Integrating High-Throughput, On-Chip Electrophoresis Analysis into PCR Diagnostics Projects

Application Note

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Abstract

Laboratory projects that require accurate analysis of PCR products can be divided into two classes: low throughput and high throughput. Small numbers of samples can be sized and quantitated on a traditional agarose ethidium gel. More precise measurements can be obtained on a microfluidics workstation utilizing Lab-on-a-Chip technology, such as the Agilent 2100 bioanalyzer. However, when the number of samples to be analyzed turns into hundreds or thousands, typical gels and 12-well chips are not sufficient to maintain a continuously productive workflow. In some cases, a particular sized band on a gel image or a peak on an electropherogram is enough to give a "yes or no" answer in a diagnostic assay. For example, screening cell lines for viral pathogens or mycoplasma can be achieved by simple PCR and gel electrophoresis. In other cases, specific band/peak sizes and concentrations are a necessary step in sequencing DNA PCR products to determine mutations. The 5100 Automated Lab-on-a-Chip Platform (ALP) from Agilent Technologies has been used in our laboratory to attain a workflow level that could not be reached by standard low-throughput electrophoresis methods. This instrument allowed rapid, sensitive quality control that relieved a bottleneck in our procedural assays. In this application note we demonstrate how this high-throughput system can be applied to four different molecular biology projects.

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Introduction

Current technology for analyzing PCR product size and quantity (concentration in ng/µL) includes conventional capillary electrophoresis instruments, the Agilent 2100 bioanalyzer, and various gel rigs, power supplies, and UV illuminators. We found that for our increasing sample influx, even 100-well agarose/ethidium gels were labor intensive, time consuming, somewhat hazardous, and not very accurate.

Agilent's 2100 bioanalyzer greatly assisted us in specifying size ranges down to 1 base pair (sizing accuracy: ± 10%) and concentrations down to 0.5 ng/µL. Unfortunately this chip instrument only analyzes 12 samples in approximately 40 minutes. Although the preparation time and sample volume needed were reduced compared to gels, the throughput was not meeting our needs.

We are currently involved in a mutation detection project, which includes 1,188 patient DNA samples aliquoted onto 15 (96-well) plates to be tested for mutations in at least four cancer genes. Each gene is divided into exon regions of 1,000 base pairs or less, and PCR primers are chosen to amplify that area. If an exon is larger than 1 kb, it is split into several PCR reactions to cover the entire length. Some genes also had more than one exon to be tested. Ultimately four gene assays evolved into nine separate PCR reactions per sample, so 1,188 samples turned into 10,692 PCRs. This does not include the initial PCR optimization we perform to find the best possible conditions for generating quality products for the ultimate step of sequencing. The initial optimization involves testing at least two DNA samples with nine of our in-house PCR cocktails at about six different annealing temperatures. This in itself is at least a plate's worth of samples per gene region to be analyzed electrophoretically.

We began analyzing optimized reactions on the 2100 bioanalyzer, sometimes running more than 12 chips per day. A technician had to be at the instrument every 40 minutes, preparing the next chip of 12 samples. It was obvious this would take over 800 chips, and approximately 75 business days (4 months) to complete, with no time allowed to commit to other pressing projects.

In a mutation detection project such as this one, PCR products must be subjected to quality control measures prior to submittal for DNA sequencing. The proper base-pair size must be determined to ensure that the primers are annealing to the specific gene region desired. The concentration must also be monitored; our high throughput sequencing process requires an optimal 50 ng/µL concentration. If at this point a sample showed no band, it would have to have its PCR reaction repeated.

Our other PCR diagnostics projects include screening cell lines for viral pathogens and mycoplasma. At certain times, both of these projects can require high sample throughput. For this testing, a definitive positive or negative result is all that is essential. Again, correct base pair sizing is needed, and concentration is an added bonus.

We are also involved in the development stages of some gene expression microarray applications. This demands preliminary checks of sizing and concentration of approximately 188 sample plates with 384 wells each (72,000 samples).

