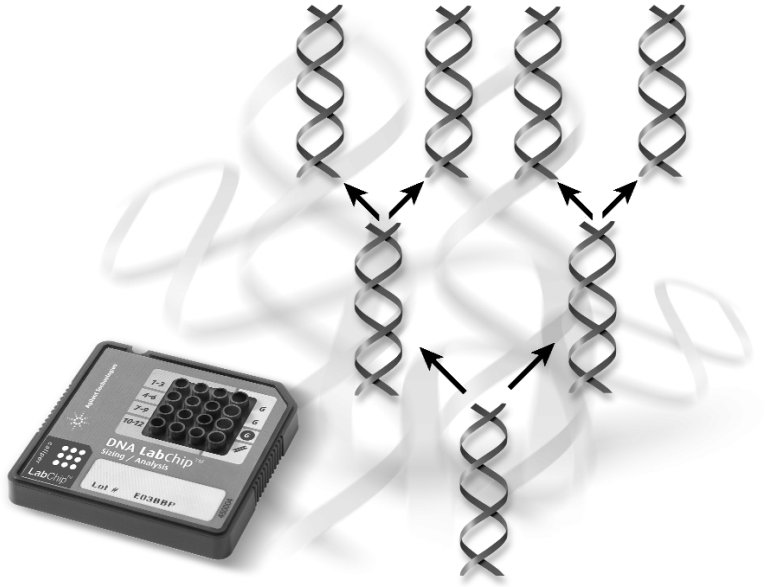
Quantitative analysis of PCR fragments with the Agilent 2100 Bioanalyzer

Application Note

Odilo Mueller



Abstract

This application note describes how the Agilent Technologies 2100 bioanalyzer can be used to analyze PCR fragments using the DNA 7500 LabChip® kit. Both size and concentration information can be obtained for fragments ranging from 100–7500 base pairs. Sizing and quantitation accuracy were determined and performance was compared to slab gel electrophoretic methods. The advantages of the DNA 7500 assay for determining PCR product purity are also described.

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Introduction

At present, slab gel electrophoresis (SGE) is the most widely used technique for the analysis of PCR fragments. While SGE is a relatively inexpensive and easy to use technique, the amount of information, which can be derived with little effort from slab gels is limited. Typically, size and concentration information is estimated by the scientist through visual comparison to appropriate size and mass ladders which are run in separate lanes on the gel. These estimations might be appropriate for experiments where a simple yes-or-no answer is adequate. However, for many experiments it is advantageous or even mandatory to gather more precise data. For example, increased data precision is important for the optimization of PCR reaction protocols or for subsequent biochemical reactions or cloning experiments. Gel-scanning systems are available for these applications. They are used to scan gels after staining with an appropriate dye. However, these systems are expensive, require manual intervention, and the use of several individual hardware components.

The limitations of slab gel electrophoresis 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 micro-fabricated channels and automates detection as well as on-line data evaluation. The Agilent 2100 bioanalyzer is connected to a PC for run control and automated data analysis. Several kits are available to analyze a variety of nucleic acid sample types. The DNA 7500 LabChip kit is especially useful for PCR product analysis where the concentration as well as the size of fragments are investigated.

Analysis of PCR products with the Agilent 2100 bioanalyzer yields several important advantages compared to traditional slab gel electrophoresis. Since a short separation channel is employed and a high electrical field strength is applied, the speed of analysis is dramatically increased compared to slab gel electrophoresis. This high speed of analysis results in an increased sample throughput. 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.

Materials and methods

Agilent 2100 bioanalyzer instrument and software

All chip-based separations were performed on the Agilent 2100 bioanalyzer which was controlled by dedicated software running on a PC. The Agilent 2100 bioanalyzer software package includes data collection, presentation, and interpretation functions. Data can be displayed as a gel-like image and/or as electropherogram(s) (see figure 1). Additionally, sizing and quantitation data is presented in tabular form and can be easily exported to various spreadsheet programs. A number of software tools are available for data manipulation and comparison.

The Agilent 2100 bioanalyzer contains high voltage power supplies, each of which is connected to a platinum electrode. These electrodes allow the instrument to perform multiple injections and other fluid manipulations from specific sample wells. The instrument uses fluorescence detection, monitoring fluorescence between 670 nm and 700 nm.

