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Improvement of low-density microelectronic array technology to characterize 14 mutations/single-nucleotide polymorphisms from

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produce a peak within 1–2 °C of the predicted T_m . This allows T_m peaks, which indicate the presence or absence of a PCR product, to be strategically placed within the typical melting curve range of 45–70 °C for hybridization probes. Over this melting curve range we feel it should be possible to clearly distinguish three peaks in a single channel. This potentially makes it possible to multiplex six mutations in two capillaries when both fluorescent channels are used. This enhances the capabilities of the LightCycler for mutation detection; similar strategies could also be used for other real-time systems.

The results presented here demonstrate the ability to carry out multiplex mutation detection by use of a combination of ARMS PCR and real-time detection. A laboratory currently using ARMS PCR in a diagnostic setting can quite easily convert the standard ARMS PCR to a real-time ARMS PCR. This could have a major advantage in time savings and reduce the handling of potential carcinogenic ethidium bromide.

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Improvement of Low-Density Microelectronic Array Technology to Characterize 14 Mutations/Single-Nucleotide Polymorphisms from Several Human Genes on a Large Scale, Sabrina Frusconi,^{1,†*} Betti Giusti,^{2,†} Luciana Rossi,² Sara Bernabini,¹ Filippo Poggi,² Irene Giotti,¹ Rosanna Abbate,² Guglielmina Pepe,² and Francesca Torricelli¹ (¹Unita' Operativa Citogenetica e Genetica, Azienda Ospedaliera Careggi, Florence, Italy; ²Dipartimento Area Critica Medico Chirurgica, University of Florence, Florence, Italy; † these authors contributed equally to this work; * address correspondence to this author at: Unita' Operativa Citogenetica e Genetica, Azienda Ospedaliera Careggi, Viale Morgagni 85, 50134 Florence, Italy; fax 39-055-4279686, e-mail genomica@ao-careggi.toscana.it)

Large-scale human genetic studies require new technologies to genotype several samples with relative ease, high accuracy, and reasonable costs. Among the available approaches, a microelectronic array technology has been developed for DNA hybridization analysis of mutations/single-nucleotide polymorphisms (SNPs) (1–4). The microelectronic array system (NanoChip[®] Molecular Biology Workstation; Nanogen) produces a defined electric field that allows charged molecules, such as nucleic acids, to be transported to any test site, or pad, on the electronic chip (NanoChip cartridge). Electronic-based molecule addressing can rapidly achieve a high concentration of amplicons on each pad of the cartridge. Control of temperature allows use of an optimal thermal stringency to characterize a SNP/mutation in all 100 pads of a cartridge simultaneously (5, 6). A thin hydrogel permeation layer overlies the pads; the presence of avidin or streptavidin in this layer allows the binding of biotinylated PCR products.

Although the technology is attractive, only a few protocols for its use have been published (7–11). We describe the development, optimization, and validation of a high-throughput method for SNPs and mutations analysis that allows performance of 1372 characterizations on each chip.

We studied samples from 150 individuals for 14 SNPs/mutations previously characterized by standard methods (restriction analysis, automatic sequencing, and allelic discrimination). Genomic DNA was isolated from peripheral blood by use of the FlexiGene DNA reagent set (QIAGEN GmbH). We analyzed 14 DNA mutations/SNPs for a total of 2100 characterizations (homozygous wild type, n = 1367; heterozygous, n = 522; homozygous mutant, n = 211). The 14 nucleotide substitutions were SNPs/mutations involving the glycoprotein Ia (*GpIa*), glycoprotein IIIa (*GpIIIa*), follicle-stimulating hormone receptor (*FSHR*), hereditary hemochromatosis (*HFE*), and α_1 chain of collagen type 6 (*COL6A1*) genes.

For each mutation/SNP we designed a set of probes consisting of a forward and a reverse PCR oligonucleotide (one oligonucleotide for each SNP/mutation was 5'-biotinylated); two reporter oligonucleotides (one labeled with Cy3, specific for the wild-type nucleotide; the other, labeled with Cy5, specific for the mutant nucleotide), and

one stabilizer oligonucleotide. Reporter oligonucleotides had Cy3 or Cy5 fluorophore at the 5' end and a SNP/mutation at the 3' end. The stabilizer was located immediately downstream from the 3' end of reporter oligonucleotides. All of the designed oligonucleotides are listed in Table A of the Data Supplement that accompanies the online version of this Technical Brief at <http://www.clinchem.org/content/vol50/issue4/>.

To genotype the 14 SNPs/mutations, we amplified seven DNA sequences by PCR. The seven amplicons were pooled and desalted with the Nucleo Fast system (96-well PCR plates) from Macherey-Nagel. We mixed 30 μ L of each 55- μ L desalted amplicon pool with 30 μ L of 100 mmol/L histidine. Four wells containing solutions of 0.3 mol/L NaOH, 0.1 mol/L NaOH, and 50 mmol/L histidine and a known heterozygous sample for each SNP/mutation analyzed were also included on the plate. The loader was programmed to electronically address each amplicon pool to specific pads on the cartridge. The 50 mmol/L histidine solution without amplicons was addressed to two pads in each cartridge as background. Each pool of samples was analyzed on one pad in two different cartridges, whereas two known heterozygous samples for each mutation/SNP were addressed to four pads in each cartridge. Each sample was amplified in a second independent PCR reaction, pooled, and addressed to one pad of a third cartridge. A second amplification reaction was also carried out for the two known heterozygous samples for each mutation/SNP, which were pooled and addressed to four pads of the third cartridge.