To increase throughput for these tasks, we used an Agilent 5100 Automated Lab-on-a-Chip Platform (ALP). The ALP provided simultaneous electrophoretic analysis of four samples with unattended analysis of up to 10 standard well plates (96 or 384 formats). We found minimal hands-on preparation time involved with the chips, user-friendly software combined with an Oracle database for data mining on the ALP, and quality data output that assisted our lab in achieving a constant workflow.

Materials and Methods

Templates

For the mutation detection project, DNA samples were sent to us previously extracted on 96-well plates. For virus testing, cell pellets had DNA extracted from them in-house. For the mycoplasma screening, cell culture supernatants were heated to 95°C and used as templates in the PCR reactions. For the gene expression array project, a panel of cell line DNAs was PCR tested for the BRAF, CDKN2A, and RB cancer genes.

PCR

The various 96-well PCR reactions were carried out, in most cases, using dNTPs, primers, 10X PCR buffer, magnesium sulfate, and Platinum Taq High Fidelity polymerase (all from Invitrogen). Total volumes were 50 µL. An initial denaturation step of 2–3 minutes at 94°C–95°C was followed by 30–40 cycles of 94°C–95°C for 30–60 seconds, specific annealing temperature for 30–60 seconds, and extension at 68°C–72°C for 20–60 seconds. A 10 minute final extension at 72°C was also employed. For the mycoplasma testing, the VenorGem kit instructions from Sigma were followed. For testing of the Epstein-Barr virus, the kit instructions from Maxim Biotech were followed. A 10 µL reaction was used for the 384-well plate PCRs. ABI/Perkin Elmer 9600 and 9700 DNA Thermal Cyclers were used.

Analysis of PCR-products

Amplified products were electrophoretically separated using a four-capillary DNA 1000 HT-4 chip on the Agilent 5100 Automated Lab-on-a-Chip Platform. The Quick Start User's Guide was referenced as a standard protocol.

Once logged onto the ALP software and connected to the instrument, a new job measurement was created. In the software, we labeled the plate name(s), specified the DNA assay and plate type used (e.g. Eppendorf 96 well Twin Tec or Agilent 384-plate), and exported the Excel file to correlate sample identification numbers with well numbers. Newly created jobs were saved and closed. In the instrument tab, the arrangement of plate and chip order in the instrument depot is displayed once the job is opened.

The reagent plate was made as described in the Quick Start Guide. One column of the reagent plate containing gel, gel-dye mix, and marker is sufficient for the electrophoretic separation of 384 (4 x 96) samples. Twenty-five to thirty microliters of PCR amplicons were aliquoted into the proper 96 well plate. All plates were briefly centrifuged and sealed with Agilent's aluminum foil seals for four seconds at 170°C on the Remp Easy Sealer. Plates and chip were placed in the depot as shown in Figure 1 with the reagent plate in D1, sample plates in B1 (B2, C1, and C2 if needed),

