

Direct PCR from Whole Blood

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Introduction:

The advent of PCR technology has transformed the clinical diagnostic laboratory. Typically, DNA used in PCR assays is extracted according to established procedures which are time-consuming and increase the overall cost and turn-around time of these clinical assays. Genetic testing directly from blood has not been routinely used with regular Taq DNA polymerases, due to the presence of multiple PCR inhibitors in whole blood^{1,2}. To counter the inhibition of heme group, immunoglobulin G (IgG), sodium citrate, heparin, or EDTA in whole blood, two main approaches have been used. KAPA Biosystems has developed a special Taq DNA Polymerase by engineering Taq that can withstand higher concentrations of blood inhibitors by selective pressure. Taq that has evolved in this environment offers the ability to amplify DNA fragments directly from blood. Others (Blood Direct™ PCR buffers and EzWay™ Direct PCR buffer) have formulated buffers with higher pH and/or with PCR additives that will stabilize Taq and protect it from inhibitors in whole blood³.

PCR buffers at a higher pH range of 8.8-9.0 with 50 mM of Tricine, 100 mM KCl, & 1.5-4 mM MgCl₂ tend to overcome the inhibitory effect of blood on PCR. But chemically modified hot start DNA polymerases, such as AmpliTaq Gold DNA polymerase (Applied Biosystems, Inc.) and HotStarTaq DNA polymerase (Qiagen, Inc.), do not work under high pH conditions. Additions of enhancer or Taq stabilizers such as glycerol, BSA, betaine, etc. help in such chemically modified hot start DNA polymerases. Increasing the amount of Taq in the PCR reaction and the number of PCR cycles to 40-45 aids in PCR amplification from whole blood.

Using previously genotyped samples, we tested two commercially available buffers, (Blood Direct™ PCR buffer and EzWay™ Direct PCR buffer) and a Whole Blood PCR kit from KAPA Biosystems on several assays performed at ARUP Laboratories. Two of the assays are commercially available (CF from Abbott diagnostics and AmpFISTR Identifier PCR Amplification from ABI). We also tested two in-house based assays (UGT1A1 and HD), using the same samples.

Materials and Methods:

Residual blood samples with either sodium citrate, heparin or EDTA additives were used. One microliter of blood was used straight for PCR or 50 ul of the blood sample was spotted on Guthrie cards and dried for at least 2 weeks at room temperature. A 6mm disc was punched out and re-suspended in 200 µl of 1X PBS buffer for 15 min with intermittent vortexing; the suspension was then boiled at 95-100°C for another 15 min. One µl of this was used for PCR. The Guthrie card samples were used for CFTR genotyping with Direct PCR buffer and for DNA sequencing with the EzWay™ Direct PCR buffer. Whole blood samples were used in the STR set-up with Direct PCR buffer, and with the KAPA Blood PCR Kit with UGT1A1 and HD assays.

CFTR Delta F508 Locus Amplification and Sequencing.

One 25 µl reaction was set up for a heterozygous delta F508 mutation using EzWay™ Direct PCR buffer as follows: 5 µl of EzWay™ Direct PCR buffer mixed with 1µl of blood, 1.25 µl of 10 µM CFTR Exon 10 M13 tagged forward and reverse primers, (10 F TGTAACACGACGGCCAGTTCATAGCAGAGTACCTGAAACAGGA & 10 R CAGGAAACAGCTAGCTGATCCATTACAGTAGCTTACCCA), 1.25 µl of 10 µM dNTPs, 0.5 µl Platinum Taq, with the volume brought to 25 µl with molecular grade water. PCR amplification was performed with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. Unincorporated primers and dNTPs were removed by incubating with 3 units of ExoSAP at 37°C for 45 minutes, followed by heat inactivation of the enzyme at 80°C for 15 minutes. Sequencing reactions were performed using M13 primers, for bidirectional sequencing on an ABI Prism 3100 ABI DNA sequencer, using the Big Dye Termination Cycle Sequencing Ready Reaction kit 1.1 and recommendation of the manufacturer. (Figure 1)

CFTR Genotyping from Guthrie Cards.

