# Performance of the Early Access AmpliSeq™ Mitochondrial Panel with degraded DNA samples using the Ion Torrent™ platform

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**Abbreviations: DI**, degradation index; **HVR**, hypervariable region; **INDEL**, insertion and deletion; **MPS**, massively parallel sequencing; **mtDNA**, mitochondrial DNA; **rCRS**, revised Cambridge Reference Sequence

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The Early Access AmpliSeq™ Mitochondrial Panel amplifies whole mitochondrial genomes for phylogenetic and kinship identifications, using Ion Torrent™ technology. There is currently limited information on its performance with degraded DNA, a common occurrence in forensic samples. This study evaluated the performance of the Panel with DNA samples degraded *in vitro*, to mimic conditions commonly found in forensic investigations. Purified DNA from five individuals was heat-treated at five time points each (125°C for 0, 30, 60, 120 and 240 minutes; total *n*=25). The quality of DNA was assessed via a real-time DNA assay of genomic DNA and prepared for massively parallel sequencing on the Ion Torrent™ platform. Mitochondrial sequences were obtained for all samples and had an amplicon coverage averaging between 66X to 2,803X. Most amplicons (157/162) displayed high coverages (452 ± 333X), while reads with less than 100X coverage were recorded in 5 amplicons only (90 ± 5X). Amplicon coverage was decreased with prolonged heating. At 72% strand balance, reads were well balanced between forward and reverse strands. Using a coverage threshold of 10 reads per SNP, complete sequences were recovered in all samples and resolved kinship and, haplogroup relations. Additionally, the HV1 and HV2 regions of the reference and 240 minute heat-treated samples (n=10) were Sanger – sequenced for concordance. Overall, this study demonstrates the efficacy of a novel forensic Panel that recovers high quality mitochondrial sequences from degraded DNA samples.

## 1 Introduction

There is value in using mitochondrial DNA (mtDNA) for ancestry and kinship analysis. This is true in cases of unsuccessful nuclear DNA typing or when there is lack of reference profiles [1]. Traditionally, mtDNA sequencing has been restricted to the hypervariable regions (HVR) that exhibit the most polymorphisms [2]. However, the control region covers only 7% of the human mitochondrial genome and limits the resolution of matrilineal and ancestry inferences [3-5]. Whole genome sequencing offers additional sequences from which mitochondrial haplogroups can be determined to the highest resolution [6, 7].

For the last forty years, chain-terminating technology has been a consensus method of DNA sequencing [8]. However, using this technology to sequence whole genomes can be highly labour intensive and costly. Alternatively, massively parallel sequencing (MPS) can retrieve billions of ssDNA molecules, amplified from panels containing targeted or whole genome markers [9]. Both targeted and whole genome panels have been used as *in-house* methods to amplify mitochondrial genomes for sequencing [7, 10-12]. However, based on panel design, the performance of *in-house* panels can be highly variable in accuracy, coverage and strand balance. This is not optimal for operational forensic laboratories that require a standard panel of optimised performance. Therefore a standard panel is required to normalise mitochondrial MPS testing across forensic DNA laboratories.

The Early Access AmpliSeq™ Mitochondrial Panel (Applied Biosystems, CA, USA) offers a solution to standardising MPS testing of mitochondrial genomes. The Panel uses 162 primer pairs to amplify whole mitochondrial genomes for MPS uses. To date, only earlier versions of the Panel have been evaluated using degraded DNA samples [13]. This study assessed the performance of the MPS panel to amplify mitochondrial genomes in reference and degraded DNA samples. Coverage, strand balance and accuracy of variant calls for familial and phylogenetic relations were evaluated and the potential of the MPS Panel to supplement the CE workflow of operational forensic laboratories is discussed.