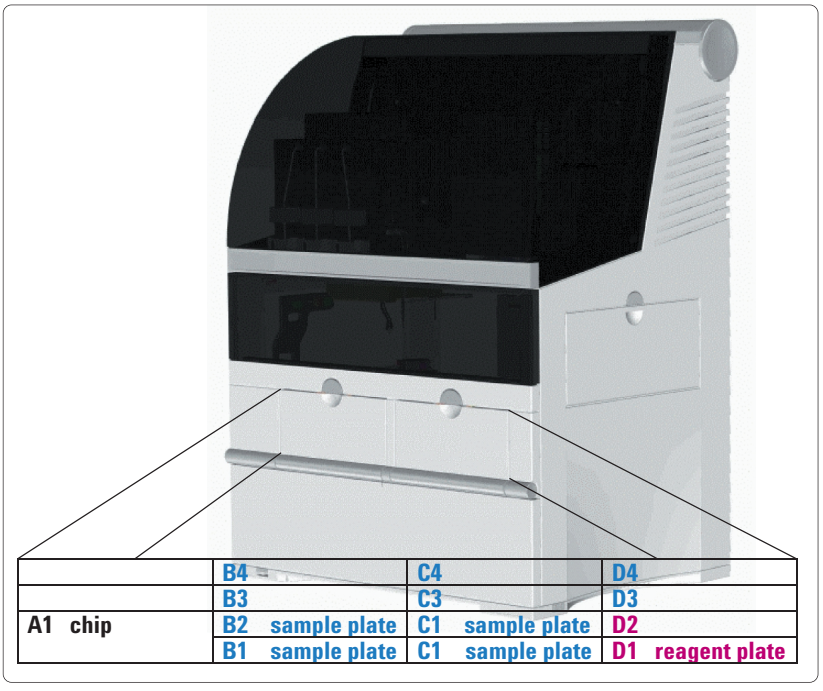

Figure 1. Depot loading. The ALP instrument contains a specific compartment (depot) for the storage of chips, reagent plates and sample plates. The depot allows maximum loading of 10 sample plates. The different depot positions as well as the depot loading used in this experimental workflow is indicated.

chip in A1. Up to 10 sample plates can be handled during one job.

The chip was removed from the storage box, and the underside wiped dry with a KimWipe. The chip was then placed in the chip carrier.

Our ladder was prepared depending on the amount of salt used in our PCR reactions. In general, 10 µL of 10X PCR buffer was added to 10 µL of the 10X ladder (supplied with DNA 1000 HT-4 reagents), 4 µL of 50 mM $MgS0_4$, and 76 µL of HPLC water. Finally, the ladder was added to the four dedicated wells of the provided ladder trough. If multiple 384 well plates were run, additional wells of the ladder trough needed to be filled with ladder.

Clicking on the "execute job" icon prompts a job start wizard. Once the depot was completely loaded, an initial verification step was induced. As each item is checked off, you proceed to the next. The chip, reagent plate, and sample plates are already approved at this point. The columns of reagents prepared on the reagent plate must be designated in the software wizard. The ladder trough must be correctly inserted into the trough holder (basin). The proper number of Agilent pipette tips must be inserted into the tip container. The reagent bottles should be as follows: Maintain at least 30 mL of storage buffer in the buffer bottle, at least 500 mL of HPLC water in the water bottle, and keep the waste bottle empty. Once this is complete, the job is started.

Results and Discussion

We found that this instrument currently takes approximately 1.5 hours to initialize/prime a chip. It takes an additional 1.5 hours to run a 96 well plate. Then, a final cleanup of the chip takes at least 30 minutes after a run. All-in-all, one plate would take 4 hours, or 2.5 minutes per sample. However, running multiple 96-well plates or even 384-well plates lessens the sample run time to 1.7 minutes/sample for 192 samples, 1.45 minutes/sample for 288 samples, and 1.3 minutes/sample for 384 samples, to even below 1 minute/sample when running 10 plates per job. Comparing this to the bioanalyzer, which has a constant run time of approximately 2 minutes per sample, this is an improvement. The "walk-away" aspect of the ALP is the other important factor. Once a job commenced, we were able to commit time to other projects; not having to constantly load chips every 30–40 minutes.

Figure 2 shows a gel-like image of BRAF exon 15 PCR products and various cancer genes, respectively. The primary goal was to verify bands in strong and clean products, then optimize primer amplification before increasing the workload with more samples. Utilizing the ALP review software, clicking on the Data tab enables views of gel-like images, electropherograms, overviews of plate run quality, and allows for custom peak table edification.

