Use of mitoPrimers™ and CleanAmp™ PCR Master Mix for Mitochondrial DNA Testing

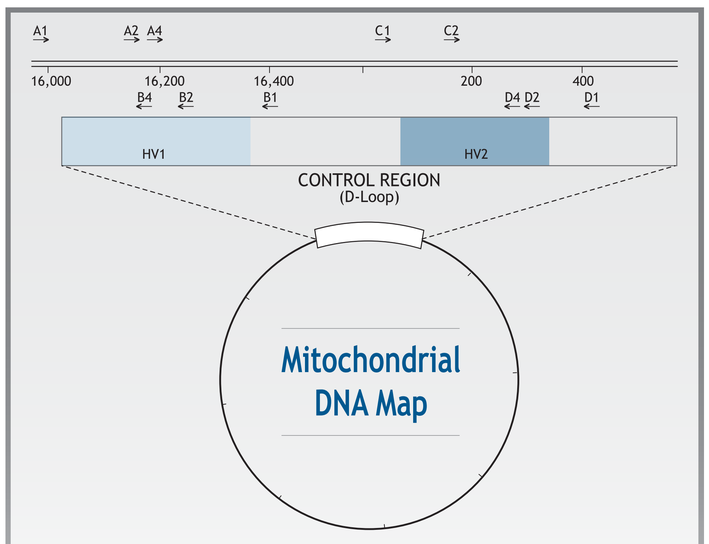
Sabrina Shore, MS[1], Natasha Paul, PhD[1], Gerald Zon, PhD[1], Rhonda K Roby, PhD, MPH[2]

[1]TriLink BioTechnologies, San Diego, CA. [2] Department of Forensic and Investigative Genetics, University of North Texas Health Science Center, Fort Worth, TX.

Abstract

Methods of identifying human biological material are continually improving and expanding: new multiplex STR kits, optimized buffers, high-performing robotics and sophisticated software. While commericially available kits for amplification of genomic DNA are established and validated by the forensic community, mitochondrial (mtDNA) amplification and sequencing reagents and protocols remain unstandardized. mtDNA amplification and sequencing protocols employ a variety of DNA polymerases, primers, other PCR accessory reagents and thermal cycling conditions, which all can influence the quality of downstream data for Sanger sequencing. TriLink has developed standardized mtDNA analysis reagents. Now, off-the-shelf primers and soon master mixes will be available for mtDNA testing. TriLink's mitoPrimers™ are the same primers the forensic community has been using for over 25 years to interrogate the control region of the mitochondrial genome. These primers are HPLC purified and pre-aliquotted into convenient, dilute-and-go vials. The primers are vacuum-desiccated, subjected to quality control testing and available by overnight delivery. Additionally, a PCR master mix, containing CleanAmp™ dNTPs (TriLink), is being developed and optimized specifically for the amplification and downstream analysis of the control region of mtDNA, the CleanAmp™ mtDNA PCR 2X Master Mix. The key component in this PCR master mix is hot start dNTPs which employ a thermolabile protecting group. This modification blocks low temperature primer extension and is released at higher temperatures to allow for more specific DNA polymerase incorporation. The goal is to optimize amplification success by improving PCR yield and specificity, decreasing potential human error from the addition of individual components and providing reagents that are quality control tested prior to release. The primers, the master mix development and the QC testing performed will be presented.

Figure 1: Quality Controlled mitoPrimers™ for Amplification and Sequencing of the mtDNA Control Region



Hypervariable Region 1 (HV1)		Hypervariable Region 2 (HV2)	
Region	For/Rev Primer	Region	For/Rev Primer
HV1 (15998-16390)	A1/B1	HV2 (49-407)	C1/D1
HV1A (15998-16236)	A1/B2	HV2A (49-284)	C1/D2
TV1B (166130/16390)	A2/B1	TV2B (178-407)	C2/D1
HV1A4 (16210-16390)	A4/B1	HV2A4 (49-265)	C1/D4
HV1B4 (15998-16162)	A1/B4		