Chip preparation

All chips were prepared according to the instructions provided with the DNA 7500 LabChip kit. Each kit includes 25 chips and the following reagents: sieving matrix, dye concentrate, DNA markers, DNA sizing ladder, syringe, and spin filters. The gel-dye mix was prepared by mixing 400 µl of the gel matrix with 20 µl of the dye concentrate and the mixture was filtered through a spin filter. The separation chip was filled with the gel matrix/dye mixture and 5 µl of the markers was added to each sample well. After adding 12 samples (1 µl each) to the sample wells and the DNA sizing ladder (1 µl) to the assigned ladder well, the chip was vortexed and run on the Agilent 2100 bioanalyzer.

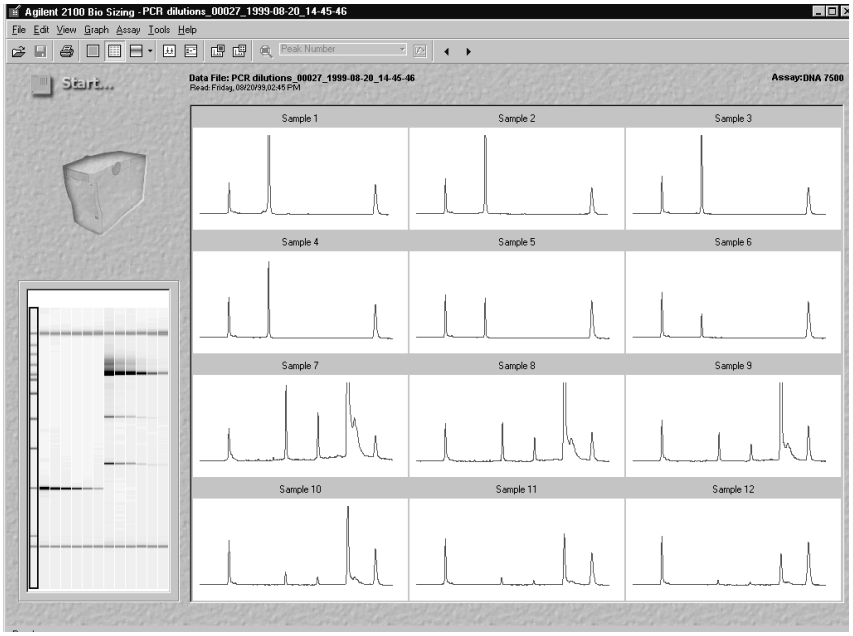


Figure 1
A DNA 7500 assay as displayed in the Agilent 2100 biosizing software. Data is presented both as electropherograms and a slab gel-like image. The screen shows a dilution series of the two PCR fragments as described in figures 2a and 2b.

Chemicals and reagents

AmpliTaq Gold DNA polymerase was purchased from PE Corp. (Foster City, CA) and PCR reactions were performed according to the supplier's recommendation. SYBR Gold nucleic acid stain was obtained from Molecular Probes Inc. (Eugene, OR) and the polyacrylamide gels were bought from Novex Corp. (San Diego, CA). The 100 base pair and low DNA mass ladders were purchased from Gibco BRL Pvt. (Rockville, MD). Vector pSP64polyA was purchased from Promega Corp. (Madison, WI). The DNA 7500 LabChip kit was from Agilent Technologies GmbH (Waldbronn, Germany). Adenovirus 2 genomic DNA was purchased from Sigma (St. Louis, MO). Primers for PCR amplification of adenovirus sequences were purchased from Operon Technologies Inc. (Alameda, CA).

Quantitation of PCR fragments before and after firing through thermal ink jet heads

Human monocyte antigen CD14 (HMA), a 1441 base pair fragment, was PCR amplified from Image clone #47679 using M13 primers. CON, a 485 base pair fragment was amplified from the pSP64polyA vector using sequence specific primers. Both fragments were amplified using standard PCR conditions. The PCR fragments were isopropanol precipitated, followed by 70 % ethanol wash. The pellets were resuspended in 1X SSC (150 mM sodium chloride and 15 mM sodium citrate). The concentration of each fragment was estimated by analysis on 1 % agarose gel electrophoresis comparing gel bands to a low DNA mass ladder. The fragments were loaded into an Agilent Technologies thermal inkjet head at a concentration of 0.25 mg/ml, and the head was fired 10-20 times into a collection tube in order to collect 1 µl of thermal inkjet fire DNA (postfired DNA). The postfired DNA was then diluted with 99 µl deionized H_20. One microliter of the 0.25 mg/ml stock DNA (prefired) was also diluted with 99 µl deionized H_20. The prefired and postfired PCR fragments were analyzed by the Agilent 2100 bioanalyzer. The same samples were also analyzed by polyacrylamide gel electrophoresis using a 5 % polyacrylamide mini gel, followed by staining with SYBR Gold and scanning with Fluorimager 595 (Molecular Dynamics, Mountain View, CA).