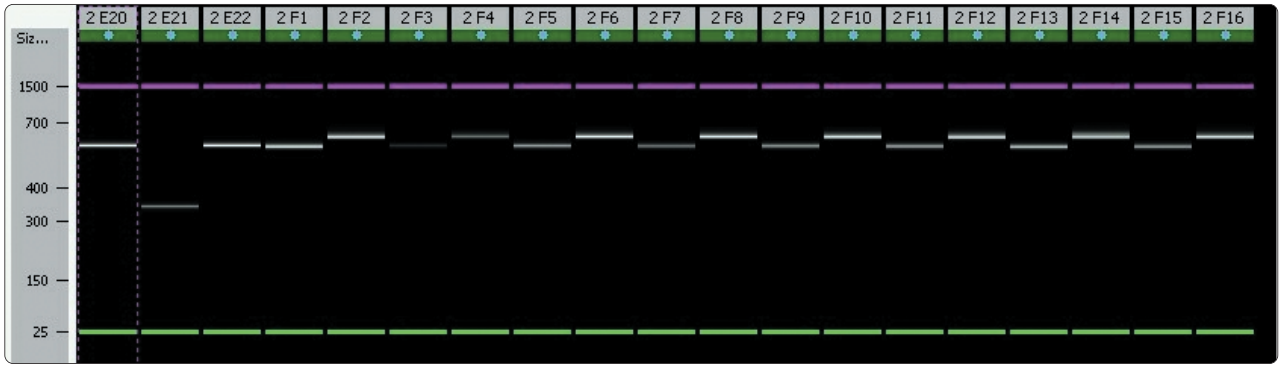

Figure 2. PCR amplicon bands of 19 samples. The gel-like image gives a first overview on the experimental results. The arrangement of the gel-like image is customizable by selecting the dedicated samples in the sample table of the software. The internal upper marker is shown at 1500 bp—the lower marker at 15 bp in an extra fluorescence window was used to calculate the green calibration mark at 25 bp. The size axis on the left gives an estimate on band sizes.

So far, approximately twenty 384-well plates and twenty 96-well plates have been run for the cancer gene study. The sizing range of the PCR products is 500–700 bp and concentrations could be determined in the range of 4–50 ng/µL. The sizing accuracy remains within 5%.

The output result flagging of 64 samples in a viral pathogen test is shown in Figure 3. Row E contains GAPDH (glyceraldehyde phosphate dehydrogenase) ubiquitous housekeeping internal control PCR. All should have been positive as indicated by the dark purple color. The samples in wells E1 and D6 show a different color-coding indicating that the positive control didn't meet the expected criteria. This example shows how the result flagging can be used for a fast initial screening of all samples.

Figure 4 is an electropherogram of a positive control for Mycoplasma. The positive control corresponds to 268 bp PCR fragment and the internal control to a 196 bp peak in the electropherogram. Within this project, 40 supernatant samples were analyzed, most of which were negative.

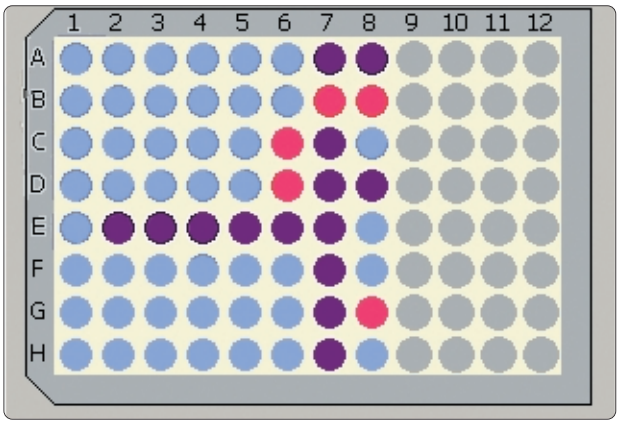

Figure 3. Result flagging for the identification of positive control bands for viruses. The different colors correspond to rules sets defined in the result flagging of the software. By applying the result flagging to the acquired and analyzed data, a fast initial screening for specific parameters such as peak number, peak size, and concentration can be performed.

