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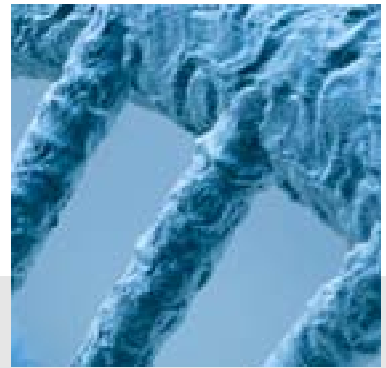

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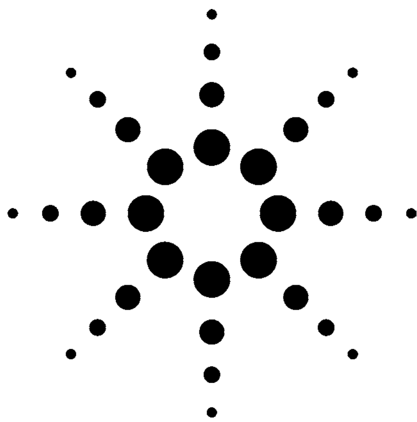

Use of the Agilent 2100 Bioanalyzer and the DNA 500 LabChip in the Analysis of PCR Amplified Mitochondrial DNA

Application

Homeland Security/Forensics

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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 descendents 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

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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].

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

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.

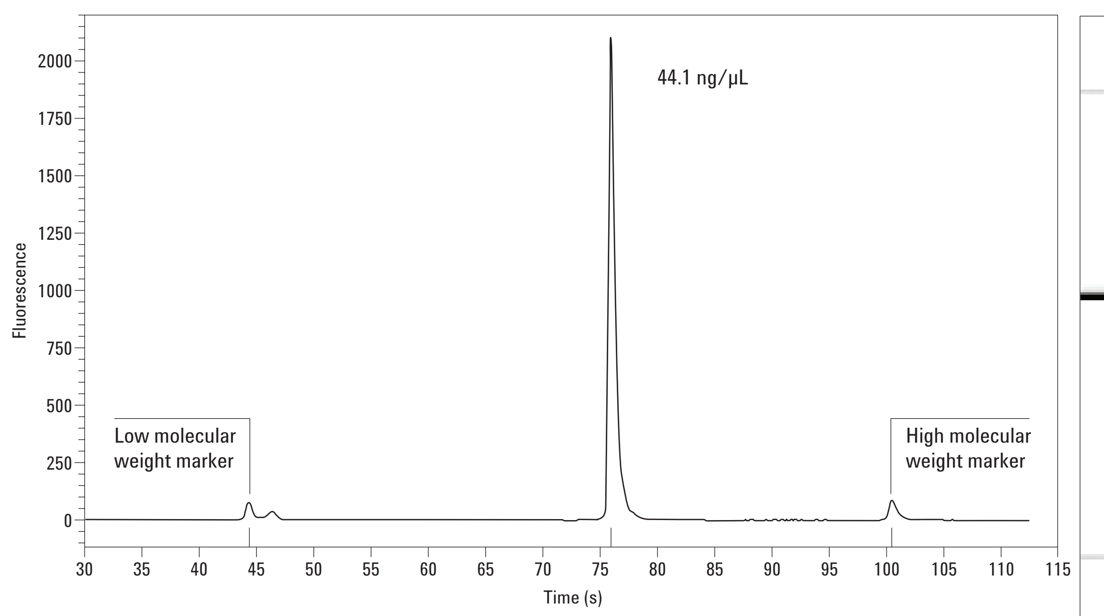

Figure 1. Electropherogram of PCR-amplified mtDNA from HV1 region analyzed on the Agilent 2100 bioanalyzer.

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 reannealled 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.