Figure 2: mitoPrimers™ Experimental Workflow

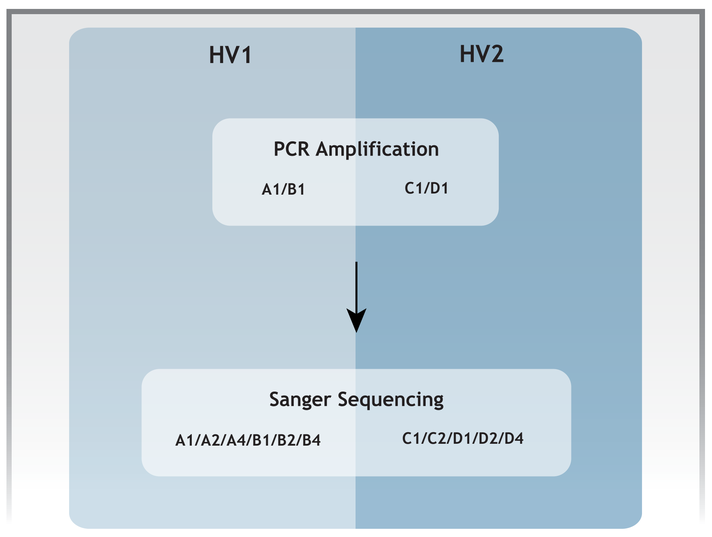


Figure 2: mitoPrimers™ are Quality Control Tested with NIST-certified DNA

Representative mitoPrimers™ QC release data

Primer	Catalog #	Lot #	Criteria 1 (PCR)	Criteria 2 (Sequencing)	Trace Score	Contiguous Read Length
A1 (L15997)	O-33001	T1-APV01A	Pass	Pass	55	384
A2 (L16159)	O-33002	T1-AME01A	NR	Pass	32	216
A4 (L16209)	O-33003	T1-AKW03B	NR	Pass	37	252
B1 (H16391)	O-33004	T1-AME02A	Pass	Pass	32	408
B2 (H16237)	O-33005	T1-AKW05A	NR	Pass	35	227
B4 (H16164)	O-33006	T1-AKW06A	NR	Pass	32	145
C1 (L048)	O-33007	T1-AKW07A	Pass	Pass	37	329
C2 (L177)	O-33008	T1-AKW08A	NR	Pass	32	153
D1 (H408)	O-33009	T1-AKW09A	Pass	Pass	36	350
D2 (H285)	O-33010	T1-AKW10A	NR	Pass	40	216
D4 (H266)	O-33011	T1-AKW11A	NR	Pass	38	210

Trace Score (TS) is the average quality value of each base in the trace after trimming. High quality TS = 35 to 100; medium quality TS = 21 to 34 (yellow); and low quality TS = 0 to 20.

Contiguous Read Length (CRL) is calculated as the number of uninterrupted bases in the trace that have a quality value greater than 20; the number of contiguous bases is dependent on the region sequenced.

NR = Not Required.

Figure 3: Primer Purity is Important for High Quality mtDNA Sanger Sequencing Data