Results and discussion

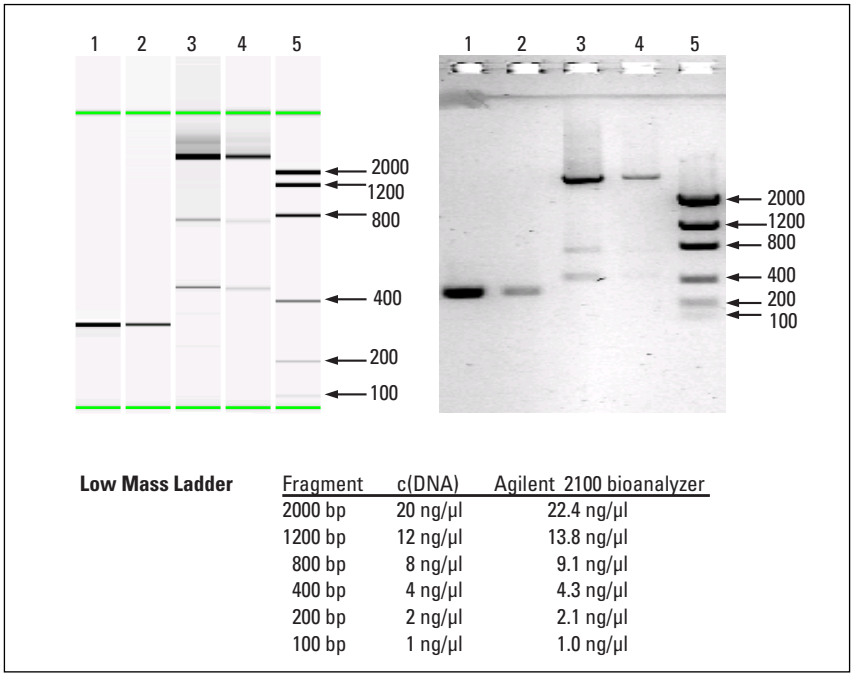
The Agilent 2100 bioanalyzer analyzes 12 DNA samples in less than 30 minutes in a sequential manner and the results for each sample can be viewed after completion of its separation. The DNA 7500 assay can be used to size and quantitate double stranded DNA fragments ranging from 100 bp to 7500 bp with sizing accuracy better than 85 % bp and a quantitation accuracy better than 70 %. The linear range of quantitation is 0.5 - 50 ng/µl ($r \geq 0.995$). Run to run and chip to chip reproducibility are ensured by means of external standards (DNA sizing ladder) and internal standards (DNA markers). The assay is compatible with commonly used PCR buffers so that no desalting or other sample pretreatment is necessary. Some PCR amplifications may require dilution of the sample, if the PCR fragment concentration significantly exceeds 50 ng/µl. The high fidelity of sizing and quantitation is especially useful for the analysis of PCR fragments, where size and quantity of the PCR product are a clear indication of the quality of the PCR reaction. Moreover, for applications requiring standardized amounts of PCR products, quantitative information can provide additional benefits.

Analysis of PCR fragments

System performance was evaluated by comparing the results obtained on the Agilent 2100 bioanalyzer to slab gel analysis of the same PCR fragments. Two different sets of primers were used to amplify Adenovirus 2 genomic DNA resulting in a 300 bp fragment and a 2966 bp fragment respectively. Both fragments were divided into two aliquots and one aliquot of each was diluted 1:4 prior to analysis. The results of the analysis using the DNA 7500 assay and a 1 % agarose gel are displayed in figures 2a and 2b. Comparing the gel images (figure 2a) shows that the agarose gel has slightly better resolution for fragments larger than 1500 base pairs, whereas the Agilent 2100 bioanalyzer separates better in the lower base pair range. A mass ladder which is run in a separate lane can be used as a visual control for the size as well as the quantity of the PCR fragments. The sizing results

are displayed in a tabular format for each sample after completion of the run. The first results can be read from the screen after seven minutes (five minutes warm-up of the instrument). For the two PCR fragments, the sizing accuracy was greater than 97 %. Typically, sizing accuracy is above 90 %. In the rare case where DNA fragments show a strong sequence dependent migration, sizing accuracy remains greater than 85 %.