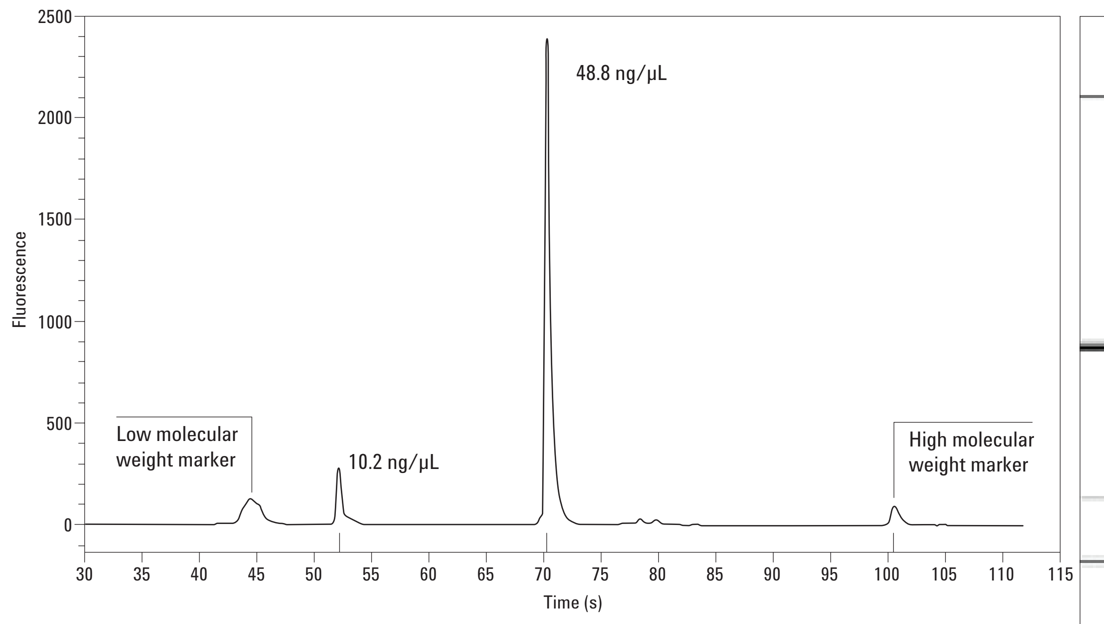

Figure 2. **Electropherogram of PCR-amplified mtDNA from HV1 region showing the presence of a second unintended PCR product analyzed on the Agilent 2100 bioanalyzer.**

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.

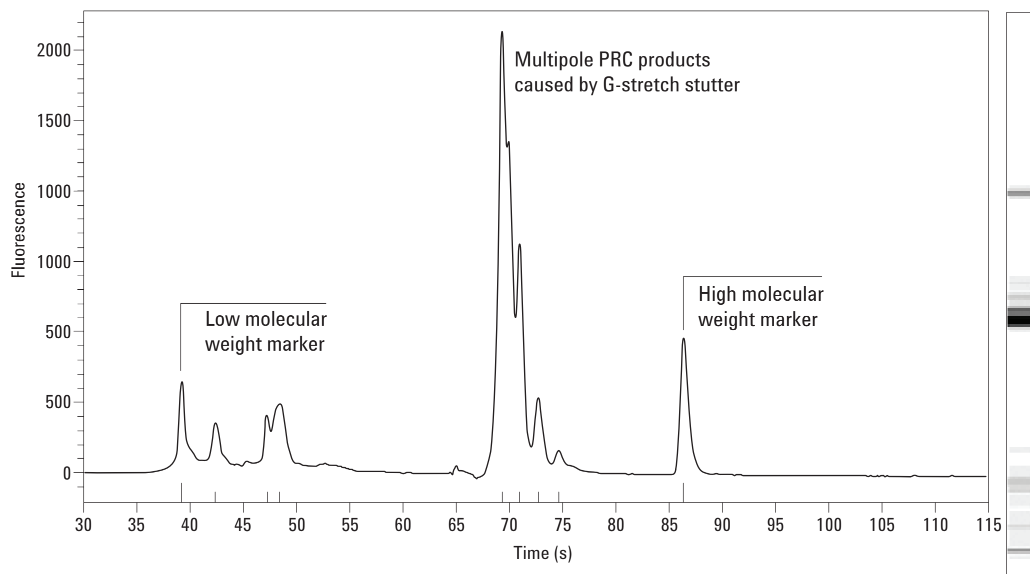

Figure 3A. Electropherogram of PCR-amplified mtDNA from the HV1 region showing the presence of multiple G-stutter products analyzed on the Agilent 2100 bioanalyzer.