## 2 Materials and methods

### 2.1 Samples

Saliva samples (Classiq Swabs™, Copan Diagnostics, CA, USA) were collected from one unrelated individual of Swiss ancestral background and four related Fijian-Indian individuals. The ancestry information was self-reported and based on the biogeographical ancestry of all five grandparents. DNA samples were collected with informed consent and handled according to ethical procedures approved by the Human Ethics Committee for the University of Technology Sydney (Approval Number: 2015000296). DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol for buccal swabs [14]. Equal volumes of each biological extract were heat-treated at 125°C for 30, 60, 120, 240 minutes. An untreated sample for each individual was used as a reference for artificial degradation (total *n*=25). The quantity and degradation of DNA was measured using the Quantifiler™ Trio DNA Quantification Kit (Life Technologies, CA, USA), on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, CA, USA), following manufacturer's protocol [15]. DNA concentrations were normalised (4 ng/µL) and stored at -20°C.

### 2.2 Library preparation

Each DNA template was amplified for 162 mitochondrial amplicons using two primer pools included in the Early Access AmpliSeq™ Mitochondrial Panel (Applied Biosystems, CA, USA) in two separate reactions. Following manufacturer's 2-in-1 method for low copy number samples, 0.1 ng gDNA for each sample was combined with 5X Ion AmpliSeq™ HiFi Mix and 10X primer pool, included in the Precision ID Library Kit (Applied Biosystems, CA, USA) [16]. Amplification included 2 minutes at 99°C followed by 21 cycles of 15 seconds at 99°C, 4 minutes at 60°C and overnight hold at 10°C on the Veriti® 96-Well Thermal Cycler (Applied Biosystems, CA, USA). Products from two primer pools were combined for each

92 respective sample and post-PCR primers were digested with FuPa reagent included in the
93 kit.

94 Amplicons were ligated to Ion P1 Adapter and Ion Xpress™ Barcode adapters and, purified
95 using AMPure™ XP reagent (Beckman Coulter, CA, USA). Size (bp) of library fragments
96 were assessed using the Agilent High Sensitivity DNA Kit on the Agilent 2100 Bioanalyser
97 (Agilent Technologies, CA, USA), following standard protocols [17]. Quantity of libraries was
98 determined using the KAPA SYBR® FAST ABI Prism qPCR Kit (Kapa Biosystems, MA, USA)
99 on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, CA, USA) [18].
100 Libraries were diluted (8 pM) and equal volumes pooled for template preparation.

101 Pooled libraries (25 µL) were fixed onto template beads and amplified using the Ion PGM™
102 Template OT2 200 Kit (Applied Biosystems, CA, USA) [19]. Amplification was carried out on
103 the Ion OneTouch™ 2 System (Applied Biosystems, CA, USA). Amplified templates were
104 enriched for target monoclonal Ion Sphere™ Particles (ISPs) on the Ion OneTouch™
105 Enrichment System (Applied Biosystems, CA, USA). Quality of ISPs were evaluated with the
106 Ion Sphere™ Assay (Life Technologies, CA, USA) on Qubit® 2.0 Fluorometer (Invitrogen,
107 CA, USA) [20].

108 **2.3 DNA sequencing**

109 25 mtDNA template libraries were loaded onto a Ion 316™ Chip using the Ion PGM™ Hi-
110 Q™ Sequencing Kit (Applied Biosystems, CA, USA), following the manufacturer's
111 recommendations [21]. Libraries were sequenced on the Ion Torrent PGM™ System
112 (Applied Biosystems, CA, USA) using the protocol for 500 nucleotide flows.

113 **2.4 DNA sequence analysis**

114 DNA sequences were reconstructed by pooling all barcoded libraries to respective samples
115 and trimming adapter sequences 20 bases from the 3' and 5' end, using Torrent Suite™
116 software (Applied Biosystems, CA, USA). Sequences were formatted to the human

mitochondrial genome by alignment to the revised Cambridge Reference Sequence (rCRS) [22, 23]. Sequence variants, SNPs, insertions and deletions (INDELs) were reported using the Ion PGM™ System: Torrent Variant Caller (Applied Biosystems, CA, USA) plugin as variant caller files. Binary alignment map files of aligned sequences and variants were inspected using Integrative Genomics Viewer (Broad Institute, MA, USA) [24, 25]. Variants were imported into MitoTool, a third party online software designed with PhyloTree Build 17, and used for assignment of mitochondrial haplogroups [26, 27]. HaploGrep 2 (v2.1.0) was used as a secondary confirmation of haplogroups [28]. A minimum arbitrary threshold of 10X coverage reads was used to call mitochondrial variants and a threshold of 0.05 was set for point heteroplasmy detection. In line with forensic convention, length heteroplasmy was reported to the most dominant allele of all detected sequences [29].

