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REVIEW ARTICLE

Analysis of SNPs and Other Genomic Variations Using Gel-Based Chips

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Application of microarrays for the analysis of point mutations and SNPs in genomic DNAs is currently under intensive development. Various technologies are being investigated, employing enzymatic, chemical, and physical tools [for review, see Tillib and Mirzabekov, 2001]. Our current approach is based on the use of IMAGE chips (immobilized microarrays of gel elements) consisting of an array of gel pads attached to a hydrophobic glass surface. The gel pads range in size from picoliters to nanoliters and are used for immobilization of oligonucleotide probes, as well as miniature test tubes for chemical or enzymatic reactions with tethered compounds. Nucleic acids are hybridized, fractionated, modified, and subjected to enzymatic reactions inside the pads. All steps of sequence analysis (PCR-amplification, activation or release of primers and products, DNA extension, hybridization, and reading of the results) can be performed within the same pad. A flexible and inexpensive technology platform enables one to monitor processes in the arrays in both real time and steady-state. Identification of SNPs, microsequencing, and other specific tasks are easily performed. In particular, stacking interactions with short oligonucleotides enhance the capability of high-throughput screening. The IMAGE chips can be analyzed using a variety of equipment, from a dedicated multi-color fluorescent microscope or MALDI-spectrometer to an inexpensive portable analyzer suitable for field conditions. Customized gel-based chips were successfully used for screening of SNPs in a broad range of biologically meaningful genes. Hum Mutat 19:343–360, 2002. © 2002 Wiley-Liss, Inc.

KEY WORDS: SNP; mutation detection; microarray; contiguous stacking hybridization; microsequencing; IMAGE; gel pad

DATABASES:
http://snp.cshl.org (The SNP Consortium)
www.ncbi.nlm.nih.gov/SNP/ (dbSNP)

INTRODUCTION

The availability of the complete sequence of the human genome calls for large-scale investigation of its diversity. In humans and other organisms, single-nucleotide polymorphisms (SNPs) are the most frequent (about one per kilobase in the human genome) and evenly spread [Kruglyak, 1997]. The initial identification of SNP, as well as other polymorphisms applicable in genomic research, is usually achieved by traditional sequencing. Further screening of these polymorphisms is based in most cases on the use of DNA microchips—arrays of oligonucleotide probes attached to solid support.

Screening of mutations using microchips consists of three major steps: preparation of target sample (performed in most cases by PCR amplification of a short fragment containing the polymorphism(s) of interest); interrogation of the target sample; and analysis of the results [Gut, 2001]. The interrogation step is based on hybridization of the target sample to immobilized probes with further discrimination between

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perfect and imperfect match either by direct quantification of the hybridized probe, or by some form of enzymatic treatment.

Commercially available arrays are usually manufactured by immobilizing DNA probes on nylon membrane (macroscopic design) or glass (microscopic design) [for review, see Schultze and Downward, 2001]. The analysis is performed by hybridization of liquid sample to the probes arranged in a two-dimensional monolayer. Although it proved to be advantageous for high throughput applications, the two-dimensional arrangement of probes has its limitations: enzymes do not always work on flat microchips; the concentration of the probe and, therefore, sensitivity, are relatively low; the use of short probes with the highest discrimination capacity is difficult; technology is rather complex and costly. Therefore, we developed microarrays of three-dimensional gel elements based on an alternative approach to study interactions between immobilized and dissolved molecules.

ARRAYS OF IMMOBILIZED COMPOUNDS AND NANOLITER TEST TUBES

We started the development of oligonucleotide microchips in the Engelhardt Institute of Molecular Biology in Moscow in 1988 [Lysov et al., 1988] and demonstrated that polyacrylamide gels can be used as convenient support for oligonucleotides [Khrapko et al., 1989]. In 1995–2000, we organized a joint multidisciplinary biochip program with Argonne National Laboratory (USA). All these efforts resulted in the development of a gel-based chip technology platform.

The chip consists of a glass slide with gel pads attached by chemical bonds to its surface [Yershov et al., 1996]. Typically, we use 0.2 nL gel pads ($100 \times 100 \times 20$ μm) separated by 200 μm space on a glass slide 18×21 mm. Chips carrying up to 4,200 (60×70) gel pads have been manufactured [Drobyshev et al., 1999]. The technologies of manufacturing the pads as small as $10 \times 10 \times 5$ μm [Vasiliskov et al., 1999], as well as using more porous and chemically stable gel support than polyacrylamide [Arenkov et al., 2000] have been developed.