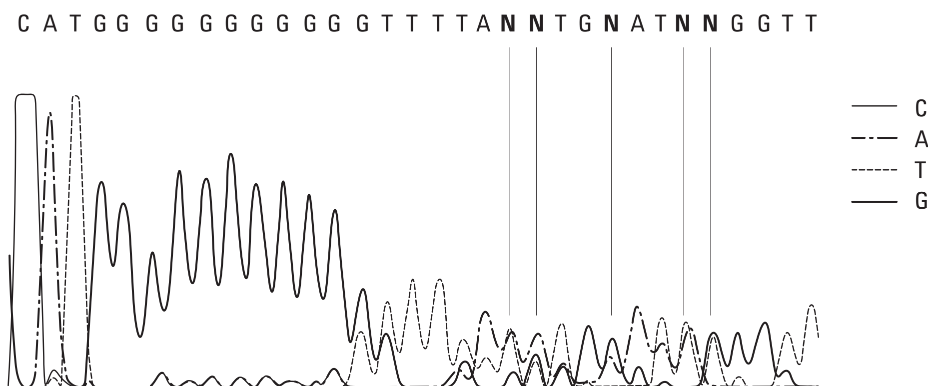

Figure 3B. Typical sequencing data output for mtDNA PCR products using amplified products with a G-stutter. Note the loss of readable sequence after the run of Gs.

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.

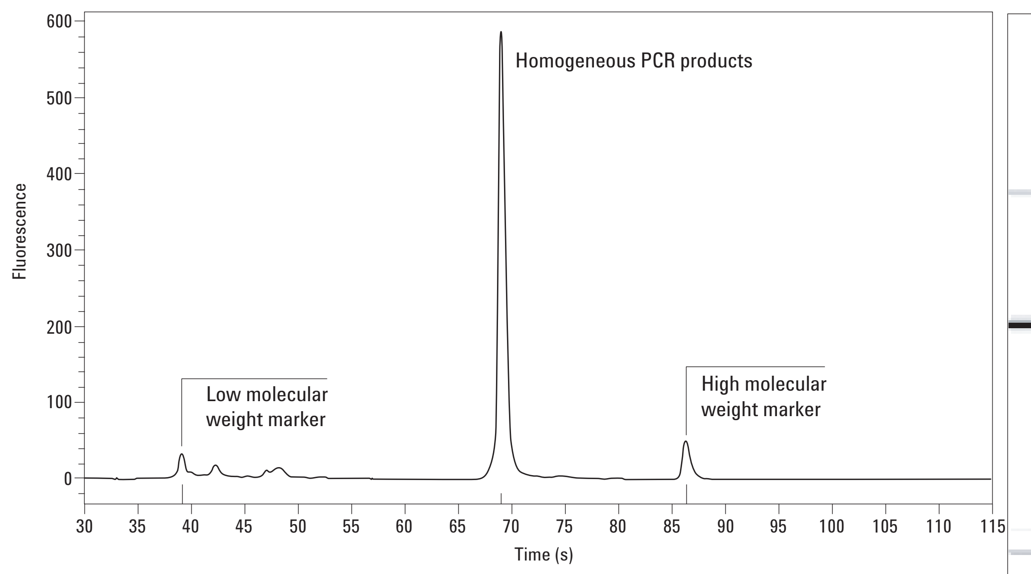

Figure 4A. Agilent 2100 bioanalyzer electropherogram of PCR amplified mtDNA sequence from the HV1 region showing a single homogeneous PCR product.