### 2.5 Statistical analysis

Coverages of mitochondrial amplicons were pooled and presented as overall reads for each treatment ($n$=5). Strand balance was calculated as a percentage of lower reads/higher reads between complementary amplicon strands [30]. ANOVA (one-way) was used to measure strand balance differences across treatments. The statistical tests ($\alpha$=0.05) were performed using Microsoft Excel 2010 (Microsoft Corporation, WA, USA).

## 2.6 CE concordant sequence

HV1 (15,971 – 16,410 rCRS) and HV2 regions (15 – 389 rCRS) of the reference and 240 minute heat-treated samples ($n$=10) were sequenced by CE systems for concordant data. PCR amplification was prepared with the HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) using 4 ng genomic DNA and 0.5 µM HV1 and HV2 M13-tailed primers [31, 32]. The Veriti® 96-Well Thermal Cycler (Applied Biosystems, CA, USA) was used for amplification with parameters set to 95°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 62°C for 45 seconds, 72°C for 1 minute and final extension of 72°C for 10 minutes [33]. Products were sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit

on the 3730XL DNA Analyser (Applied Biosystems, CA, USA) [33]. Raw DNA sequences were analysed and aligned using DNA Sequencing Analysis Software (Applied Biosystems, California, USA).

## 3 Results and discussion

### 3.1 DNA quality

The quality of DNA was decreased with heat-treatment, marked by the higher degradation indexes (DI) (Fig. 1). The DNA quantities (ng/µL) used to derive DI's for each treatment group is provided in Supporting Information Figure 1. Little to no degradation was observed in reference samples (4 ng/µL), with a DI 0.75 ± 0.03 (mean ± S.E.M.). Mild degradation was seen in the heat-treated DNA samples at 30 minutes (DI 1.02 ± 0.07, 3.52 ng/µL) and 60 minutes (DI 1.74 ± 0.21, 2.46 ng/µL). Prolonged heat-treated samples at 120 minutes (DI 5.64 ± 0.67, 1.41 ng/µL) and 240 minutes (DI 18.18 ± 3.22, 0.44 ng/µL) were highly degraded, indicating occurrence of putative DNA fragmentation.

Note in this study, degradation refers to the fragment quality of DNA. While heat-treatment was used for *in vitro* degradation, it is recognised that the method is limited as it does not truly mimic forensic DNA samples. Other factors such as the quantity of DNA and presence of co-inhibitors in extracts can also be encountered in compromised samples. Consequently it is strongly recommended that these variables can be assessed in separate studies.

Degradation of DNA has been previously shown to start at 100°C and become completely degraded with longer exposures [34]. In these cases, heat was found to degrade nucleic acids into fragments due to DNA depurination, and a break of glycosidic and phosphodiester bonds [35]. Therefore these findings support the progressive degradation of heat-treated DNA samples that were determined from a real-time assay. However, there is a limitation to infer mtDNA quality from genomic real-time assays as mtDNA has been shown to be more

stable to degradation [36, 37]. Hence, a real-time duplex assay for mtDNA targets of which few are available, would offer a more accurate assessment of DNA quality [38, 39].

### 3.2 Sequencing metrics

A total of 173 megabases of MPS data was generated for 25 samples using 500 flow runs. An average live ISP density of 47% (1,503,921 reads) was obtained and 131 million bases were correctly aligned to the rCRS. Mean coverage depth for alignment quality was reported to 7,875X at mean read lengths of 99 bp fragments.

