

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.



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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 Na₂HPO₄, 0.0018 M KH₂PO₄, 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

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

of the buffers is shown in Table 1.

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.

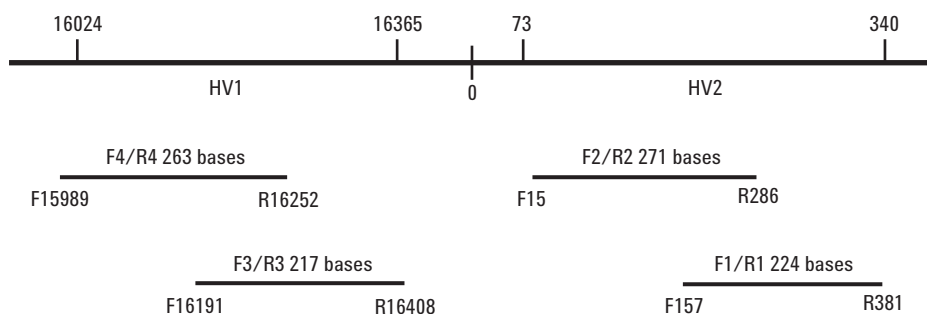


Figure 1. Primer site selection: Base position is assigned from the Anderson reference sequence [5].

Each of the four amplifications was evaluated for yield of primary target and secondary artifact products. Optimum primer concentration, 0.4 μ M, and annealing temperature, 55 $^{\circ}$ 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.

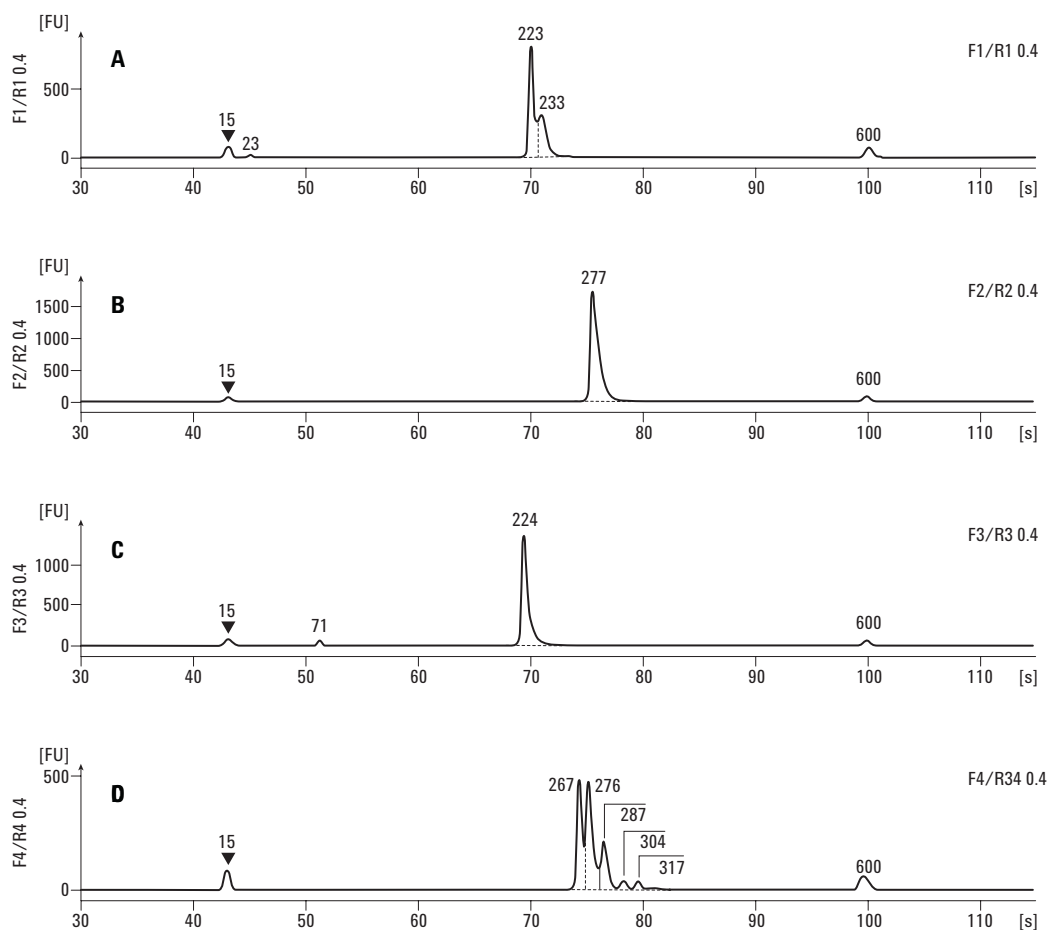


Figure 2. 2100 Bioanalyzer electropherograms of the four mtDNA amplifications; (A) F1/R1 - 224 bp, (B) F2/R2 - 271 bps, (C) F3/R3 - 217 bps and (D) F4/R4 - 263 bps.

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.