A 10 µl reaction was set up for 10 samples using 1µl of blood processed from the Guthrie cards, 2.5 µl each of 5X BloodDirect Buffer 1 and 5X BloodDirect Buffer A, 2.5µl of CF v3 Genotyping kit (Abbott/Celera), 0.5 µl Platinum Taq and 1.0 µl of molecular grade water. PCR conditions were those recommended by the manufacturer with a slight change to the first initial denaturation hold cycle, with reactions held at 95°C for 5 min instead of 94°C for 12 mins. There were no changes to the OLA part of the assay. Two µl of PCR products were mixed with 0.5 µl of ROX 90 and 10 µl of HiDi. These were heated at 95°C for 2 min before loading onto an ABI 3100. The CF products were injected at 15 sec and separated at 5 kvolts for 1900 sec with a run temperature of 60°C using the 3100 POP™-6 polymer, 1X GeneticAnalyzer Buffer with EDTA, and a 36 cm array (Applied Biosystems). (Figure 3)

UGT1A1 Genotyping.

Six 25 µl PCR reactions were set up using 1.0 µl of 10µM of each primer UGT1A1_F 5' GTCACGTGACACAGTCAAAACATT 3' (FAM labeled), UGT1A1_R 5' CTC CACAGCCATGGCCGCTTT 3', 5.0 µl each of 5X KAPA 2G Buffer A & 5X KAPA Enhancer 1, 2.5 µl of 25 mM MgCl₂, 0.2 µl of KAPA Blood DNA Polymerase, 1 µl of undiluted whole blood, 200 µM of each nucleotide and 5% of glycerol, and the volume brought to 25 µl with molecular grade water.

Amplification was performed with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 30 sec and a final cycle of 72°C for 15 min. PCR products were diluted 1:20. One µl of this was mixed with 0.5 ROX 350 and 10 µl of HiDi and injected into an ABI 3100. The UGT1A1 alleles were injected at 15 sec and separated at 3 kvolts for 2600 sec with a run temperature of 60°C using the 3100 POP™-6 polymer, 1X Genetic Analyzer Buffer with EDTA, and a 36 cm array (Applied Biosystems). (Figure 4)

AmpFISTR Identifier PCR amplification.

A 25-µl master mix reaction consisting of 10.5 µl of AmpFISTR PCR Reaction 5.5 µl of AmpFISTR Primer Set mix, and 0.5 µl of Platinum taq DNA polymerase) was set up for three samples. Fifteen µl of above mixture were added to a mixture of 11 µl of 5.0 µl each of 5X BloodDirect Buffer 1 and 5X BloodDirect Buffer and diluted whole blood samples (1 µl blood:9 µl molecular grade water).

Amplifications were carried out at the following cycles: 1 cycle at 94°C for 5 minutes, 28 cycles at 94°C for 1 minute, 59°C for 1 minute, 72°C for 1 minute, 1 cycle at 60°C for 45 minutes and 25°C forever. Each sample was analyzed on the 3100 by adding 1 µl of PCR product to 19 µl of Hi-Di formamide containing 0.5 µl LIZ 500(Applied Biosystems). The STR alleles were injected 20 sec and separated at 1 kvolts for 2600 sec with a run temperature of 60°C using the 3100 POP™-6 polymer, 1X Genetic Analyzer Buffer with EDTA, and a 36 cm array (Applied Biosystems). (Figure 5)

Huntington Disease (HD) Genotyping

Five separate PCR amplifications were done in a 25 µl reaction containing 1.25 µl of 10µM of primer set HD_F 5' CCTTCGAGTCCTCAAGT CCTTC 3' (FAM labeled) and HD_R 5' CGGTGGCGGCTGTGCTG 3', 12.5 µl of KAPA Blood PCR Kit B with MgCl₂, 1 µl of whole blood, 10 % DMSO and volume brought to 25 µl with molecular grade water.