### 3.3 Coverage

The Panel shows high amplicon coverage for reference mtDNA samples, though coverage reads are non-uniformly distributed, with some reads as low as 66X (amplicon 3473-3596 rCRS) and other reads as high as 2803X (amplicon 10,482-10,577 rCRS) (Fig. 2). Overall amplicon coverage (*n*=5) was 468 ± 21 reads (mean ± S.E.M.) for time 0 samples, 348 ± 8 reads for 30 minute samples, 395 ± 12 reads for 60 minute samples, 339 ± 12 reads for 120 minute samples and 274 ± 11 reads for 240 minute samples (Fig. 3).Ninety-seven percent of amplicons (157/162) demonstrated reads greater than 100X on both strands. Only five amplicons (3473-3596 rCRS, 10,394-10,492 rCRS, 12,352-12,459 rCRS, 13,686-13,789 rCRS and 14,276-14,367 rCRS) were under-reported with reads below 100X. While there was a marginal decrease in reads for prolonged heating times, the decrease did not affect the detection of variants. This trend agrees with a previous study that recovered full mitochondrial genomes for DNase-treated samples (at 50 reads) [13]. In comparison, at a coverage threshold of 50 reads, 92% (23/25) of samples in this study displayed complete genomes and reported all variants. When lowered to 10 reads, all samples were sequenced for complete genomes (Supporting Information Table 1).

The difference in recovery of genomes may be explained by sequencing samples of varying qualities on a single chip. The multiplexing of different quality samples has been considered

to reduce read quality as there is a preferable amplification towards high quality DNA samples [40]. Consequently separating samples of pristine and compromised quality may improve sequence recovery. Alternatively reducing the number of samples may also improve coverage and sequence quality as more reads can be assigned to each sample [41, 42]. Though chip density was not maximised (ISP, 47%) which indicates manual library preparation and chip loading could be further optimised or automated. The highest number of samples multiplexed in a sequence run has been previously reported to be 15 samples on a single Ion 316™ Chip, albeit using a different custom MPS panel [43]. Here it is demonstrated that chip capacity can be extended to at least 25 samples, using this whole genome Panel. Therefore it is suggested that the pooling of samples of similar qualities as well as reducing the number of samples in sequence runs, may result in overall higher coverages.

The low reads reported for two samples may indicate potential nucleotide sites (14,766 and 14,783 rCRS) which are difficult to sequence. Both sites follow a 4-mer poly-A-stretch (AAAA) region. Studies have suggested that it is the homopolymer stretch that lowers the efficiency of reads in this region [7, 43]. Low coverage in homopolymer regions of the Ion PGM™ or other MPS platforms has been described, as have high coverages in amplicons with few homopolymeric repeats [44, 45]. Bragg et al. [46] suggest the most likely explanation to be an inaccurate flow-call of the proton-based system. As well as homopolymer stretches, G/C content and sample quality are also known to affect the accuracy of flow-calls [44]. The over- and under- calling of nucleotides would also explain the non-uniform distribution of amplicon reads that were observed in homopolymeric regions. It seems that the amplicon reads were mostly affected by sequence composition over other variables such as base size. The two samples which failed to reproduce complete genome sequences were both heat-treated at 125°C for 240 minutes. Naturally, DNA samples of poor or degraded qualities are likely to record low to no reads, because of the fragmented state of the DNA [12, 13, 47]. Therefore the observed low coverages most likely arise from a

complication in the assigned flow-call algorithm and DNA quality as opposed to the design of the Panel.

Point heteroplasmy was detected in all samples (ranges, 1-16,569 rCRS) at a threshold of 0.05. Three point heteroplasmic variants were each detected in the Swiss individual (214R, 750R, 16221Y) and the Fijian-Indian father (750R, 10586R, 15043R), while the same point heteroplasmy was shared between the Fijian-Indian mother, son and daughter (750R, 15043R, 15258Y). Manual inspection of mitochondrial variants showed insertion and deletion of nucleotides, especially in homopolymer regions. Length heteroplasmy was also observed in these homopolymer regions. All reference samples were found to contain an uninterrupted C stretch in the range of 303-315 rCRS as 310C, 315.1C and 315.2C. Additionally one sample also showed deletions, 514- at 214 reads and 515- at 212 reads (Supporting Information Figure 2).

### 3.4 Strand balance

Amplicons of the Panel were well balanced between forward and reverse strands, though balance was non-uniform across the mitochondrial genome. For five reference samples, strand bias (<50%) was observed in 19/162 (12%) amplicons. In particular, three amplicons (299-411 rCRS, 13,686-13,789 rCRS and 14,276-14,367 rCRS) showed extreme strand bias (<10%) (Fig. 4).

