Use of the Agilent 2100 Bioanalyzer and the DNA 500 LabChip in the Analysis of PCR Amplified Mitochondrial DNA

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Abstract
Sequence analysis of PCR-amplified mitochondrial DNA (mtDNA) is quickly becoming an accepted tool for forensic testing. Establishing that these amplified products possess the proper size, purity, and concentration to be suitable for sequence analysis is a critical first step for forensic mtDNA applications. The Agilent 2100 bioanalyzer and DNA 500 LabChip have been shown to accurately and precisely measure both size and concentration of DNA fragments. In this application note, the capability of the bioanalyzer to rapidly and efficiently analyze mitochondrial DNA fragments amplified by PCR prior to sequence determination is demonstrated.

Introduction
The utility of mtDNA sequence analysis has seen enormous growth in recent years. In 1995 mtDNA analysis was certified by the Department of Defense as a reliable forensic tool [1]. Since that time, identification of American war casualties has been done primarily through mtDNA sequence analysis. The remains of victims from the September 11 terrorist attack on the World Trade Center bombing are also currently being analyzed by mtDNA sequence. In addition to these forensic applications, mtDNA is used in the study of both historical and anthropological samples. These studies include such samples as the heart of Louis XVII [2], the remains of Czar Nicholas II [3], 7000-year-old brain tissue, and the remains of a Neanderthal man.

Mitochondria are subcellular structures found in the cytoplasm of virtually all eukaryotic cells. These structures are believed to be the descendants of bacterial cells incorporated by primitive eukaryotic cells. Over time the bacterial cells developed a symbiotic relationship with their hosts and eventually became part of the hosts essential biochemical machinery. This is believed to be the reason mitochondria contain their own unique DNA, which encodes some of the proteins found in these structures.

Mitochondrial DNA was first sequenced in the lab of Fredrick Sanger in 1981. Human mtDNA is a small circular genome containing 16569 base pairs (bp). The current reference human mtDNA sequence is known as the Anderson or Cambridge reference sequence (CRS) (Genebank accession: M63933). This genome encodes for numerous polypeptides that are subunits of proteins involved in oxidative phosphorylation. Nucleotide sequences for 2 ribosomal RNAs and 22 transfer RNAs are also found in this genome. In addition to these coding sequences, the mitochondrial genome contains a noncoding region of 1100 bp known as a D-loop or control region. The D-loop contains sufficient sequence variation to be useful in human identity testing. The variation in sequence between unrelated individuals in this region ranges from
1%–3%. Most of the sequence variation is found in two sections of the control region, hypervariable region 1 (HV1) and hypervariable region 2 (HV2).

Mitochondrial DNA sequences are inherited directly from the mother. Unless a mutation has occurred, siblings and all maternal relatives have the same mtDNA sequence. Since mtDNA does not undergo recombination, maternal relatives dating back several generations can provide useful genetic samples. Such sequence information is often quite helpful in missing persons cases. In forensic cases mtDNA sequence is more often used for exclusion rather than identification of suspects, because multiple members of the same family all have the same mtDNA sequence.

Unlike the cell nucleus, which contains only one copy of genomic DNA, the cell cytoplasm may contain up to 1000 copies of mtDNA. Since each cell contains multiple mtDNA copies, only a few cells are required for sequence analysis. To carry out a sequence analysis on a mtDNA sample, it is usually necessary to extract the DNA and amplify the variable regions by polymerase chain reaction (PCR). Because the amplification process can convert even a few copies of target DNA into billions of copies, it is possible to do a sequence analysis from limited or badly degraded samples such as bone, teeth, or hair.

**Analysis of PCR Amplified mtDNA**

The number of PCR required to amplify the mtDNA HV regions depends on the age and condition of the sample. When mtDNA degradation is minimal, each HV region is amplified in a single PCR. These reactions yield products that are roughly 450 bp in length. Prolonged exposure to moisture, heat, and bacteria can result in significant degradation of the DNA, whereby the DNA is broken into smaller pieces. The average mtDNA fragment size in such degraded samples may be considerably shorter than 450 bp. Since the size of the amplification product cannot exceed the initial DNA target, amplifications on highly degraded samples are carried out in a series of shorter PCR steps, generally ranging in length from 100–200 bp.