Amplification was performed with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec and extension at 72°C for 2 min and a final cycle of 72°C for 15min. Ten µl of the PCR products were run on a 2% agarose gel (Figure 6). PCR products were then diluted 1:20. One µl of this was mixed with 0.5 µl of ROX 500 and 10 µl of HiDi and heated at 95°C for 2 min. The HD products were injected at 10 sec and separated at 3 kvolts for 5100 sec with a run temperature of 60°C using the 3100 POP™-6 polymer, 1X Genetic Analyzer Buffer with EDTA, and a 36 cm array (Applied Biosystems), before loading onto anABI 3100, (Figure 7).

Results:

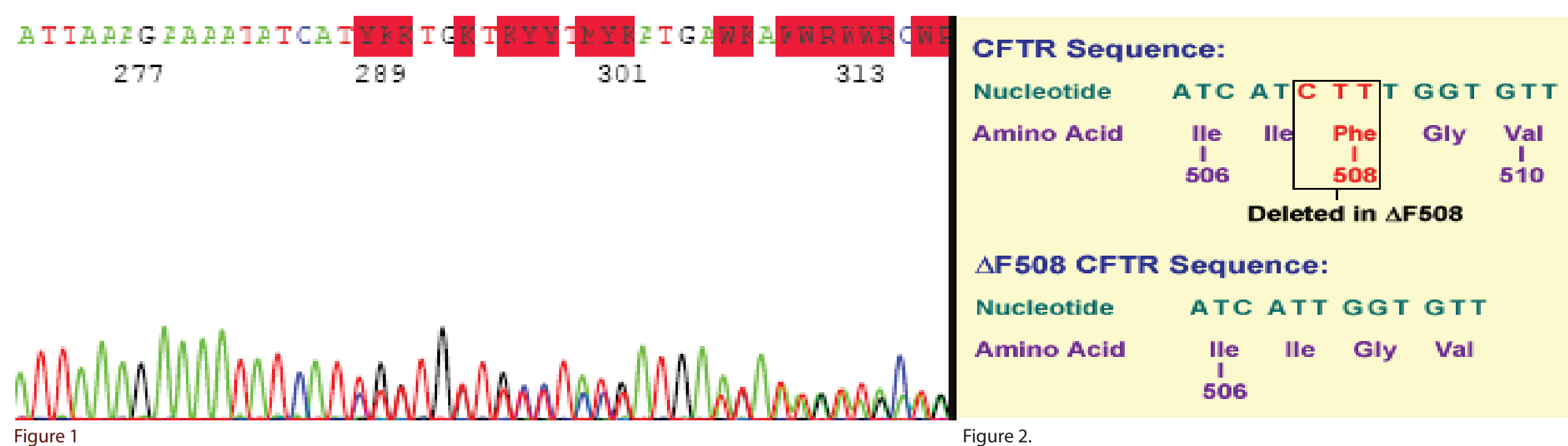


Figure 1. CF sequencing electropherogram of sample #1 amplified using EzWay™ Direct PCR buffer showing heterozygous delta F508 mutation characterized by the deletion of three base pairs in the CFTR gene sequence, resulting in the loss of phenylalanine located at position 508 of the CFTR gene see Figure 2.