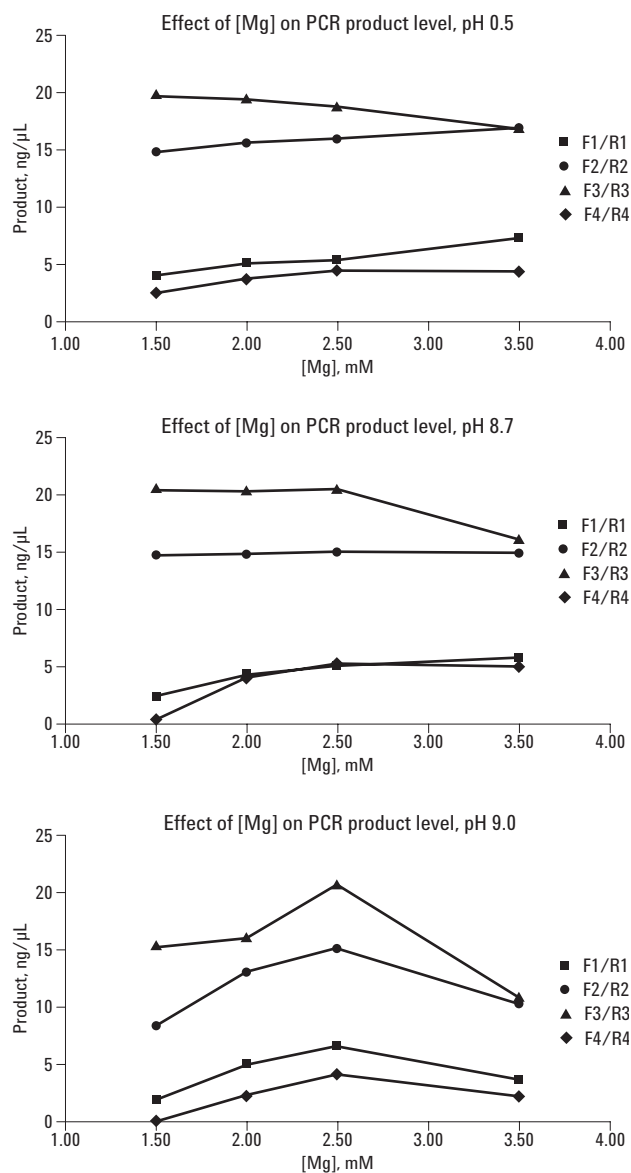


Figure 3. Influence of Mg level on product yield.

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.

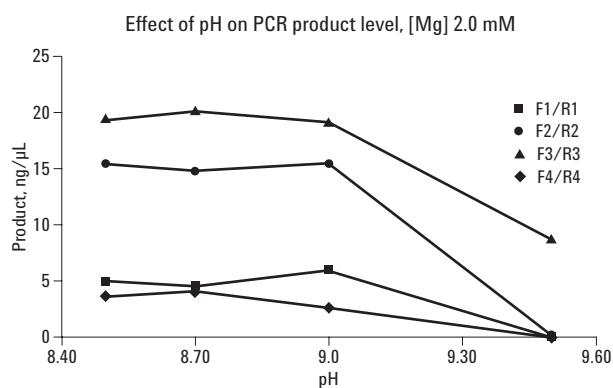


Figure 4. Reaction buffer pH level influence on product yield.

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.

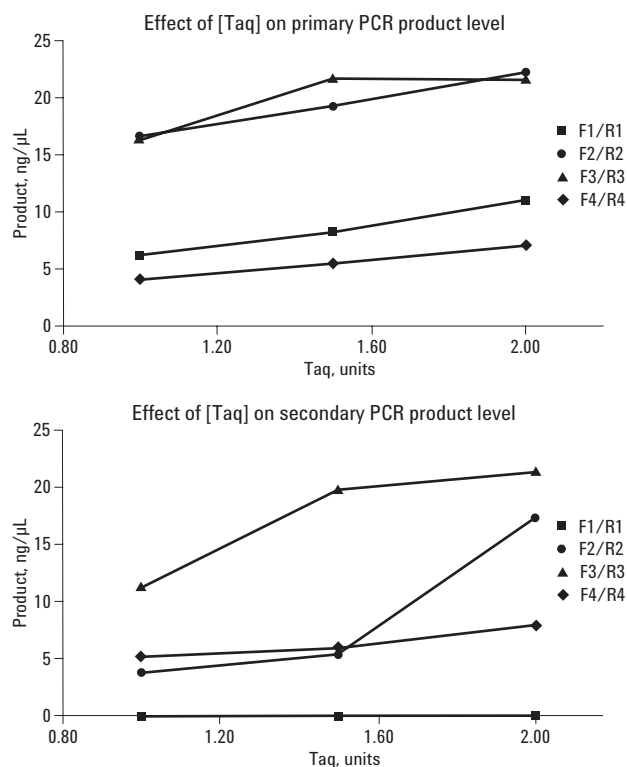


Figure 5. Influence of Taq activity on primary and secondary product yields.

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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