One set of stabilizer and reporter oligonucleotides at a time, specific for one SNP/mutation, was hybridized to the cartridge. The hybridization mixture was then removed, and the chip was rinsed twice with high-salt buffer. The cartridge was then placed in the reader, where the temperature was increased to the optimum temperature for discrimination (see Table A of the online Data Supplement). After each hybridization and scanning procedure, a stripping step was performed to remove reporters and to allow hybridization with another set of probes. The same protocol was applied to analyze each mutation/SNP. To study all 14 SNPs/mutations, we performed 14 hybridization/stripping steps. A fluorescence scan was

performed with the system reader. The software directly assigned the genotype to each sample (Table 1). Known heterozygous control samples were used as Cy3: Cy5 fluorophore ratio references for signal normalization. For background subtraction, fluorescence signals in the histidine pads were used. The mean values for Cy3 and Cy5 fluorescence were determined by averaging the amount of fluorescence in each pad. The Cy5: Cy3 ratio for each pad in the heterozygous control set was averaged together to build the scale. The scaled mean was determined by multiplying the scale and the mean. The ratios of the scaled means were used to make the genotype designation. For this purpose the Nanogen-recommended criteria were used: heterozygous if the Cy5: Cy3 ratio was between 3:1 and 1:3; homozygous for the wild-type and mutant alleles if the ratios were >1:5 and >5:1, respectively. Ratios between 1:3 and 1:5 for the wild-type or between 3:1 and 5:1 for the mutant alleles indicated a need to repeat the analysis. For pad exclusion, the signal-to-noise ratio was set to a default at 5:1.

Fluorescence intensities were similar for the same PCR products addressed to different pads of the same cartridge and in different pads of a different cartridge. Major differences in signal intensities were observed in the case of the second amplification addressed to a different cartridge. After the normalization procedure, all heterozygous samples showed ratios very close to 1, ranging from 1.0 to 1.53 [mean (SD), 1.11 (0.13)] in adherence with data relative to the known heterozygous samples shown in Table B of the online Data Supplement.

No background increase was detected after the 14th hybridization. The background signal ranged from 1.5 to 15 independent of the SNP/mutation analyzed and was not related to the number of hybridization/stripping steps performed.

We found 100% concordance between the results obtained by the approach described here and those obtained by standard techniques (automated sequencing, allelic discrimination, and restriction analysis) either within or between assays. This concordance was observed for all of the samples independent of the genotype (homozygous wild type, 1367 of 1367; heterozygous, 522 of 522; homozygous mutant, 211 of 211), the SNPs/mutations stud-

Table 1. Example of all data generated by the analysis software for a heterozygous, a homozygous wild-type, and a homozygous mutant sample.^a

Sample	Red	Green	Pads	Ratio (R::G)	Genotype	Background	Mean SNR	Mean	SD	CV, %	Scaled mean
H101	2.5	399.5	2	1::159.8	Wt/Wt	1.5/ 5	2.67::1/ 80.9::1	2.5/ 399.5	0/ 12.02	0/ 3.01	2.5/ 399.5
H107	183	211.5	2	1::1.16	Mut/Wt	1.5/ 5	123::1/ 43.3::1	183/ 211.5	3.54/ 12.02	1.93/ 5.68	183/ 211.5
H154	408	43.5	2	7.63::1	Mut/Mut	1.5/ 5	273::1/ 9.7::1	408/ 43.5	7.78/ 2.83	1.91/ 6.5	408/ 53.49

^a R, red (Cy5 fluorescence); G, green (Cy3 fluorescence); SNR, signal-to-noise ratio.

ied, and the comparison method. In 7% of the samples the signal-to-noise ratio was low. The software failed to assign a genotype because of a PCR failure in 5% of samples and because of an insufficient amount of PCR product in 2% of samples.

Our data are in agreement with other reports on the Nanogen technology (7, 9, 10), but our approach reduced the time for the detection of a SNP/mutation from 5 min (manufacturer's protocol) to ~0.7 min. The manufacturer's protocol required 8 h for loading of 98 PCR products, one hybridization step, one washing step, and 98 detections. The longest time (~7 h) was required to address the 98 samples to the pads. Our protocol took 7 h for the addressing of 98 samples, each containing 7 amplicons, and 8 h to perform 14 hybridizations, 14 washing steps, and 14 × 98 detections. An additional factor to be considered is the cost per assay: reagents in the protocol suggested by the manufacturer cost approximately €9 for each detection (DNA extraction, PCR, purification, chip, and probes). The simultaneous analysis of multiple SNPs/mutations on each pad provided by our protocol reduces the price to approximately €2 for each SNP/mutation. This reagent cost is similar to or less expensive than the reagent costs for the standard techniques (allelic discrimination, restriction analysis, and sequencing), with reagent cost ranges of €2–25. In addition, the shorter analysis time for our protocol reduces technician time and provides increased numbers of results over which to amortize the cost of the instrument. Finally, our protocol allows optimization of procedures in only 1 h for each SNP/mutation.

To the best of our knowledge, this is the first report describing the multiple analysis of 14 SNPs/mutations involving 5 human genes (7 amplicons) on each pad of the same microelectronic chip without affecting the accuracy of the analysis.

The easy preparation and analysis of samples, the low

cost per sample, the reduction in the time required for the analysis, and the concordance of the results with the comparison methods demonstrate that this approach improves the throughput of microelectronic chip technology. We believe that the Nanogen technology is a promising tool for molecular diagnosis, pharmacogenetics, and research.

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