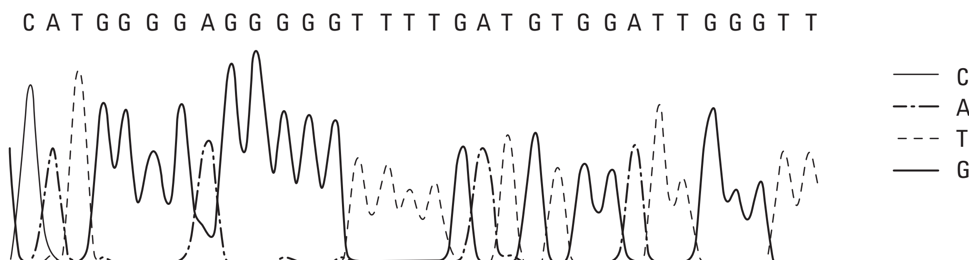

Figure 4B. Typical sequencing data output for mtDNA PCR products using amplified mtDNA with a single homogeneous product. Note that the sequencing data output shows no loss of readable sequence after the run of Gs.

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.

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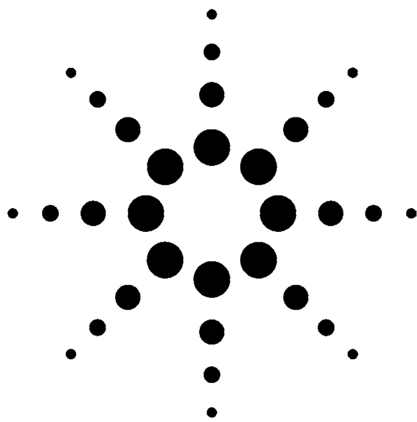

Using the 2100 Bioanalyzer to Optimize the PCR Amplification of Mitochondrial DNA Sequences

Application

Genomics

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Abstract

In polymerase chain reaction (PCR) product analysis, a comparison of target amplicon yield to such PCR artifacts as primer-dimers and misprimed secondary products is essential in PCR assay optimization. This process requires accurate and reproducible measurement of amplified DNA products over a wide range of sizes and concentrations. The 2100 Bioanalyzer is ideally suited to provide rapid quantitative analysis of all products and byproducts in a PCR reaction, thereby facilitating the development of an optimized PCR amplification.

Introduction

Optimization of PCRs can often be a tedious and time-consuming process, particularly when the amplified segments contain long stretches of high or low melting temperature sequences. The objective of the optimization process is to minimize secondary products, such as primer-dimers and mispriming events, while maximizing the yield of the target amplicon. Analysis of the amplification products by gel electrophoresis is not particularly helpful because it is difficult to obtain accurate quantitative information about secondary PCR products. The absence of such quantitative information can delay and confound the optimization process and can even result in the final PCR reaction being run under conditions that do not produce the maximum selectivity and yield.

The 2100 Bioanalyzer is an ideal tool to address problems of PCR process optimization. Not only can secondary products be accurately and reproducibly measured, but secondary products that are similar in size to the desired target can also be resolved and quantified [1]. The ability to quantitate all of the reaction products provides the user with the opportunity to accurately characterize the mathematical relationship between specific reaction parameters and the overall efficiency of the PCR reaction. These relationships can then be used to facilitate the identification of the optimal reaction conditions.

Mitochondrial DNA (mtDNA) is a good example of a target sequence that can present a number of PCR amplification difficulties. Human mtDNA is a small (16,569 bp) circular genome containing 37 genes found in cellular cytoplasm. The mitochondrial genome contains a noncoding region of 1100 bp known as a D-loop or control region. Within the D-loop is found two sections of highly variable DNA sequence. These regions, hypervariable region 1 (HV1) and hypervariable region 2 (HV2), contain sufficient sequence variation to be useful in human identity testing [2, 3].

When a sufficient amount of intact genomic DNA (gDNA) cannot be extracted from a sample, mtDNA becomes the next analysis tool because the cytoplasm of a single cell may contain up to 1000 copies of mtDNA. MtDNA is often extracted from small or badly degraded samples such as bone, teeth, or hair and amplified by PCR. The amplified product is used for DNA sequence analysis.

Since sample degradation decreases the mean DNA fragment size, the number of PCRs required to amplify the HV regions depends on the age and condition of the sample. If the DNA degradation is minimal, each HV region can be amplified in a single PCR. Highly degraded samples require the HV regions to be amplified in a series of PCR reactions of 100 bps–200 bps. Since these samples require a variety of amplification strategies, amplification of mtDNA sequences is a relevant and important example of how the 2100 Bioanalyzer may be used to facilitate the optimization of specific PCR amplifications.