Analysis of PCR amplicons has typically been done with agarose or acrylamide gels. These yield-gels require the user to run the unknown PCR sample adjacent to a set of predetermined standards of known concentration. The user attempts to match the band intensity of the PCR sample to one of the standards. The range of standard concentrations usually covers at least three orders of magnitude because it is also necessary to quantitate any secondary unintended PCR products. The accuracy of this type of analysis is minimal at best because errors in estimating concentration often exceed 100%. For this reason, the use of yield-gels to assess the ratio of target to nontarget PCR product is quite problematic.

The gel electrophoresis limitations can be overcome with the Agilent 2100 bioanalyzer, which is the first commercially available chip-based nucleic acid separation system. The Agilent 2100 bioanalyzer separates nucleic acid fragments in microfabricated channels and automates detection as well as online data evaluation. The Agilent 2100 bioanalyzer is connected to a PC for run control and automated data analysis.

Analysis of PCR products with the Agilent 2100 bioanalyzer has several important advantages compared to traditional gel electrophoresis. With a short separation channel and the application of a high electrical field, the speed of analysis is dramatically increased compared to gel electrophoresis. The instrument is equipped with a fluorescence detection system resulting in superior detection sensitivity. The prepackaged reagents and kits are used in conjunction with standardized protocols, and result in more reproducible data. These kits also help to improve the overall reproducibility between different runs, chips, and instruments. Compared to data assessment with gel-scanning systems, the amount of manual work is significantly reduced and even data analysis is performed in an automated manner. Sample and reagent consumption in the range of one to a few microliters minimizes exposure to hazardous materials and reduces the amount of waste material.

Several kits are available to analyze a variety of nucleic acid sample types. Because of its 25–500 bp size range, the DNA 500 LabChip® is well suited for the rapid determination of amplicon concentration and quality, as required for accurate mtDNA sequencing. The DNA 500 assay is capable of 5-bp resolution for 25 to 100-bp fragments, and 5% resolution for 100 to 500-bp fragments. The sizing accuracy has an error of less than 10% over this entire size range. Previous studies have shown that the
bioanalyzer is capable of detecting DNA fragments that cannot be identified on agarose gels. A comparison with gels stained with SYBR gold or ethidium bromide demonstrated that the bioanalyzer was 5 times more sensitive than SYBR gold and 25 times more sensitive than ethidium bromide staining. The bioanalyzer has consistently detected DNA at the 20 pg level [4].

The quantitative capability of the bioanalyzer is illustrated in Table 1. A DNA mass ladder containing three fragments with sizes of 100, 200, and 400 bp was used to verify quantitation accuracy. Concentrations of the ladder components were guaranteed by the supplier (Low DNA Mass™ ladder, Life Technologies, USA). For all three fragments the error in concentration measurement was less than 10% with a coefficient of variation of less than 15% [5].

**Requirements for PCR Amplified mtDNA**

High quality sequencing data requires a single homogenous PCR product in a concentration range of 10–100 ng/mL. Laboratory procedures, in use in the FBI regional labs, currently stipulate that the amplified target mtDNA must be present in 10-fold excess above any unintended PCR products. Failure to meet that purity requirement means that the underlying sequence data from the secondary PCR products may render the target sequence unreadable or may even result in erroneous nucleotide base assignments. For this reason, an accurate determination of the concentrations of all the PCR products is critical in assessing the quality of the PCR sample.

Figure 1 shows an example of an amplified mtDNA sequence made from the HV1 region. Aside from a small primer dimer peak adjacent to the low molecular-weight marker, the PCR product contains only a single homogeneous 273 bp PCR product at a concentration of 44.1 ng/μL. Such a product is clearly suitable for mtDNA sequence analysis.

| Table 1. Quantitation Accuracy and Precision of the Agilent 2100 Bioanalyzer |
|---------------------------------|--------|--------|--------|
|                                | 100 bp | 200 bp | 400 bp |
| Average [ng/μL]                | 0.73   | 1.53   | 3.02   |
| Target [ng/μL]                 | 0.80   | 1.60   | 3.20   |
| Percent error                  | -8.28  | -4.56  | -5.62  |
| STDV                           | 0.08   | 0.11   | 0.20   |
| CV                             | 10.73  | 7.46   | 6.73   |