With the Agilent 2100 bioanalyzer quantitative results are available at the completion of each sample run. No scanning or other procedures are required. The results of the quantitative analysis are shown in figure 2b. As compared to the labelled concentration values of the mass ladder the quantitation error was between 0 and 15 %. Typically, quantitation of PCR fragments can be performed with an accuracy greater than 70 % with a linear range of concentration between 0.5 to 50 ng/µl. The linear range of detection makes the assay useful to detect the presence of non-specific PCR products. In the given example, virtually no non-specific products were detected in the smaller fragment sample (impurity level <2%) whereas the larger fragment sample showed a significant amount of two non-specific PCR fragments (impurity level >50%).



Low Mass Ladder

Fragment	c(DNA)	Agilent 2100 bioanalyzer
2000 bp	20 ng/µl	22.4 ng/µl
1200 bp	12 ng/µl	13.8 ng/µl
800 bp	8 ng/µl	9.1 ng/µl
400 bp	4 ng/µl	4.3 ng/µl
200 bp	2 ng/µl	2.1 ng/µl
100 bp	1 ng/µl	1.0 ng/µl

Figure 2a
Comparison of gel-like image (Agilent 2100 bioanalyzer) and gel-scan

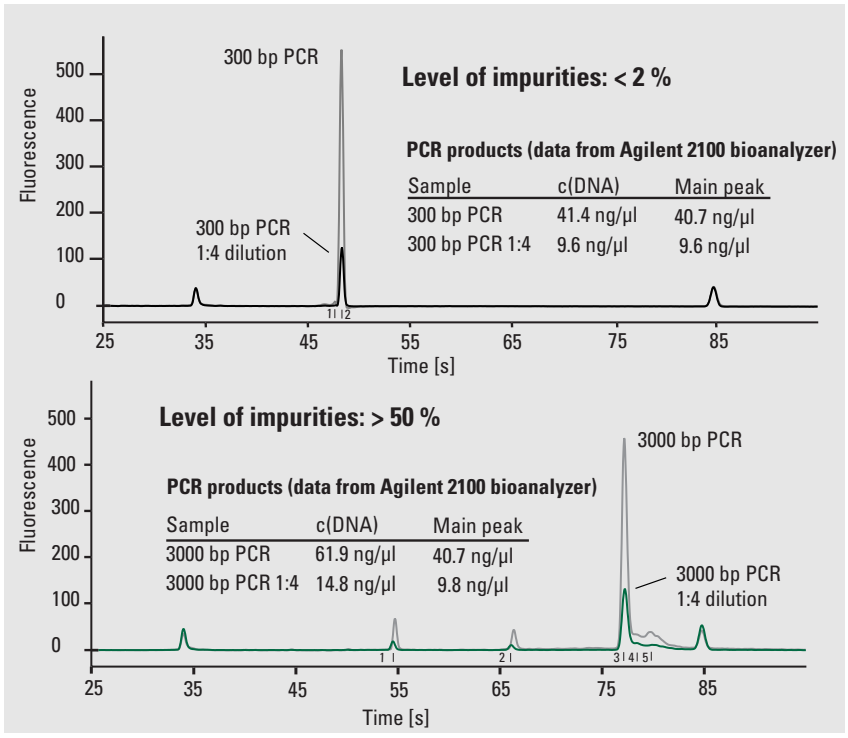


Figure 2b
Quantitative data obtained from the Agilent 2100 bioanalyzer

Quantitation of PCR fragments before and after firing through thermal ink jet heads

The importance of accurate quantitation of PCR fragments can also be exemplified in the context of DNA array technology. DNA arrays are produced by deposition of PCR fragments in a predefined manner on a solid surface. Prior to deposition onto the arrays, the PCR reactions must be checked to ensure that the correctly sized product is present and that no non-specific fragments have been amplified. Furthermore, it is important to determine the concentration of the PCR products which will be spotted onto the arrays. The method of deposition can have a significant influence on the quality of DNA on the array. Thermal inkjets are a convenient means to supply minimal but well-defined amounts of DNA in a precisely defined position. There is, however, the risk that there might be a loss of DNA through thermal degradation, dilution of the DNA inside the inkjet head, mechanical shearing, or incompatibility with the inkjet head materials.

The Agilent 2100 bioanalyzer was used to correctly size and quantitate two separate PCR fragments before and after deposition by thermal inkjet heads as a method to ensure DNA integrity. These results were then compared to those obtained using standard acrylamide gel electrophoresis. Figure 3a shows the electropherogram of a 485 base pair fragment before and after deposition through the inkjet head. It was found that the fragment remained intact after deposition, with no evidence of thermal degradation or mechanical shearing of the DNA. In addition, there was no significant change in the DNA concentration after firing, indicating that the DNA did not irreversibly bind to the inkjet head or become diluted once loaded inside the head. Similar results were obtained with the larger PCR fragment (figure 3a), although there was a slight decrease in the concentration after firing.