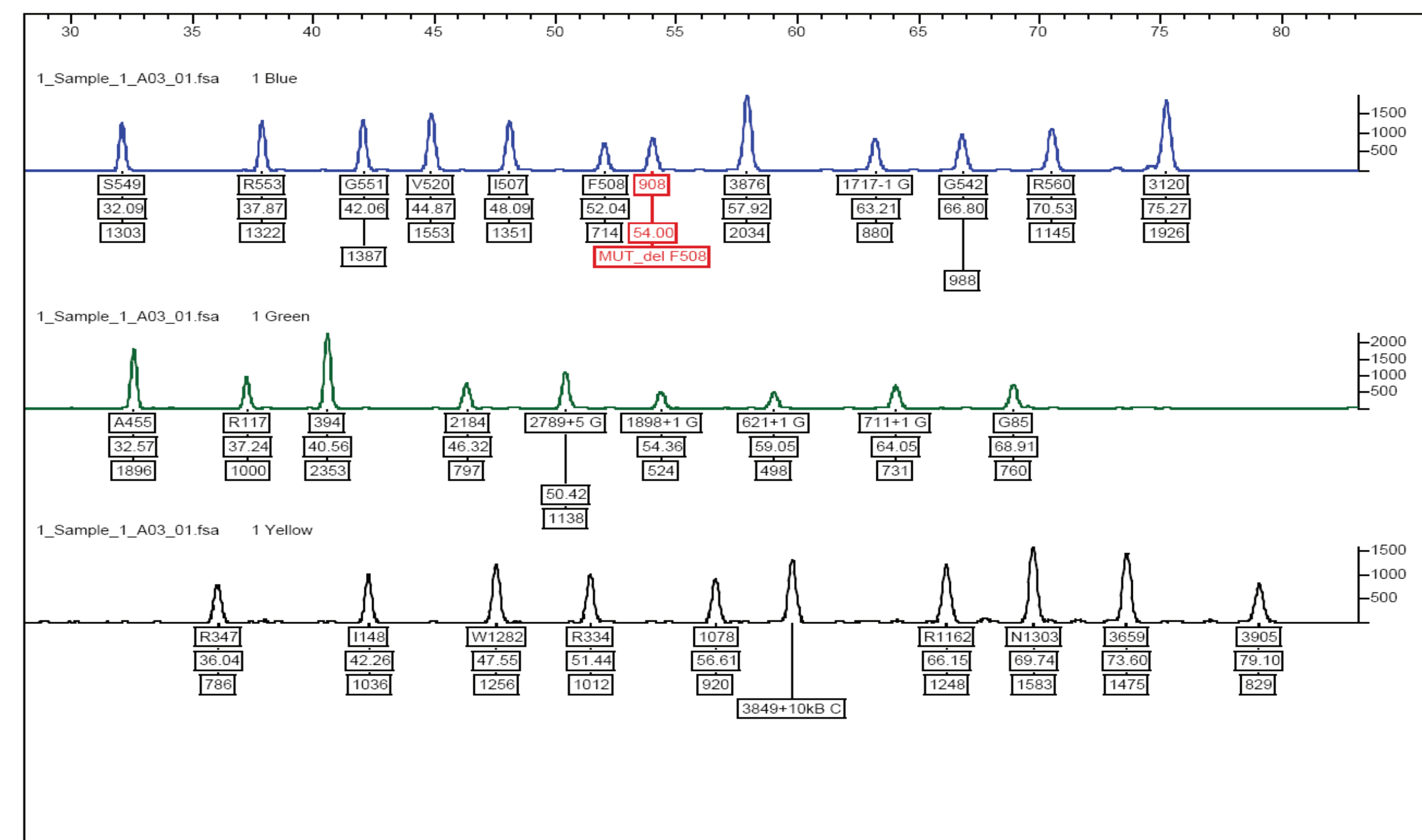


Figure 3. Electropherogram of CF mutation panel for sample #1 using Blood Direct™ PCR buffer and Abbott/Celera CF v3 Genotyping kit showing heterozygous delta F508 mutation.