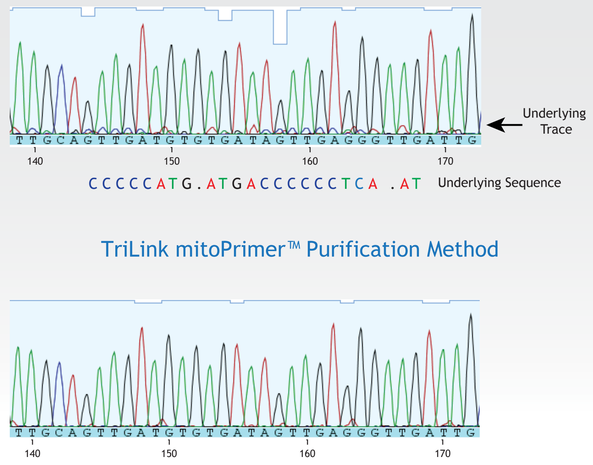


Figure 4: CleanAmp™ Hot Start dNTP Activation Mechanism

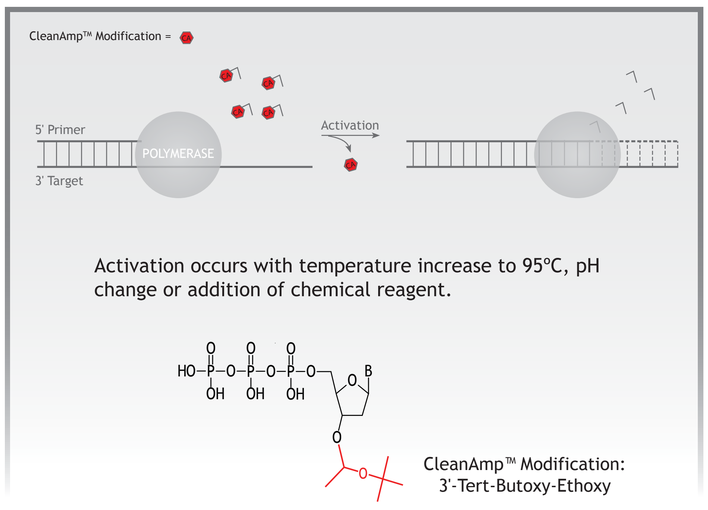
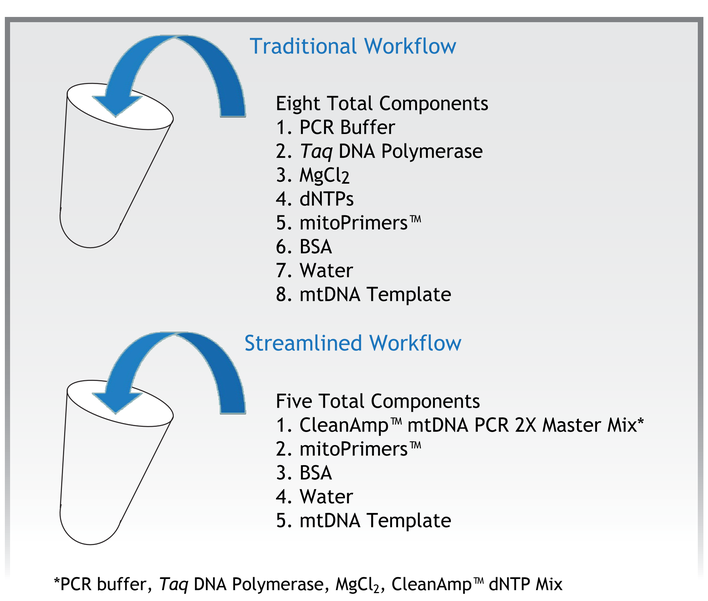
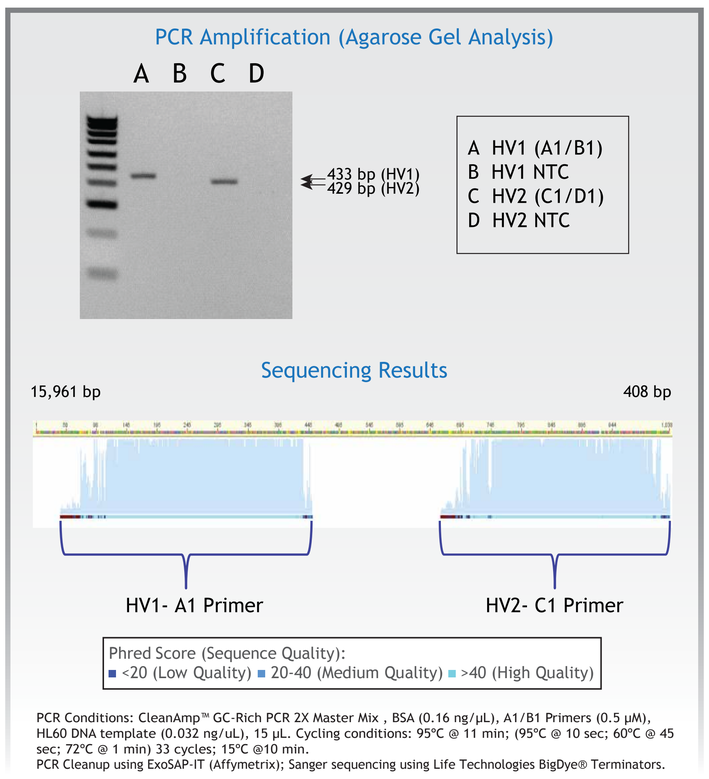


