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Evaluation of DNA Fragment Sizing and Quantification by the Agilent 2100 Bioanalyzer, *Nicholas J. Panaro,[1] Po Ki Yuen,[1] Taku Sakazume,[1,2] Paolo Fortina,[1,2] Larry J. Kricka,[1] and Peter Wilding[1*]* ([1] Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 3400 Spruce St., Philadelphia, PA 19104; [2] Department of Pediatrics, The Children's Hospital of Pennsylvania, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; * author for correspondence: fax 215-662-7529, e-mail pwilding@mail.med.upenn.edu)

Capillary electrophoresis (CE) achieves efficient separation of molecular species by the application of high voltages to samples in solution *(1)*. Commercial CE units, available for slightly more than a decade, have found numerous applications *(2–6)*, but are expensive (~$60 000) and require substantial user training and experience. Recent advances have allowed CE to be performed on microchip devices *(7–11)*. We evaluated the Agilent 2100 Bioanalyzer (Agilent Technologies), which represents a new generation of CE instruments that use this technology.

The Bioanalyzer is relatively inexpensive (~$18 000) and is simple to operate, requiring only routine pipetting and basic computer skills. Typically, 12 nucleic acid samples can be sized and quantified on a disposable chip within 30 min. Chips are fabricated from glass and comprise an interconnected network of fluid reservoirs and microchannels, which must be filled with a gel-dye mixture. Each chip contains 16 wells: 3 for loading the gel-dye mixture, 1 for a molecular size ladder, and 12 for experimental samples. The movement of nucleic acids through the microchannels is controlled by a series of electrodes, each of which is independently connected to a common power supply. The Bioanalyzer displays data as both migration-time plots and as computer-generated virtual gels. Traditional CE operating variables [temperature, voltage, capillary material, and pH, ionic strength, and viscosity of buffer *(12)*] cannot be modified. The instruments costs ~$18 000, and chips cost ~$12–18 per chip ($1–1.50 per sample).

The gel-dye mixture consists of a linear polymer and a fluorescent, intercalating dye. The marker mixture consists of a buffer along with lower and upper molecular size markers, which the Bioanalyzer uses as references when sizing DNA fragments. The upper marker is also used as a reference for calculating the concentration of DNA fragments in each sample. Each reagent set also contains a molecular size ladder for sizing experimental DNA fragments.

We evaluated the ability of the Agilent 2100 Bioanalyzer (Biosizing software, Ver. A.01.05) to generate consistent results with respect to sizing and signal quantification on a well-to-well, chip-to-chip, and day-to-day basis, using PCR fragments and commercially available plasmid digests.

Whole blood was obtained from healthy volunteers and drawn into EDTA-coated tubes. Genomic DNA was purified directly from whole blood using the QIAamp DNA Blood Mini reagent set (Qiagen) and resuspended in DNase-free water. Gene-specific primers for endothelial nitric oxide synthase (eNOS) *(13)* yielded a 379-bp fragment (forward primer, 5′-GTG ATG GCG AAG CGA GTG AAG-3′; reverse primer, 5′-GAC ACC ACG TCA TAC TCA TCC-3′). Amplification of eNOS DNA was achieved using a GeneAmp 2400 PCR System (Perkin-Elmer) and AmpliTaq Gold (Perkin-Elmer) under the following conditions: 10-min activation of Taq enzyme at 95 °C, followed by 45 cycles of 94 °C for 30 s (to denature), 65 °C for 30 s (to anneal), and 72 °C for 30 s (to extend). All reaction volumes were 50 μL. PCR product was concentrated via precipitation using two volumes of cold ethanol (−80 °C, overnight), centrifuged at 14 000*g* for 5 min, washed once with cold ethanol (950 mL/L), aspirated, allowed to dry under ambient conditions, and resuspended in DNase-free water. Removal of primers, nucleotides, and salts was accomplished using the QIAquick PCR purification method (Qiagen). The final elution of PCR product was performed with Tris-EDTA buffer to a final concentration of ~40 ng/μL. The concentrations of eNOS PCR samples were determined via absorbance readings at 260 nm using a SmartSpec 3000 spectrophotometer (Bio-Rad).

DNA 7500 Lab Chips (Agilent Technologies) were loaded with samples as recommended by the manufacturer with minor modifications. Briefly, microchannels were filled by pipetting 9 μL of gel-dye mixture into the appropriate well and then forcing the mixture into the microchannels by applying pressure to the well via a 1-mL syringe. The ladder well and sample wells were subsequently loaded with 5 μL of marker mixture plus 1 μL of either molecular size ladder or sample, respectively, using one of four protocols: (protocol A) 5 μL of marker mixture was loaded into each sample well, followed by 1 μL of sample; chips were vortex-mixed at setting 5 for 1 min in a Fisherbrand Vortex Genie-2 (Fisher Scientific); (protocol B) same as (A) except that the chips were vortex-mixed at setting 4; (protocol C) 66 μL of marker mixture and 13 μL of sample were mixed in a microfuge tube; 6 μL of this mixture was loaded into each sample well; chips were vortex-mixed at setting 4 for 1 min; (protocol D) marker mixture was loaded into each sample well, followed by 1 μL of sample; the contents of each well on the chip were gently pipetted three times in situ using a P10 Pipetman; chips were vortex-mixed at setting 4 for 1 min. After being vortex-mixed, chips were immediately

inserted into the Bioanalyzer and processed. The marker mixture for the DNA 7500 Lab Chip contains lower and upper molecular size markers of 50 and 10 380 bp, respectively.