The glass surface between the pads is hydrophobic, and the content of individual pads is completely isolated when the chip is either exposed to the air or covered with a water-insoluble solvent. Therefore, each pad behaves as an individual nanoliter or smaller test tube. This is important for pad-specific reactions such as chemical immobilization of individual compounds within each gel pad, PCR-amplification, or detachment of oligonucleotides.

Tethering the molecules within a three-dimensional gel significantly, 10^2 to 10^3 times, increases the immobilization capacity as compared with equivalent glass surface. Actual concentration of immobilized oligonucleotides within the pads can reach 10 mM [Yershov et al., 1996]. Interactions of macromolecules with gel-immobilized compounds occur in the surrounding homogeneous liquid, and the physics of these processes is more similar to water solution than heterophase interactions with glass-immobilized compounds [Forman et al., 1998].

MANUFACTURING

Our protocol to produce the first generation of gel-based microchips consisted of five steps [Khrapko et al., 1991]. The micromatrix of square gel pads (blank microchip) was manufactured by photoinduced polymerization of monomer solution, usually acrylamide and bisacrylamide. The mold consisted of an assembly of a glass slide, Teflon spacers, and a quartz mask with 1-μm-thick chromium film. The film was nontransparent and had windows in the shape of gel pads. The assembly was illuminated with UV-light to induce polymerization, and the microchips were briefly treated with Repel-Silane to render the space between the pads hydrophobic. These chips can be dried and stored.

The gel pads of a blank microchip were activated to generate reactive chemical groups, usually aldehyde or hydrazide ones, for chemical coupling [Timofeev et al., 1996; Proudnikov et al., 1998]. Then oligonucleotides or DNAs containing complementary chemical groups (amino- or aldehyde) were applied to each gel pad by a robot and the coupling reaction took place in the air. Finally, aldimine bonds between the gel and oligonucleotides were stabilized by reduction. Reducing compound could be delivered to the pads by covering the chip with chloroform containing the agent.

This method is rather inefficient for mass production. First, it is technically difficult to apply nanoliter drops on gel pads with high enough precision; second, the compounds are applied on the surface of gel pads and tend to be immo-

bilized there, instead of being evenly distributed throughout the volume of the pads.

The second generation of the microchips employs the reaction of copolymerization performed in three steps [Vasiliskov et al., 1999; see also Rehman et al., 1999]. A solution of monomers is applied by a robot on a glass slide. This solution also contains the compound to be immobilized in the pad modified by attaching a monomer group similar to one of the components of the gel itself. The monomer can be incorporated into oligonucleotides during their synthesis, or it can be attached to DNA or protein molecules in a separate chemical reaction. The copolymerization of free monomers with a monomer attached to the compound of interest is induced by UV-irradiation or by a chemical initiator.

A simpler two-step procedure has been developed to satisfy the needs of mass production of custom oligonucleotide and protein microchips even in small academic laboratories. To manufacture these so-called IMAGE chips (immobilized micro arrays of gel elements), the copolymerization procedure is carried out without a casting mold. Photoinduced reaction takes place in drops applied by a robot on a pre-treated hydrophobic glass surface. The resulting semispherical pads are approximately 80 μm in diameter and 0.1 nL in volume (see below).

Three different robots have been tested for the production of gel-based microchips. A custom-built model [Yershov et al., 1996] and a piezoelectric dispenser (Packard BioScience, Meriden, CT) are suitable for all immobilization methods. A high output four-pin robot (Affymetrix, Santa Clara, CA) works well when the drop-gel polymerization method is used.

As discussed above, specific groups have to be introduced in DNA molecules to attach them to the gel. Alternatively, PCR-amplified DNA can be prepared using synthetic primers already carrying such groups.

In many situations, the only way to deliver substances into the pads is by diffusion. Standard gels seem to be permeable for DNA up to 150 to 200 bp long, and porous gels up to 400 bp. A systematic study of the permeability of the last generation gels is under way. These gels allow for more efficient diffusion and amplification and are stable enough to sustain repetitive thermal cycling [Strizhkov et al., 2000; Tillib et al., 2001].