While most strands were balanced in this study, extreme strand bias has led to the erroneous designation of SNPs in other MPS panels [48]. Other studies using different *in-house* mitochondrial panels have also observed imbalance at similar positions suggesting that strand bias in these regions is inherent in the sequence of DNA [43]. Since strands are equal in length and complementary, strand bias can quite possibly be sequence and platform dependent [49, 50]. Previously, strand bias has been attributed to the multiple continuous stretches of homopolymers in the DNA region [43]. This is plausible as homopolymers have been shown to reduce reads more so in strands with poly-C-stretches than any other repeats [30].

Overall strand bias was 72% ± 14% (mean ± S.E.M.) for reference samples and 60% ± 3% for heat-treated samples at 30, 60, 120 and 240 minutes ($n$=5) (data not shown). Strand bias was significantly increased in degraded samples ($P$=0.000, one-way ANOVA). Originally it was hypothesised that strand bias would remain unaffected by the nature of the sample (degraded or non-degraded) as forward and reverse strands undergo the same treatment. However, random breakage of covalent bonds in strands is increased with heat-treatment and thereby may account for the imbalanced reads [34, 51].

### 3.5 Variant detection

As expected more variants were detected in the whole mitochondrial genome than the HV1 and HV2 regions. In non-treated samples, the number of HVR variants (16,024-576 rCRS)

compared to the whole mitochondrial genome (1-16,569 rCRS) were 7/14 for sample 1, 11/33 for sample 2, 10/34 for sample 3, 11/33 for sample 4 and 11/33 for sample 5 (Table 1). These variants were mostly concordant to the HV1 and HV2 CE sequences of reference and 240 minute heat-treated samples. Compared to the typing of single DNA molecules in CE systems, MPS platforms have a greater sensitivity to detect intra-individual sequences as it types a multitude of DNA fragments [52]. The greater sensitivity accounts for the differences in base calls that were mostly found in heteroplasmic positions, such as 214R and 16043R. Other sequence differences were due to unreported bases by the CE system, mostly likely arising as artifacts of sequencing. A remaining 97 variants were called in addition to HV1 and HV2 and highlight the increased resolution of a whole genome MPS panel over HV1 and HV2 sequencing.

Variant assignment of mitochondrial haplogroups was consistent and accurate between reference and degraded samples of the same individual. However, not all archetypical variants of haplogroups were present in each individual as might be expected (Table 1). It is common for individuals to acquire mutations that differ to the overall variants of historical haplogroups because of the high mutation rate of mtDNA [30, 53-55]. Nonetheless, it has been shown that a majority of detected variants will allow haplogroups to be assigned with a sufficient reliability. Specifically, mtDNA sequences of high scoring quality (>90% quality, HaploGrep) have been shown to correctly assign haplogroups [30]. Herein, the quality scores of variants were >90% and thereby indicates a reliable and, accurate alignment of variants to haplogroups.

*3.5.1 Kinship analysis*

Kinship relations were correctly assigned to individuals of the same maternal lineage. Within the five individuals, twenty one unique variants were detected for Individual 1 and seven unique variants for Individual 2 which could be used for kinship exclusion. All heat-treated samples were used for kinship analysis at 10X minimum coverage. The results

demonstrated that even the prolonged-treated samples can be used for exclusion of the Swiss individual and Fijian-Indian father as maternal lineages of the Fijian-Indian mother, son and daughter, who all shared the same haplogroup (M30d1) variants (Table 1).

*3.5.2 Phylogenetic analysis*

Haplogroup phylogenies were accurately identified to declared ancestries (Table 1). Although haplogroups for four related Fijian-Indian individuals were distributed across South/SE Asia and South Africa, the admixed ancestry has been previously observed [56]. Most likely the admixture results from a migration of the M haplogroup from Asia into the pacific islands, started by the African expansion and continued from British colonisation [56]. Nonetheless, the Panel shows diverse coverage of haplogroups of different ancestries, in this case, of European and Fijian-Indian ancestries. Previously the earlier version of this Panel has inferred genetic ancestries in South Africa, Russia, Israel, New Guinea, Algeria, China, Italy, China, Australia, Thailand and the Netherlands [13]. The additional inference of samples from Swiss and Fijian-Indian heritage in this study shows the Panel can be further applied to worldwide haplogroups.