With chip-loading protocol A, sample loss from the wells led to poor electrode contact with the remaining sample. Liquid loss could be eliminated by using protocol B. Identical 1-μL samples of eNOS PCR product (379 bp; 12.6 ng/μL) were loaded into each sample well. This experiment was performed three times, with a chip being run on each of 3 successive days. With chip-loading protocol B, we observed significant well-to-well variations in signal quantification (CV = 12–31%), whereas DNA sizing results remained consistent (CV <1%). Specifically, samples in wells 1–3 of each chip yielded much lower concentrations than samples in other wells (Table 1A). The CV for signal quantification for the entire 12 wells was 12–31% (Table 1B). CVs for sizing were <1% for all chips with no result differing by >6 bp from the expected 379 bp (Table 1C).

Consistent well-to-well signal quantification results could be obtained with chip-loading protocol C. Day-to-day CVs for signal quantification were 5.9–7.9% (Table 1B). Sizing results were almost identical to those results for chip-loading protocol B (Table 1C). This suggested that well-to-well variations in signal quantification were attributable to poor mixing and/or staining of samples while on the chip.

Chip-loading protocol D yielded consistent well-to-well and day-to-day results for sizing and signal quantification. CVs for signal quantification were 4.0–5.4% (Table 1B) with no single quantification value differing from the predicted value (12.6 ng/μL) by more than 21% and no average value for a chip differing from the predicted value by more than 13%. Again, CVs for sizing results were <1% (Table 1C) for all chips with no individual sizing result differing from the predicted size of 379 bp by >9 bp. Similar results were observed using unpurified PCR product.

We investigated cross-contamination between wells by loading 1 μL of sample containing a single component (eNOS cDNA) in odd-numbered wells while loading 1 μL

Table 1. Variation of sizing and quantification for single and multiple DNA fragments.

A. Concentration for a single PCR fragment using sample preparation method B (ng/μL)

Well	Chip 1	Chip 2	Chip 3
1	3.4	1.2	7.3
2	11.8	8.9	12.4
3	12.2	10.3	11.3
4	11.7	13.8	11.3
5	10.5	11.6	12.0
6	13.2	13.1	11.7
7	13.3	11.4	11.7
8	13.0	11.8	10.9
9	12.5	12.8	11.3
10	12.3	13.8	12.7
11	12.6	12.8	12.1
12	11.1	12.6	10.9

B. Intrachip statistics for signal quantification of eNOS PCR fragment as a function of sample preparation method

Method	Concentration, ng/μL (CV, %) Chip 1	Chip 2	Chip 3
B	11.5 (23)	11.2 (31)	11.3 (12)
C	11.8 (5.9)	11.4 (7.9)	11.4 (7.9)
D	11.0 (4.7)	11.6 (4.0)	12.6 (5.4)

C. Intrachip statistics for molecular sizing of eNOS PCR fragment as a function of sample preparation method

Method	Molecular size, bp (CV, %) Chip 1	Chip 2	Chip 3
B	383 (0.4)	382 (0.5)	383 (0.5)
C	382 (0.5)	383 (0.4)	383 (0.4)
D	384 (0.6)	385 (0.5)	383 (0.5)

D. Intrachip statistics for molecular sizing of ϕX174 plasmid/*Hae*III digest[a,b]

Predicted size, bp	Day 1		Day 2	
	Chip 1	Chip 2	Chip 1	Chip 2
118	109 (1.3)	109 (0.7)	110 (0.7)	110 (0.9)
194	187 (1.0)	187 (0.8)	187 (0.5)	188 (0.3)
234	224 (0.8)	224 (0.6)	224 (0.6)	225 (0.4)
271	265 (0.7)	265 (0.7)	266 (0.6)	265 (0.2)
281	276 (1.0)	276 (0.9)	276 (0.6)	277 (0.5)
310	302 (0.7)	302 (0.6)	303 (0.6)	304 (0.9)
603	606 (0.9)	606 (0.9)	608 (0.6)	608 (0.4)
872	867 (0.7)	868 (0.8)	867 (0.4)	869 (0.3)
1078	1001 (1.7)	1000 (1.2)	1006 (1.9)	1002 (1.6)
1353	1232 (2.0)	1237 (1.5)	1232 (1.9)	1228 (2.1)