| Sample # | CFTR results (extracted DNA) | CFTR results (direct blood amplification from Guthrie card) |
|----------------|------------------------------|---|
| CF Sample # 1 | Heterozygous F508 | Heterozygous F508 |
| CF Sample # 2 | Heterozygous I148T | Heterozygous I148T |
| CF Sample # 3 | Heterozygous+1G>T | Heterozygous+1G>T |
| CF Sample # 4 | Heterozygous R117H | Heterozygous R117H |
| CF Sample # 5 | Heterozygous N1303K | Heterozygous N1303K |
| CF Sample # 6 | Heterozygous G551D | Heterozygous G551D |
| CF Sample # 7 | Heterozygous 3849+10kB C>T | Heterozygous 3849+10kB C>T |
| CF Sample # 8 | Heterozygous W1282X | Heterozygous W1282X |
| CF Sample # 9 | Homozygous F508 | Homozygous F508 |
| CF Sample # 10 | Wild Type | Wild Type |
| Neg | Negative | Negative |

Table 1 a. CFTR genotyping results from extracted DNA and whole blood (Guthrie cards)

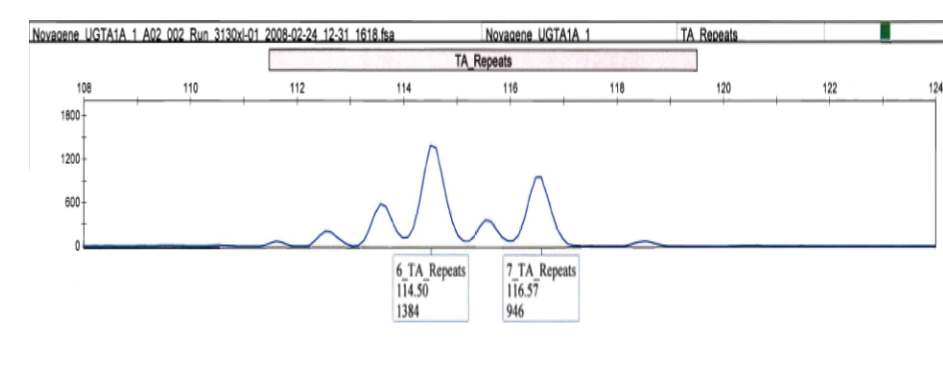


Figure 4. An example of UGT1A1 genotype showing 6/7 TA repeats in the UGT1A1 gene using KAPA blood kit.

| Sample # | UGT1A1 results (extracted DNA) | UGT1A1 results (direct blood amplification) |
|------------|--------------------------------|---|
| Sample # 1 | 6/7 | 6/7 |
| Sample # 2 | 6/6 | 6/6 |
| Sample # 3 | 6/7 | 6/7 |
| Sample # 4 | 6/6 | 6/6 |
| Sample # 5 | 7/7 | 7/7 |
| Neg | Negative | Negative |