In the peak table view, we were able to create a custom grid of Plate ID, well number, product size, concentration, molarity, and sample name. This helped us especially with having to adjust concentrations of samples to reach the optimal 50 ng/µL concentration for sequencing, as well as

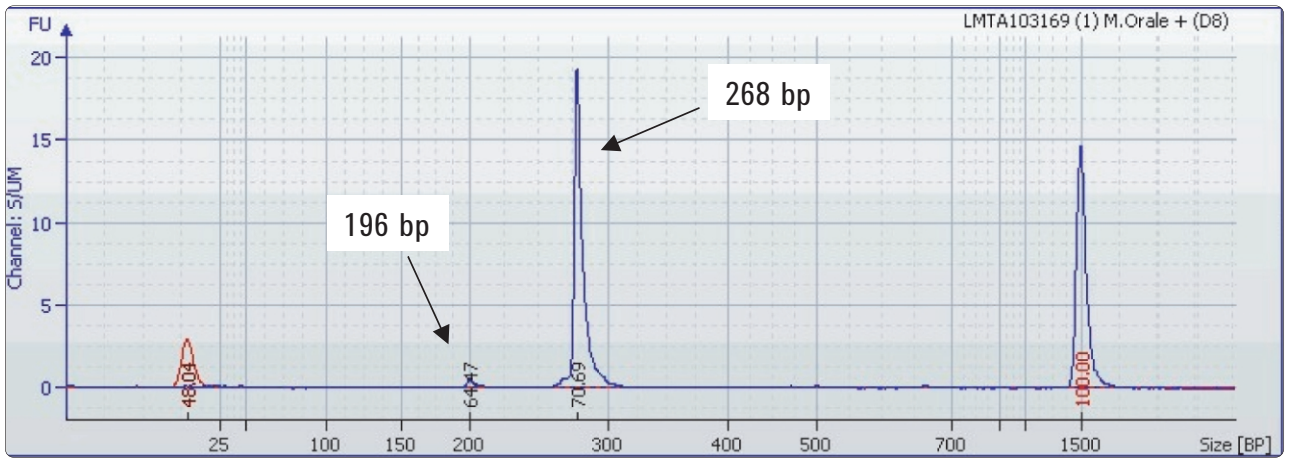

Figure 4. Electropherogram displaying internal and positive control peaks in a Mycoplasma orale sample. The positive and the internal control correspond to the large 268 bp peak and the 196 bp peak, respectively.

manufacturing equimolar samples to test on a resequencing chip. The peak table is similar to Microsoft Access databases, and can be exported to a Microsoft Excel file. Table 1 is an example of our PCR optimization peak table before sequencing. The table refers to a mutation detection project for a cancer gene where the sample number is low throughput, but the number of exons investigated for each sample makes it a high throughput test.

Conclusion

The data obtained from the Agilent 5100 ALP instrument enabled our laboratory to perform increased numbers of on-chip electrophoresis analyses for sizing and quantitation. The number of samples tested at our facility had gone well beyond the capacity of simple gels, and even the Agilent 2100 bioanalyzer. The accuracy of sample concentration and specificity of base pair sizing allowed us to adjust samples to be optimal for DNA sequencing, as well as allowed us to be certain that we were targeting the proper gene region of interest. All of this was achieved at a level of high throughput in order to maintain the workflow demanded by our lab setting. This makes for a rapid and sensitive quality control process necessary in PCR diagnostics and mutation detection projects. These data suggest that the Agilent 5100 ALP platform can be used in labs requiring high throughput microfluidics analysis of 1kb or less amplicons produced from any gene.

Table 1. The customizable peak table provides the user with information necessary to perform post-amplification analysis procedures. The peak table shown in this example contains information on the plate-name, plate-ID, sample well, sample name, size, concentration (ng/µL) and molarity. Here, the concentrations determined how much volume to use in subsequent DNA sequence reactions.

Plate	Plate ID	Well	Sample Name	Size [bp]	Concentration	Molarity
vhlexon1opts_092804-	1	A1 (4)	M1R51	641	1.1	2.7
vhlexon1opts_092804-	1	A2 (5)	M2R51	615	1.2	2.9
vhlexon1opts_092804-	1	A5 (8)	M5R51	640	2.7	6.4
vhlexon1opts_092804-	1	A9 (12)	M9R51	644	1.6	3.7
vhlexon1opts_092804-	1	B5 (8)	M5V51	645	2.5	5.9
vhlexon1opts_092804-	1	B9 (12)	M9V51	426	3.7	13.1
vhlexon1opts_092804-	1	B9 (12)	M9V51	645	26.8	62.9

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