**3.6 Workflow**

The Panel workflow was completed in 5 days (from library preparation to sequencing) for two Ion 316™ chips. Manual preparation of the Panel required significant hands-on time and has been shown to cause variability in chip loading and densities which are difficult to reproduce [57, 58]. However, the protocol can be automated onto the Ion Chef™ (Life Technologies, CA, USA)and favour the standard workflow of forensic laboratories. An automated protocol facilitates reproducibility and has also been identified to increase through-put capacities and reduce turnaround times and processing costs compared to Sanger sequencing workflows [57]. For forensic use, issues such as contamination, in particular the carry-over of ‘ghost’ barcodes as well as the unprecedented volume of MPS data and associated bioinformatics expertise required for analysis relative to CE platforms, have been raised [50]. More-so the

logistics of storing MPS files in secured formats are considered. To address contamination, laboratories are considering the preparation of forensic and reference samples in separate sequence runs (private communication). While this may reduce sample contamination, it also results in inter-run variability between different chip uses [40]. Considering this, it is best to multiplex with barcode adapters and pool samples based on chip capacity and samples of similar qualities. This will be most cost-effective and achieve even coverages, as supported by this study. All these challenges have been raised by European laboratories and seconded by Australian laboratories [59]. Australian forensic DNA laboratories are yet to implement MPS workflows, however ongoing laboratory validations will likely lead to the introduction of MPS and associated panels in the foreseeable future. It is likely that MPS of the HV1 and HV2 regions will first be introduced into the workflow of mtDNA analyses, as this is readily compatible with existing techniques such as dideoxynucleotide sequencing.

## 4 Concluding remarks

The Early Access AmpliSeq™ Mitochondrial Panel (Applied Biosystems, CA, USA) is able to target and amplify whole mitochondrial genomes using the Ion Torrent™ technology. The Panel amplifies genomes at coverages that can reliably call variants for haplogroups, even in highly degraded samples. The Panel is amendable to the forensic identification of kinship and phylogenetic relations. In view of this performance, it is concluded that the Panel can potentially translate as a commercial and standard workflow into operational forensic laboratories that consider introducing MPS.

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## Figure Legends

**Figure 1.** Quality of genomic DNA in heat-treated DNA extracts. Mean (*n*=5) degradation index (DI) of heat-treated DNA samples determined using the Quantifiler™ Trio DNA Quantification Kit (Life Technologies, CA, USA). DI calculated as ratio, small autosomal target/large autosomal target [15]. Error bars represent S.E.M. between replicates.

**Figure 2.** Amplicon Coverage. Average coverage (in reads) of 162 amplicons spanning the mitochondrial genome in reference samples (n=5). Amplicons part of the Early Access AmpliSeq™ Mitochondrial Panel (Applied Biosystems, CA, USA). Thresholds for variant calling were arbitrary set at 10X minimum (- - - - -) and 100X maximum (——).

**Figure 3.** Amplicon Coverage for Degraded DNA. Mitochondrial sequencing amplicon coverage (in reads) of DNA samples (*n*=5) heated at 125°C for 0, 30, 60, 120 and 240 minutes. Amplicons (*n*=162) part of the Early Access AmpliSeq™ Mitochondrial Panel (Applied Biosystems, CA, USA). Error bars represent S.E.M.

**Figure 4.** Overall amplicon strand bias for five reference samples. Average strand bias (*n*=5) of mitochondrial amplicons part of the Early Access AmpliSeq™ Mitochondrial Panel (Applied Biosystems, CA, USA). Strand bias (%) between forward strand and reverse strands was calculated as lower reads/higher reads. **a)** amplicons 16-119 rCRS to 5,455-5,574 rCRS, **b)** amplicons 5,564-5,688 rCRS to 11,175-11,301 rCRS, **c)** amplicons 11,292-11,384 rCRS to 16,542-166,496 rCRS.