Chemical or enzymatic reactions can be carried out simultaneously within all gel pads on a microchip, or in each gel pad separately. In the first case, the microchip is covered with reaction solution and reactants diffuse into the pads [Dubiley et al., 1997]. In the second case, wet gel pads are separated from each other by air or water-insoluble solvents [Vasiliskov et al., 1999; Tillib et al., 2001]. Reagents can be applied to each pad by diffusion [Timofeev et al., 1996; Tillib et al., 2001] using a robot pin or piezoelectric dispenser.

The amount of probes bound within the gel pads can be determined by staining with specific dyes. For instance, oligonucleotides can be quantified using SYBR Green dyes (Molecular Probes, Eugene, OR). The resulting image can be stored as a quantitative reference to normalize hybridization signals [Battaglia et al., 2000].

COMMERCIAL DEVELOPMENT OF GEL-BASED MICROCHIPS

The technology of gel-based microchips is currently being developed by several companies. In 1998, Motorola Corp. founded the Biochips Systems subsidiary in partnership with Packard BioScience Co., and licensed the rights to use our chip technology from Argonne National Laboratory (Chicago, IL) and Engelhardt Institute of Molecular Biology (Moscow, Russia). Packard brought to this collaboration their unique non-contact piezoelectric technology to load the probes on the chips. Pilot commercial chips consisted of a continuous layer of Packard's proprietary HydroGel™, not separate pads of polyacrylamide. Since then, Motorola acquired access to a number of chip-manufacturing and processing technologies through licensing, partnerships, and acquisitions including collaborations with SurModics (Eden Prairie, CA) that is using for its chips a proprietary three-dimensional gel and photo-activated amino-linkage of the probe (PhotoLink); NEN, whose AcycloPrime™ reagents will become part of the future bioarray system for SNP analysis using fluorescence polarization technology; Axon Instruments Inc. (Union City, CA), which provides optical scanners; Compugen (Tel-Aviv, Israel), which specializes in chip design and analysis software; and Iconix Pharmaceuticals (Mountain View, CA), which develops and implements database software for mass genomic applications. Motorola was the

first non-pharmaceutical corporation to join the so-called SNP Consortium to study human genetic diversity and create a public database of polymorphisms (http://snp.cshl.org).

Another corporate entity that offers gel-based chips and related reagents on a commercial basis is Mosaic Technologies (Waltham, MA). They also offer a pre-fabricated chip for bacterial screening of platelets used in clinical transfusions. The technology employs electrophoresis-enhanced hybridization and immobilization of acrylamide-modified oligonucleotides [Rehman et al., 1999].

DATA ACQUISITION: FLUORESCENT MONITORING

Our current technology is based on the labeling of target molecules with fluorescent dyes. There are many advantages of using fluorescent detection: high sensitivity and spatial resolution, the possibility of real-time monitoring, wide dynamic range of signal, the possibility of using several dyes in one hybridization on a single microchip, and laboratory safety.

There are several alternative ways to attach fluorescent moieties to nucleic acids [Proudnikov and Mirzabekov, 1996]. One procedure is based on the generation of aldehyde groups in DNA by partial depurination or in RNA by 3′-terminal oxidation, in which fluorescent dyes carrying hydrazine group are coupled to the aldehydes, and the bond is further stabilized by reduction. In another scheme, DNA is cleaved at depurinated sites with ethylenediamine, the aldimine bond is stabilized by reduction, and the introduced primary amine group is coupled with isothiocyanate or succinimide derivative of the dye. Our newer approach is based on simultaneous fragmentation of DNA and coupling of a fluorescent dye by treatment of DNA with *bis*-(*o*-phenantroline)-copper (I) complex [Bavykin et al., 2001].

The choice of the fluorescent labels is determined by the design of the study, especially in thermodynamic analysis. The intensity of ideal labels should not change too much with temperature, and emission spectra should display minimal overlapping for simultaneous processing of several labels. In addition, some conjugated fluorescent compounds were found to affect the stability of duplexes. Taking into account these considerations, we compared a number of widely used fluorescent labels [Kotova et al., 2000a,b]. The fluorescence intensities of Texas Red (TR), Bodipy-FL (FL), and tetracarboxyphenylporhyn (TCPP) proved to be the least dependent on temperature. A combination of up to four labels such as TCPP, FL, TR, and Cy5 can be used on the same chip. At the same time, TR and TCPP increase melting temperatures of perfect duplexes, probably because of their intercalating ability, and therefore, experiments with these labels must include carefully designed controls [Barsky et al., 2002].