Methods

For the purpose of this study, the effect of pH, Mg concentration, and Taq polymerase activity, on the amplification efficiency of four mitochondrial DNA sequences was evaluated. To insure that variations in sample matrix did not influence the results, a bulk extraction of DNA was made at the beginning of the study. This extracted DNA was diluted and aliquoted out to provide all the DNA samples for the evaluation. The extraction protocol is described below.

DNA Preparation

1. Add 10 hairs with roots intact to 0.2 mL of extraction buffer and incubate for 3 hours at 56 °C.
2. Vortex and heat to 95 °C for 8 minutes.
3. Centrifuge mixture for 5 min at 15,000g.
4. Remove 100 µL and combine with 900-µL PBS for PCR amplification.
5. Use 5 µL of diluted extracted sample/PCR

Extraction Buffer

The components of the extraction buffer were as follows: 6.0% Chelex 100 in 0.137 M NaCl, 0.0027 M KCl, 0.010 M Na_2HPO_4, 0.0018 M KH_2PO_4, at pH 7.4.

The Invitrogen PCR Optimizer Kit was used for the PCR optimization evaluation. This kit contains 10 buffers ranging in pH from 8.5 to 9.5 and Mg levels from 1.5 to 3.5 mM. These individual reaction buffers were evaluated in the amplification reactions of four mtDNA segments. The description of the buffers is shown in Table 1.

Table 1. Buffer Composition

Component	1× Composition
Buffer A	1.5-mM $MgCl_2$, pH 8.5
Buffer B	2.0-mM $MgCl_2$, pH 8.5
Buffer C	2.5-mM $MgCl_2$, pH 8.5
Buffer D	3.5-mM $MgCl_2$, pH 8.5
Buffer E	1.5-mM $MgCl_2$, pH 9.0
Buffer F	2.0-mM $MgCl_2$, pH 9.0
Buffer G	2.5-mM $MgCl_2$, pH 9.0
Buffer H	3.5-mM $MgCl_2$, pH 9.0
Buffer I	1.5-mM $MgCl_2$, pH 9.5
Buffer J	2.0-mM $MgCl_2$, pH 9.5

In addition to the reaction buffers, the amplification reactions were also evaluated at three levels of Taq polymerase: 1, 1.5, and 2 units/reaction.

Primer Selection

The primer site selection was based on the Armed Forces DNA Identification Laboratory suggested sites for amplification of badly degraded remains [4]. In some cases the priming site was shifted slightly to better match the melting temperature, T_M, of all the primers. The primer locations are located in the HV regions of the D-loop in the mtDNA genome. Two sets of primers were used to cover HV1 and an additional two sets to cover HV2. The primer site locations are illustrated schematically in Figure 1.

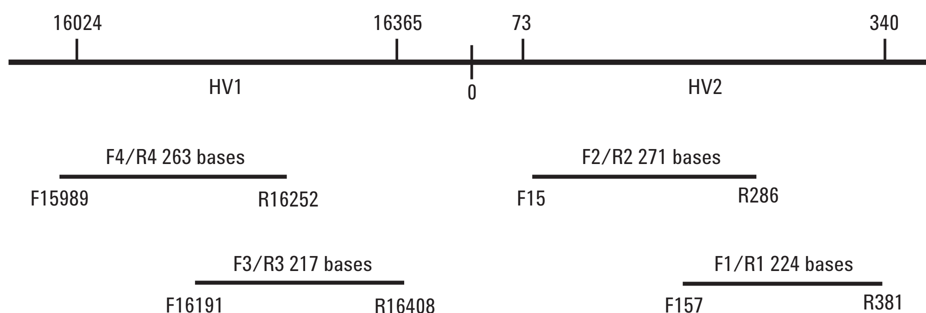

Figure 1. Primer site selection: Base position is assigned from the Anderson reference sequence [5].