Figure 1. Electropherogram of PCR-amplified mtDNA from HV1 region analyzed on the Agilent 2100 bioanalyzer.
Figure 2 shows a mtDNA amplification product that is contaminated with a second PCR amplicon. The target PCR product is a 222 bp fragment with a concentration of 48.8 ng/μL. The unintended secondary product has a concentration of 10.2 ng/μL with a size of 69 bp. Since the concentration of the second product is greater than 10% of the target fragment, this PCR amplification would not be suitable for mtDNA sequencing under the current FBI 10:1 guideline.

Another problem frequently encountered in the amplification of mtDNA sequences occurs when a region that is rich in adenine (A) and thymine (T), is followed by a long string of guanines (G). When this sequence region is amplified the DNA strands can partially melt and then reanneal. With a long string containing \( n \) number of Gs, the reannealed DNA will occasionally lose its reference frame thereby incorporating an additional G. If this happens multiple times over the amplification process, significant concentrations of amplicons containing \( n+1 \) or \( n+2 \) Gs will be produced. This behavior is referred to as a G-stutter. When PCR products showing this G-stutter phenomenon are used in sequencing reactions, data downstream from the Gs is usually unreadable.

Figure 2. Electropherogram of PCR-amplified mtDNA from HV1 region showing the presence of a second unintended PCR product analyzed on the Agilent 2100 bioanalyzer.
In Figure 3A an electropherogram of a PCR amplified HV1 sequence showing this G-stutter behavior is shown. A fluorescence labeled sequencing product made from this PCR product will contain a mixed population of sequences containing fragments with n, n+1, and n+2 Gs. This mixed population will make the sequence data downstream from the G region virtually unreadable. Figure 3B shows an example of how the sequence data is degraded. A nucleotide base designation of N indicates that no sequence information could be obtained.

Figure 3A. Electropherogram of PCR-amplified mtDNA from the HV1 region showing the presence of multiple G-stutter products analyzed on the Agilent 2100 bioanalyzer.

Figure 3B. Typical sequencing data output for mtDNA PCR products using amplified products with a G-stutter. Note the loss of readable sequence after the run of Gs.
In Figure 4A an electropherogram of a PCR amplification of the same HV1 sequence relatively free of artifacts is shown. This PCR amplicon is a single homogeneous product and is quite suitable for DNA sequence analysis. Figure 4B shows a section of sequence data generated from such a PCR product. Note that unlike the sequence from Figure 3B, the sequencing output downstream from the consecutive string of Gs is clear and unambiguous.

**Figure 4A.** Agilent 2100 bioanalyzer electropherogram of PCR amplified mtDNA sequence from the HV1 region showing a single homogeneous PCR product.

**Figure 4B.** Typical sequencing data output for mtDNA PCR products using amplified mtDNA with a single homogeneous product. Note that the sequencing data output shows no loss of readable sequence after the run of Gs.
Conclusion
Mitochondrial DNA analysis is quickly growing in popularity as evidenced by the proliferation of the technique within the forensic community. The Agilent 2100 bioanalyzer facilitates this analysis by rapidly providing accurate size and concentration profiles of mtDNA amplification products. Through the use of internal and external DNA markers, the Agilent 2100 bioanalyzer provides excellent quantitative analysis and sizing of PCR products. This technology enables the identification of high quality amplified mtDNA fragments and quantifies the yield of these fragments to assure reliable results in the subsequent sequencing analysis. The DNA 500 assay reliably performs DNA separations in the range of 25 to 500 base pairs. Because of the bioanalyzer’s excellent sensitivity and resolution, it is capable of resolving and accurately quantitating secondary PCR products. This feature is particularly important when the user is required to demonstrate that the target mtDNA amplicon is present in 10-fold excess above any secondary unintended PCR products.

References
5. O. Mueller, “High resolution DNA analysis with the DNA 500 and DNA 1000 LabChip® kits”, Agilent Technologies publication 5988-3041E www.agilent.com/chem

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