Table 1 b. UGT1A1 results from extracted DNA and whole blood

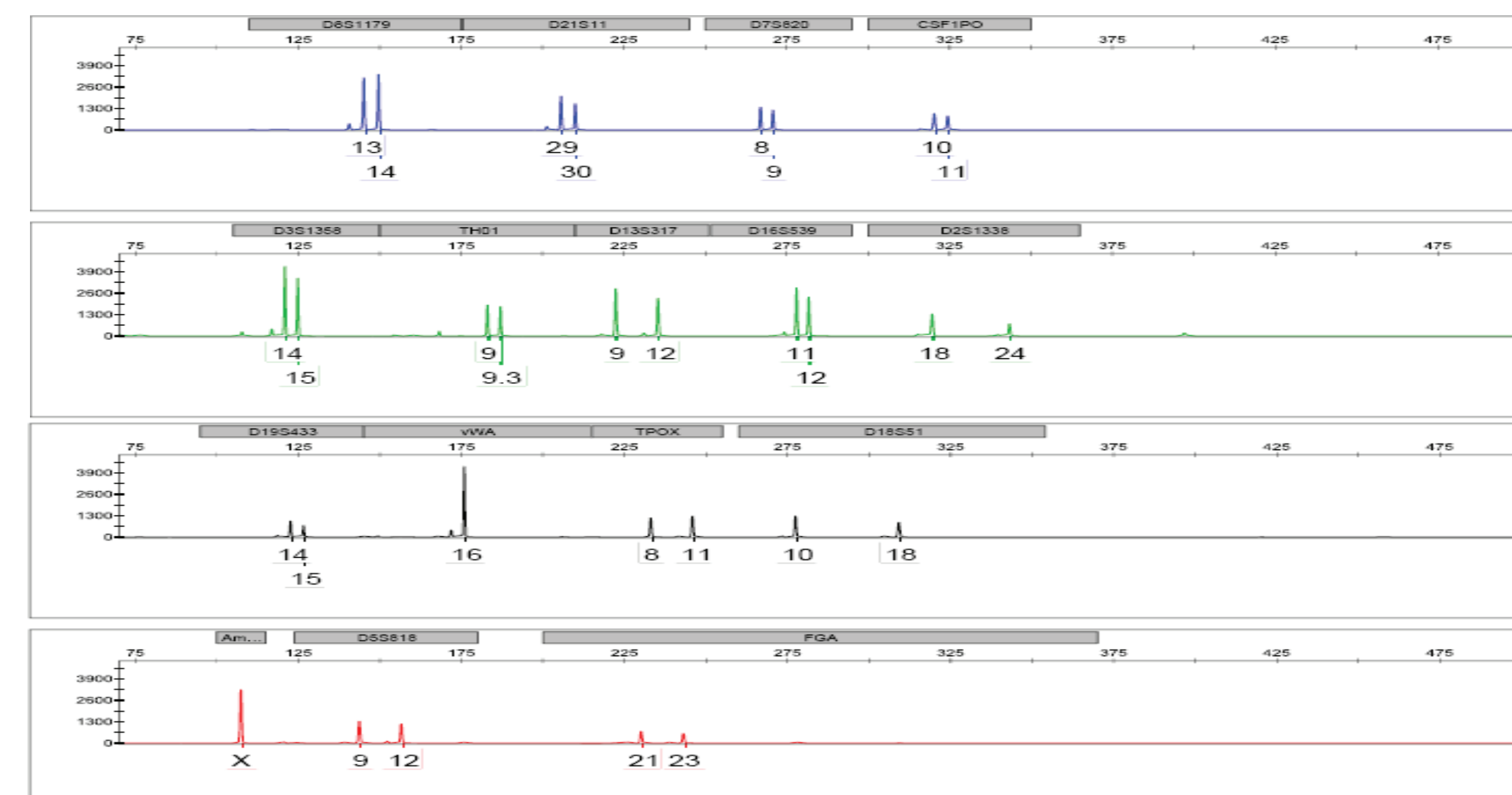


Figure 5. Electropherograms of 15 STR loci using AmpF/STR Identifier kit with BloodDirect Buffer from whole blood.