Each of the four amplifications was evaluated for yield of primary target and secondary artifact products. Optimum primer concentration, 0.4 µM, and annealing temperature, 55 °C, were determined in a previous study and will not be described in this note.

Results

Electropherograms showing typical product profiles of the four mtDNA amplification reactions are shown in Figure 2.

In Figure 2A two peaks can be seen in size region of the expected target. This phenomenon is known as C-heteroplasmy and is indicative of two sequence groups with a one base variation in the length of a run of Cs within a single mtDNA sample. Figures 2B and 2C both contain single homogenous amplification products and are suitable for sequence analysis. In Figure 2D, an even more extreme case of C-heteroplasmy is seen. Sequencing of amplicons 2A and 2D would not yield any useful sequence data downstream from the C-heteroplasmy.

Since the C-heteroplasmy is the result of a mixed sequence population within a single sample, this condition cannot be improved by any of the modifications in amplifications conditions discussed in this application note. This problem is typically overcome by selecting an additional priming site downstream from the run of Cs.

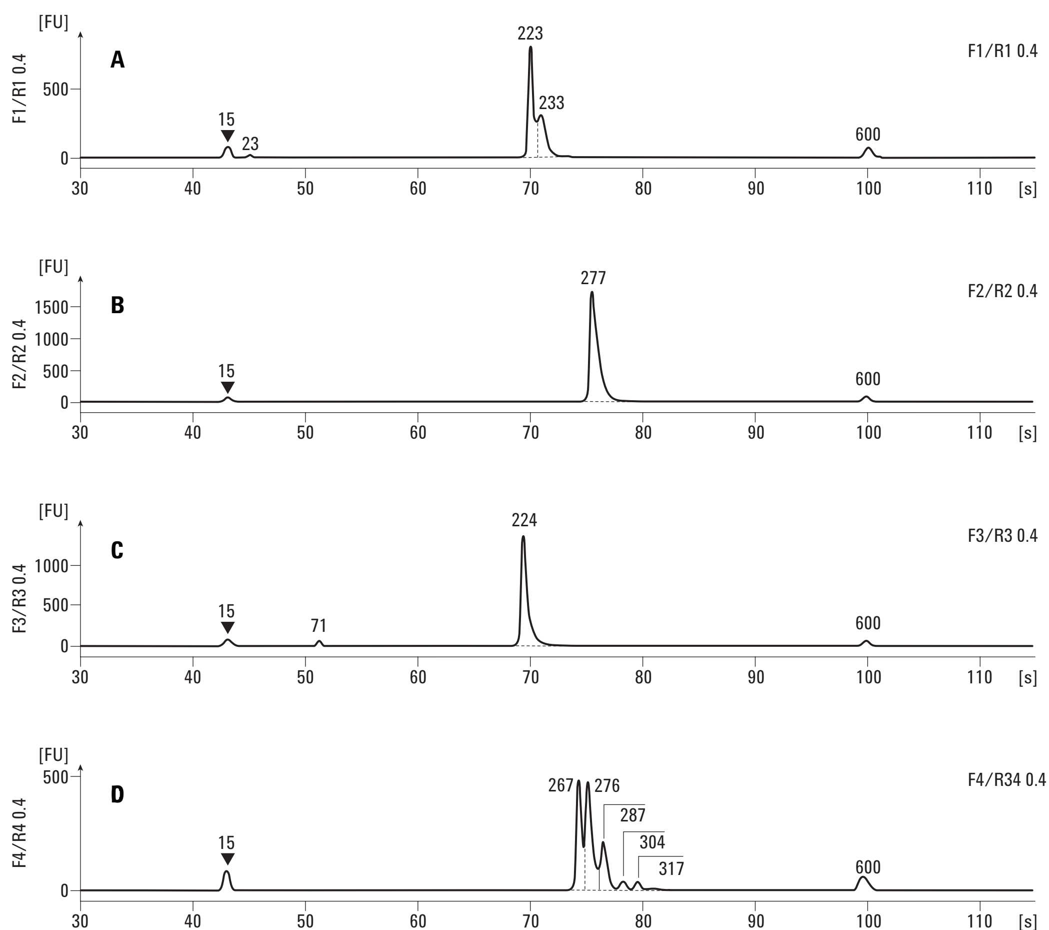

Figure 2. 2100 Bioanalyzer electropherograms of the four mtDNA amplifications; (A) F1/R1 - 224 bp, (B) F2/R2 - 271 bps, (C) F3/R3 - 217 bps and (D) F4/R4 - 263 bps.