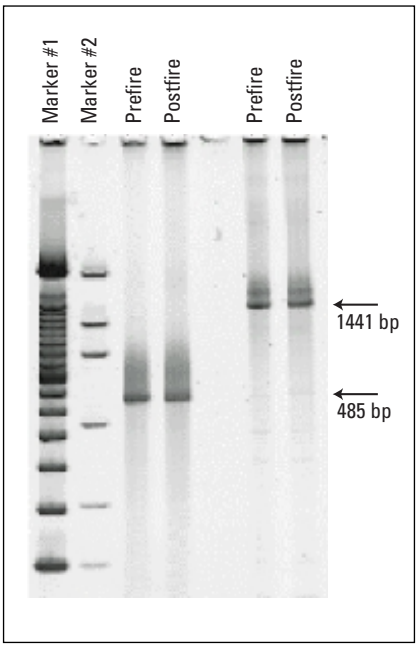


Figure 3b
Prefired and postfired fragments analyzed on a 5 % polyacrylamide gel stained with SYBR Gold nucleic acid stain

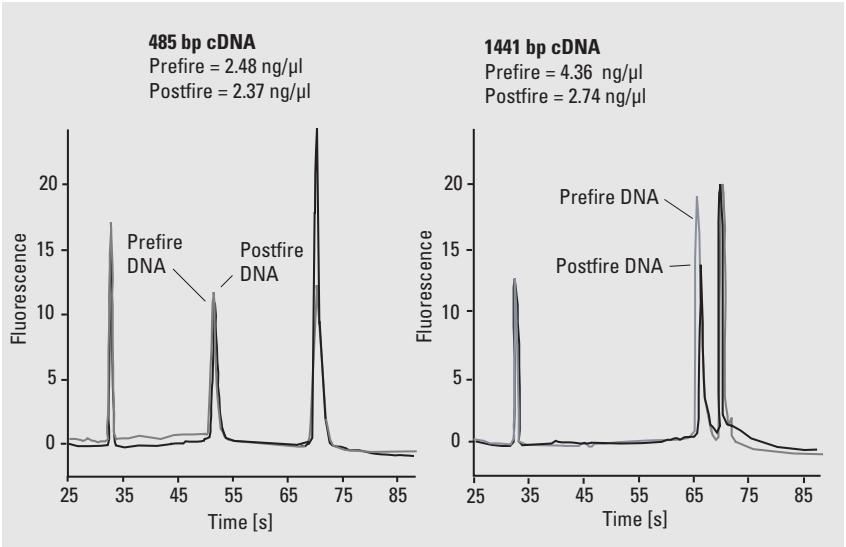


Figure 3a
Prefired and postfired PCR fragments sized and quantitated using the DNA 7500 assay

These fragments were also analyzed using traditional gel electrophoresis (figure 3b). The sizing and concentration of both fragments were similar to those obtained with the Agilent 2100 bioanalyzer. However, it was difficult, if not impossible, to detect the slight decrease in the concentration of the larger fragment. While the dilution was not significant in the context of pro-

ducing DNA arrays, this example illustrates the difference in the sensitivity between the Agilent 2100 bioanalyzer and slab gel electrophoresis. The Agilent 2100 bioanalyzer allows for detection of small changes between two samples that may not be detected using traditional gel electrophoresis. In addition, the Agilent 2100 bioanalyzer gives more precise sizing of the fragment compared to the rough estimate obtained from gel electrophoresis.

Conclusion

The Agilent 2100 bioanalyzer shows excellent performance for quantitative analysis and sizing of PCR fragments. The use of internal and external DNA markers allows analysis of multiple samples through a single separation channel with very high reproducibility and reliability. Data precision is comparable or superior to slab gel analysis, whereas analysis times are greatly reduced. Automation of both separation as well as data analysis makes the Agilent 2100 bioanalyzer versatile and easy to use. The superior system performance makes the Agilent 2100 bioanalyzer the ideal tool for sizing and quantitating PCR fragments. In addition to quantitative analysis of PCR fragments with the DNA 7500 kit, the instrument platform can be used for other nucleic acid analyses. Initial kits are available for sizing of restriction fragment digests (DNA 12000 LabChip kit) and for quantitation and integrity/purity check of total RNA and mRNA samples (RNA 6000 LabChip kit).

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