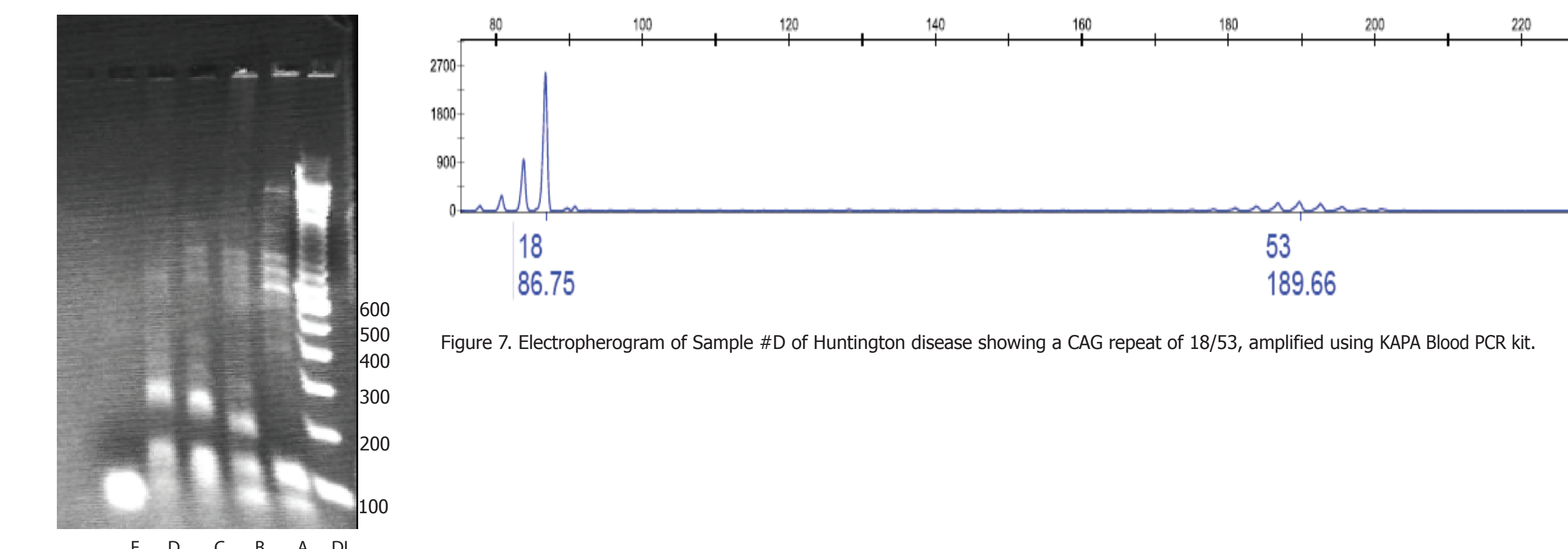


Figure 6. Photograph of a 2% agarose gel of samples A-D of various CAG repeats in Huntington gene.
DL=DNA Ladder, A=16/17 CAG repeats
B=17/40 CAG repeats, C=18/48 CAG repeats
D=18/53 CAG repeats, E=Negative Control

Conclusions:

All samples tested on the four assays performed very well from whole blood using the 2 different buffers and the KAPA Blood PCR kit, giving 100 % concordance with results from extracted DNA. We found these two buffers and blood kit to be compatible with fresh, stored, and dried blood samples on Guthrie cards. The process requires very little human blood and was compatible with a variety of commercially available DNA polymerases, (except for AmpliTaq DNA polymerases with Blood Direct™ PCR buffer).

Of great interest was the amplification of Huntington disease samples. These samples contain a variable number of CAG repeats, which could be amplified directly with the KAPA Blood PCR Kit B. The highest repeat we tested was 18/53 but it will be interesting to see how long of a CAG repeat can be amplified. The highest CAG repeats we have been able to amplify from extracted DNA has been in the mid 90's.

The STR analysis of 3 whole blood samples produced the expected DNA profiles. The STR profiles generated from whole blood exhibited peak height balance across all loci as expected for high quality DNA. There were no differences between the quality of DNA profiles obtained from extracted DNA and those obtained from whole blood amplified samples.

The direct amplification from whole blood and Guthrie cards made this attractive for large scale newborn screening for early disease detection and intervention.

These three procedures provide alternatives to the tedious DNA purification processes and may prevent possible errors inherent to the DNA extraction procedure, such as sample switches or contamination. We found that the buffers offered a convenient way to PCR, especially on a limited amount of sample, and allowed one to test different types of Taq as the buffers are compatible with a variety of commercially available DNA polymerases. The only drawback to this method is that, when loading samples for analysis on an ABI 3100 instrument, the samples must first be spun down and only supernatant can be used for loading as other negatively-charged products would compete for injection.

Acknowledgement:

The authors thank ARUP Institute for Clinical and Experimental Pathology for funding this project, Maryke Appel and Jason Steel of KAPA Biosystems for reagents and technical support, and Jacquelyn McCowen-Rose for help with poster design.

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