To characterize the effect of [Mg] on amplification yield, concentrations of Mg ranging from 1.5 to 3.5 mM were examined at three pH levels, 8.5, 8.7, and 9.0. The pH 8.7 reaction buffer was prepared by mixing equal amounts of pH 8.5 and 9.0 reaction buffer at a fixed Mg level. The influence of Mg concentration on the amplification yield for the four mtDNA amplification products can be seen in Figure 3.

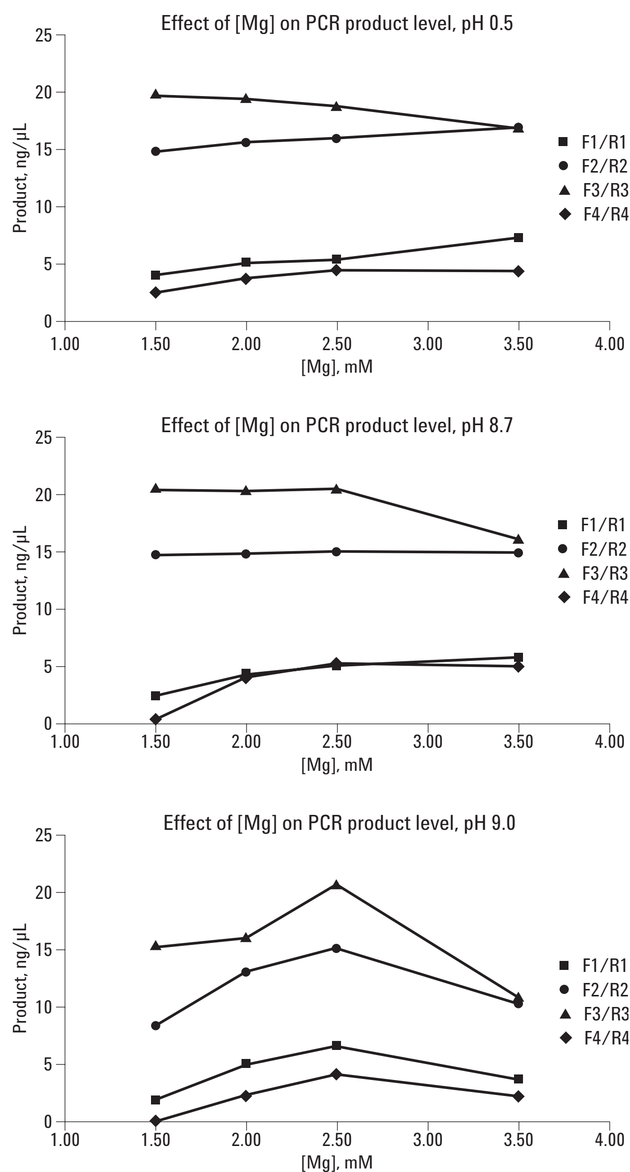

Figure 3. Influence of Mg level on product yield.

It is readily apparent from the yield graphs that the effect of Mg is both pH and sequence dependent. At pH 8.5 three of four mtDNA products show a continuous increase in yield as the Mg level is increased. For two of these amplification reactions, F3/R3 and F4/R4, the level of artifact products increases even more rapidly over the same Mg concentration range (data not shown). When the pH is raised to 8.7, only the F3/R3 and F4/R4 amplicons show increased yields with increasing Mg concentration. At pH 9.0 all four amplicons show a distinct maximum yield at 2.5-mM Mg.

The effect of pH at a single Mg level was evaluated for a pH range of 8.5 to 9.5. Figure 4 shows the effect of this pH range at a Mg level of 2.0 mM.