Figure 5: CleanAmp™ mtDNA PCR 2X Master Mix Improves Workflow



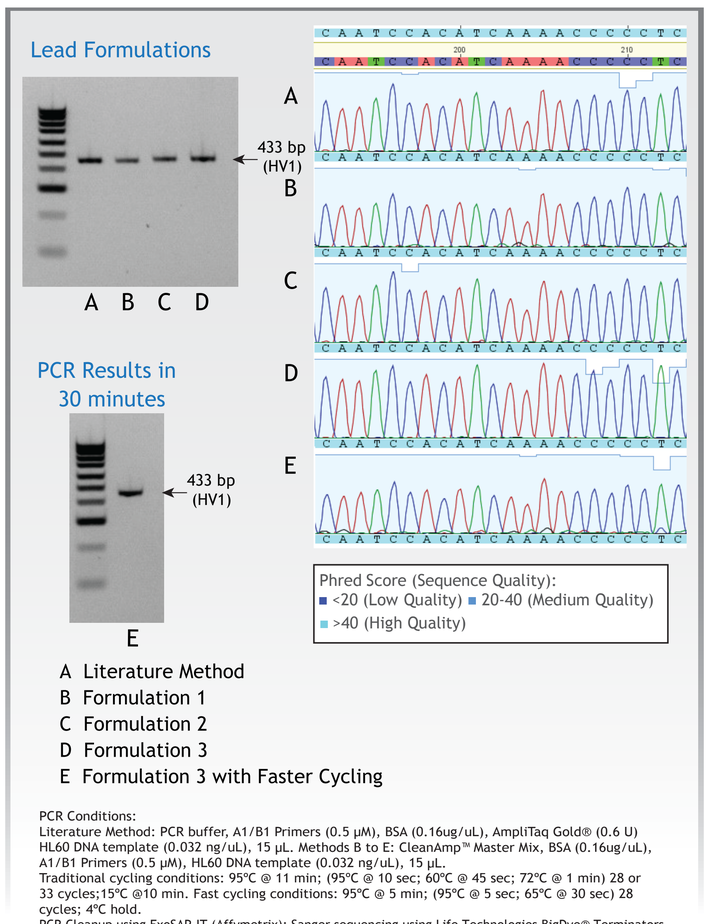
*PCR buffer, *Taq* DNA Polymerase, $MgCl_2$, CleanAmp™ dNTP Mix

Figure 7: Feasibility of CleanAmp™ PCR Master Mix to the Complete PCR and Sanger Sequencing Workflow



PCR Conditions: CleanAmp™ GC-Rich PCR 2X Master Mix , BSA (0.16 µg/µL), A1/B1 Primers (0.5 µM), HL60 DNA template (0.032 ng/uL), 15 µL. Cycling conditions: 95°C @ 11 min; (95°C @ 10 sec; 60°C @ 45 sec; 72°C @ 1 min) 33 cycles; 15°C @10 min.
PCR Cleanup using ExoSAP-IT (Affymetrix); Sanger sequencing using Life Technologies BigDye® Terminators.

Figure 7: Developing a CleanAmp™ mtDNA PCR 2X Master Mix that Will Increase Target Yields and Save Time



A Literature Method
B Formulation 1
C Formulation 2
D Formulation 3
E Formulation 3 with Faster Cycling

PCR Conditions:
Literature Method: PCR buffer, A1/B1 Primers (0.5 µM), BSA (0.16ug/uL), AmpliTaq Gold® (0.6 U) HL60 DNA template (0.032 ng/uL), 15 µL. Methods B to E: CleanAmp™ Master Mix, BSA (0.16ug/uL), A1/B1 Primers (0.5 µM), HL60 DNA template (0.032 ng/uL), 15 µL.
Traditional cycling conditions: 95°C @ 11 min; (95°C @ 10 sec; 60°C @ 45 sec; 72°C @ 1 min) 28 or 33 cycles;15°C @10 min. Fast cycling conditions: 95°C @ 5 min; (95°C @ 5 sec; 65°C @ 30 sec) 28 cycles; 4°C hold.
PCR Cleanup using ExoSAP-IT (Affymetrix); Sanger sequencing using Life Technologies BigDye® Terminators

Conclusion

- mitoPrimers ™ undergo a proprietary purification process and are QC validated to ensure high quality Sanger dideoxy sequencing results.
- The use of CleanAmp™ mtDNA PCR 2X Master Mix streamlines the PCR workflow, thereby reducing the possibility of human error, saving on cost and shortening preparation time.
- mitoPrimers™ are quality control tested with NIST-certified DNA. This allows for their use in the PCR amplification and Sanger sequencing of mtDNA samples.
- Future developments include a formulation which can be employed in fast PCR cycling protocols to acheive high quality data in as little as 30 minutes.

Acknowledgements: Richard Hogrefe, David Combs, Terry Beck, Jessica Stewart, Angela Asuncion, Elizabeth Hill, Anton McCaffrey, Julie Powers

Contact: Natasha Paul, npaul@trilinkbiotech.com

trilinkbiotech.com/products/mtDNA
trilinkbiotech.com/cleanamp