E. Intrachip statistics for signal quantification of ϕX174 plasmid/*Hae*III digest[a]

Predicted size, bp	Concentration, ng/μL (CV, %) Day 1		Day 2	
	Chip 1	Chip 2	Chip 1	Chip 2
118	5.3 (1.8)	5.4 (4.9)	5.6 (6.4)	5.5 (3.3)
194	8.2 (2.3)	8.3 (4.0)	8.7 (6.7)	8.4 (4.2)
234	10.0 (2.6)	10.1 (3.9)	10.7 (6.3)	10.3 (5.0)
271	11.6 (2.7)	11.7 (5.3)	12.2 (6.5)	12.0 (2.2)
281	10.9 (3.4)	11.0 (4.9)	11.7 (6.0)	11.3 (4.7)
310	12.1 (2.6)	12.1 (4.3)	12.9 (5.2)	12.5 (4.5)
603	24.2 (2.3)	23.9 (5.3)	25.9 (5.4)	25.3 (4.0)
872	36.5 (4.2)	35.7 (6.6)	40.3 (4.6)	38.9 (4.8)
1078	45.0 (3.5)	44.2 (6.5)	50.5 (4.3)	49.2 (4.7)
1353	53.0 (4.2)	52.5 (6.6)	62.1 (3.8)	60.0 (5.0)

[a] Each average was calculated from four replicate samples analyzed on each chip.
[b] Values in parentheses are CV, %.

of sample containing multiple components (pBR322/*Msp*I plasmid digest; Sigma) in even-numbered wells (chip-loading protocol D). No interwell contamination was observed during chip analysis. Thus, the improved consistency of signal quantification observed was not attributable to interwell contamination but rather to enhanced intrawell sample mixing. Chip-loading protocol D was used for all subsequent experiments.

To evaluate the ability of the Bioanalyzer to measure absolute concentrations, concentrated eNOS PCR product (~40 ng/μL) was diluted in Tris-EDTA buffer to yield solutions containing 2.6, 5.1, 13.0, and 28.4 ng/μL. Each sample was loaded twice on each chip, with one sample being loaded in wells 1–6 and a duplicate sample being loaded into wells 7–12. Concentration values generated by the Bioanalyzer differed from concentration values obtained spectrophotometrically by 6–16%. Well-to-well and chip-to-chip results differed by similar amounts. DNA sizing results were found to be independent of the cDNA concentration.

To evaluate the ability of the Bioanalyzer to size samples containing multiple DNA fragments, commercially available plasmid digests were analyzed. To reduce the concentration of DNA fragments <100 bp in size, plasmid digests were spin-purified using the QIAquick PCR purification method, and samples were eluted in 1× Tris-EDTA buffer and diluted to yield appropriate concentrations for analysis. pUC18/*Msp*I digest (Sigma) samples were loaded into wells 1, 4, 7, and 10; ϕX174 RF DNA/*Hae*III fragments (Gibco) samples were loaded into wells 2, 5, 8, and 11; λ DNA/*Eco*RI marker (Promega) samples were loaded into wells 3, 6, 9, and 12. This experiment was performed twice on each of 2 successive days. Table 1, D and E, shows sizing and signal quantification results, respectively, for ϕX174 RF DNA/*Hae*III. The CV for DNA sizing of fragments was ≤2.1%, whereas the CV for DNA signal quantification was ≤6.7%. Similar CV values were observed for pUC18/*Msp*I digest with respect to sizing and quantification. CVs for DNA sizing and quantification were ~8% and 7%, respectively, for λ DNA/*Eco*RI markers. Similar results were observed for unpurified plasmid digests.

In summary, we recommend a modification to the manufacturer's protocol for chip loading: namely, gentle pipetting of samples with the marker mixture after loading into the sample wells, followed by vortex-mixing for 1 min at the highest setting, which does not cause liquid loss from the sample wells. Lower concentration DNA fragments may not be detected if poor chip preparation leads to weak sample staining. This is particularly crucial with respect to the manufacturer-supplied molecular size ladder. The Bioanalyzer cannot calculate size and concentration values for the experimental samples if it fails to detect all bands in the ladder. In addition, improper staining of the upper molecular size marker may lead to poor quantification of experimental DNA fragments.

We found the Agilent 2100 Bioanalyzer to be an easy-to-use, time-efficient substitute to conventional CE. It was effective at sizing and quantifying multiple DNA fragments in a variety of experimental samples, including plasmid digests and PCR samples.

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Evaluation of a Nucleic Acid-based Cross-Linking Assay to Screen for Hereditary Hemochromatosis in Healthy Blood Donors, *Christiane Wylenzek,[1] Martina Engelmann,[1] Dirk Holten,[1] Reuel Van Atta,[2] Michael Wood,[2] and Birgit Gathof[1*]* ([1] Division of Transfusion Medicine, University Hospital of Cologne, Joseph-Stelzmann Strasse 9, 50924 Cologne, Germany; [2] NAXCOR, 4600 Bohannon Dr., Suite 220, Menlo Park, CA 94025; * author for correspondence: fax 49-0221-478-6179, e-mail Birgit.Gathof@medizin.uni-koeln.de)

Hereditary hemochromatosis (HH) is a common autosomal recessive disorder (frequency, 1 in 300–500 in the Northern European population) characterized by overabsorption of iron with consequent multiorgan failure secondary to iron overload *(1, 2)*. Because early diagnosis and therapy can entirely prevent clinical complications, HH presents a model system for presymptomatic detection at the molecular level. *HFE*, the disease-causing gene of HH, encodes a 343-amino acid protein with high