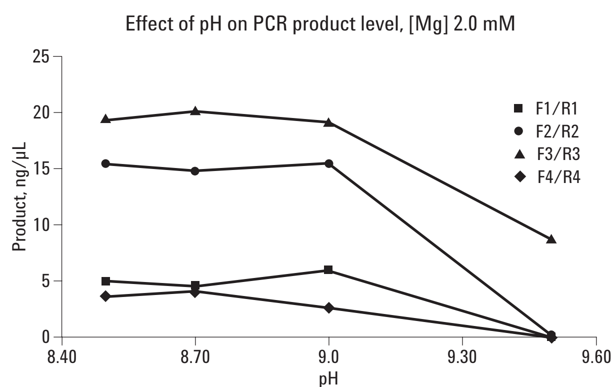

Figure 4. Reaction buffer pH level influence on product yield.

For 2.0-mM Mg the maximum yield for all four amplicons is seen at both at pH 8.5 and 8.7. A significant decrease in yield of one amplicon, F4/R4, is observed at pH 9.0. At pH 9.5 all four amplicons show a profound loss in yield.

The effect of Taq activity on the production of target and artifact amplicons was studied in buffer C, which contains 2.5-mM Mg at pH 8.5. The results are shown in Figure 5.

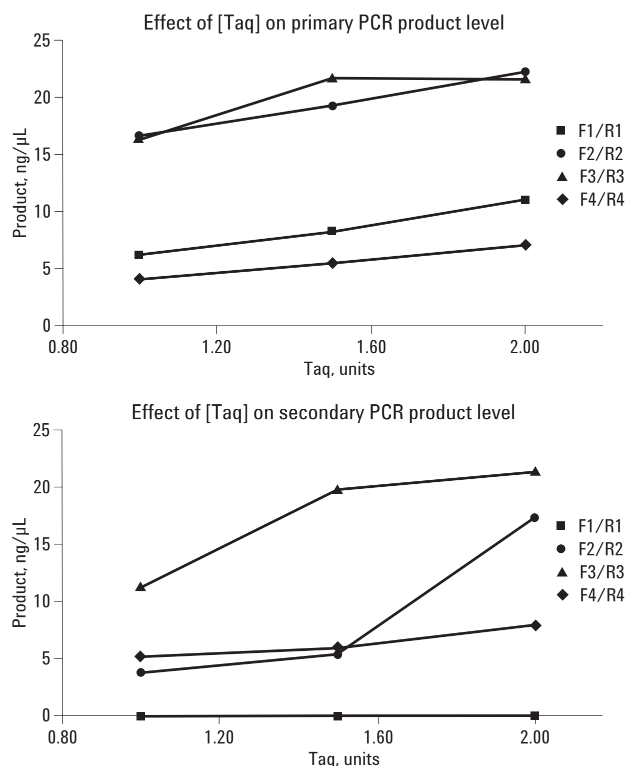

Figure 5. Influence of Taq activity on primary and secondary product yields.

The observation that target yield increases with increasing Taq activity is not surprising. However, note that the rate of artifact production increases even more rapidly for three of the amplification products. An examination of the target:artifact ratio shows that the highest ratio is obtained when only one unit of Taq is used per reaction.

Conclusion

In optimizing reaction conditions for a group of amplifications, some compromise of reaction parameters will invariably be required. This compromise requires an accurate determination of both target and artifact products for all of the PCR reactions. For the four amplicons in HV1 and HV2 of the mtDNA genome, the highest quality target amplification was produced at pH 8.7 with a Mg level of 2.0 mM, using one unit of Taq polymerase per reaction.

The Agilent 2100 Bioanalyzer facilitated this PCR optimization process by providing rapid quantitative analysis of both mtDNA amplification products and PCR artifacts. The large dynamic linear range made it possible to compare target and artifact yields even when their concentrations were more than a decade apart. Accurate characterization of the relationship between such reaction parameters as pH, Mg concentration and Taq activity, and the relative yields of target products and artifacts, made it possible to identify the reaction conditions that produce the highest relative yield of sequencing quality PCR target amplicon.

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