8
TH01	6	134	3198	23.9
TH01	9.3	150	3618	24.1
D21S11	29	60	1528	25.5
D21S11	31.2	62	1465	23.6
D18S51	16	234	5676	24.3
D18S51	18	126	3025	24.0
Penta E	7	122	3121	25.6
Penta E	14	119	2658	22.3
D5S818	12	363	7461	20.6
D13S317	9	169	4041	23.9
D13S317	11	124	2569	20.7
D7S820	8	116	2550	22.0
D7S820	11	79	1965	24.9
D16S539	9	173	4075	23.6
D16S539	13	359	7687	21.4
CSF1PO	12	355	7171	20.2
Penta D	12	179	4976	27.8
Penta D	13	99	2658	26.8
vWA	16	89	1947	21.9
vWA	19	96	2220	23.1
D8S1179	14	73	1716	23.5
D8S1179	15	135	3011	22.3
TPOX	11	233	5269	22.6
FGA	20	53	1292	24.4
FGA	23	65	1565	24.1
Amelogenin	X	193	4297	22.3
Amelogenin	Y	123	2828	23.0

Conclusion: Amplicon Rx™ Post-PCR treatment of PowerPlex 16 amplified Positive Control 2800M sample provided average RFU boost of 23.4 fold. Amplicon Rx™ Post-PCR treatment did not introduce any new alleles that were not present in the original sample.

In order to test the effect of Amplicon Rx™ Post-PCR treatment on samples amplified with Y-filer, Positive Control 007 and Negative Control (blank that contained PCR primer mix, buffer and enzyme, but no DNA template) previously PCR amplified with Y-filer, were mixed 1:10 as follows: 2.5 µL of Positive Control 007 were mixed with 22.5 µL of Negative Control. 1.5 µL of this sample were used for capillary electrophoresis as per standard protocol (Figure 8A), while the rest of the volume was treated with Amplicon Rx™ before electrophoresis (Figure 8B).

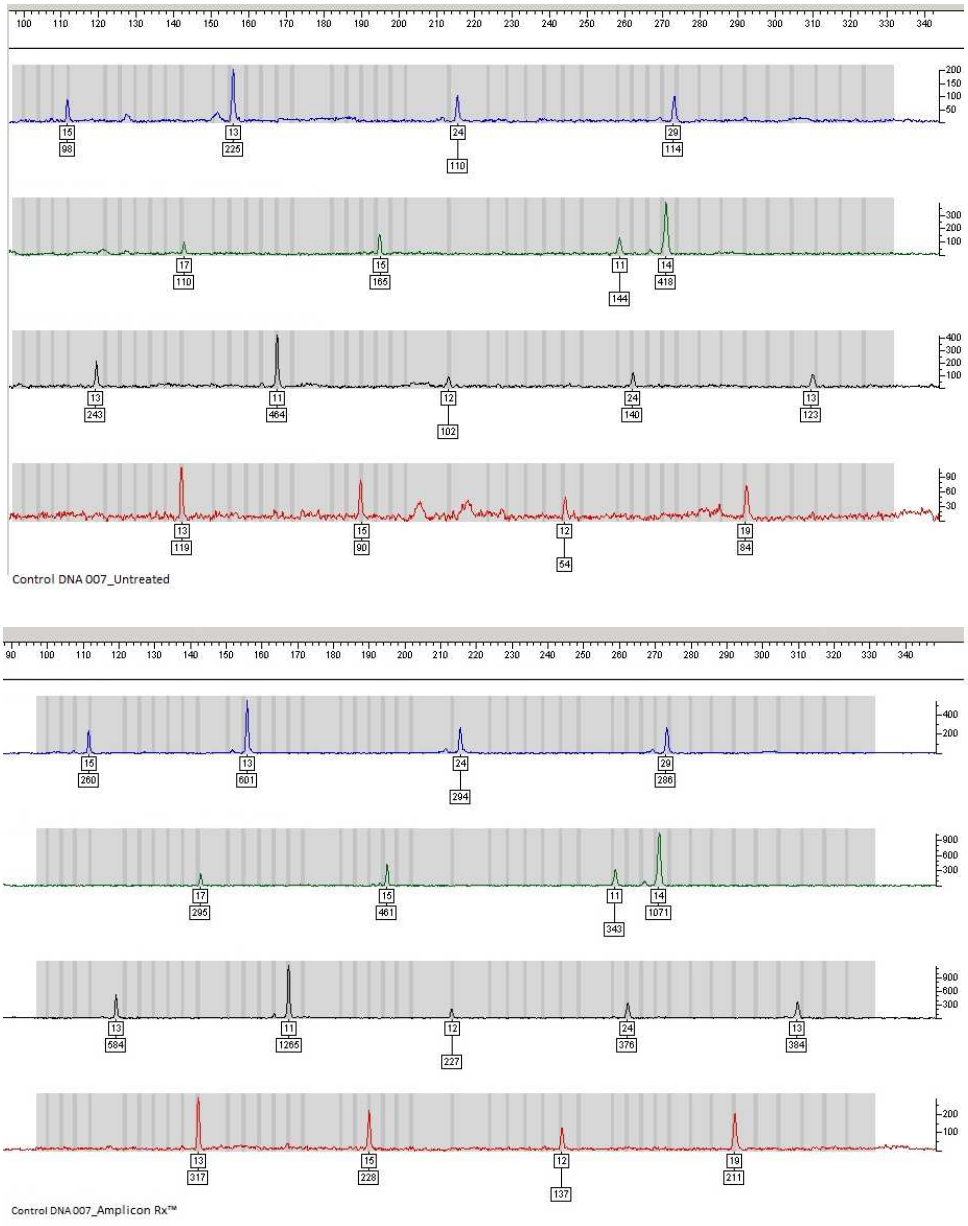

Figure 8. RFU boost after Amplicon Rx™ treatment of Y-filer amplified Positive Control 007 sample
A - Untreated Control sample; B - Amplicon Rx™ treated sample. Note different RFU scales in (A) and (B).

Table 7 summarizes the RFU signal boost observed as the result of Amplicon Rx™ post-PCR treatment.

Table 7. RFU boost after Amplicon Rx™ treatment of Y-filer amplified Positive Control 007 sample

Marker	Alleles	Unterated sample RFUs	Amplicon Rx sample RFUs	Signal Boost (times)
DYS456	15	98	260	2.7
DYS389 I	13	225	601	2.7
DYS3890	24	110	294	2.7
DYS389 II	29	114	286	2.5
DYS458	17	110	295	2.7
DYS19	15	165	461	2.8
DYS385 a	11	144	343	2.4
DYS385 b	14	418	1071	2.6
DYS393	13	243	584	2.4
DYS391	11	464	1265	2.7
DYS439	12	102	227	2.2
DYS635	24	140	376	2.7
DYS392	13	123	384	3.1
Y GATA H4	13	119	317	2.7
DYS437	15	90	228	2.5
DYS438	12	54	137	2.5
DYS448	19	84	211	2.5

Conclusion: Amplicon Rx™ Post-PCR treatment of Y-filer amplified Positive Control 007 sample provided average RFU boost of 2.6 fold. Amplicon Rx™ Post-PCR treatment did not introduce any new alleles that were not present in the original sample.