One of the major advantages of the gel chip technology is a unique opportunity to follow reactions in individual gel pads in real time. Our set of equipment to perform this task includes a specially designed fluorescent microscope with wide field (12 mm in diameter), long focus, and high-aperture objective [Barsky et al., 1998]. A high-pressure mercury lamp is the source of epi-illumination. The microscope works at up to four wavelengths simultaneously [Barsky et al., 2002]. We use routinely fluorescein, TR, and tetramethylrhodamine derivatives. The microscope is equipped with charge-coupled device (CCD) camera, controllable thermal table, and proper computer software [Mikhailovich et al., 2001; Fotin et al., 1998]. This setup enables us to monitor the reactions in individual pads at precisely regulated temperatures. In addition, we built a simple, inexpensive, large-field fluorescence microscope in which the microchip is illuminated by a laser from beside rather than through the objective.

For conventional end-point measurements of dried microchips, we use a commercial laser scanner (General Scanning Inc., Bedford, MA). It provides higher sensitivity [Lipschutz et al., 1995] and dynamic range than the fluorescence microscope, but does not allow for real-time measurement, which sometimes is necessary for the discrimination of point mutations (see below). Generally, laser-scanning platforms offer low background and excellent uniformity of fluorescence excitation and detection. However, most commercial scanners lack such features as temperature control of the sample and are closed-architecture instruments designed to readout surface-immobilized microchips. Because of these limitations, we developed a customized scanner with a 2 mW HeNe laser with excitation wavelength of 594 nm matching the absorption of TR. A low-noise PIN photodiode

is used as a detector. Working distance of a miniature objective lens is 3 mm, long enough to scan wet chips without disassembling the hybridization chamber [Barsky et al., 2002].

In principle, because of the very high concentration of the probe and, therefore, reaction products in the gel pads, accumulation of fluorescent label is sufficient to acquire data without removing the buffer solution. However, for most routine measurements it is preferable to suppress the background by briefly washing microchips. For some experiments, the microchip is subjected to a low-voltage electrophoresis for a few minutes before measurements to further diminish the background [Dubiley et al., 1999].

PORTABLE ANALYZER

The design of most setups for the identification of DNA polymorphisms requires expensive and bulky instruments for data acquisition. In some cases it becomes a major shortcoming, for instance, in field conditions or for clinical applications in developing countries. To overcome this hindrance, we developed a simple portable fluorescent analyzer [Bavykin et al., 2001; Barsky et al., 2002]. It is powered by batteries and combines a laser-diode with a Polaroid camera or a digital camera and a wide-field high-aperture objective lens at a total cost of under $2,000. The camera has sufficient resolution to capture a pattern of hybridization on a dried chip containing up to 180 individual probes. In pilot experiments these chips reliably identified "life" in general, eubacteria as a group, and discriminated between bacteria from *B. subtilis* and *B. cereus* groups.

This technology is especially handy for rapid diagnostics of TB resistance. Working toward this goal, Mikhailovich et al. [2000, 2001] developed a simple reliable chip that can be analyzed by mere visual inspection.

DATA ACQUISITION: MALDI MS

When the hybridization of target probes with biochips is analyzed by fluorescent readouts, the resulting patterns are indiscriminate. Monitoring the hybridization results of individual elements on a chip using matrix-assisted laser desorption ionization mass-spectrometry (MALDI MS), on the contrary, yields important information on the nature of the molecules bound in individual elements. In addition to precise identification of bound compounds, MALDI spectrometers are potentially high-throughput devices. Taking into account the large capacity of gel pads as compared with solid-phase immobilization, we investigated the possibility of analyzing pentamers bound to target sequences within the gel pads by MALDI MS [Stomakhin et al., 2000]. The binding of the 5-mers with the existing partially single-stranded structure, the so-called contiguous stacking hybridization (CSH; Fig. 1A), is defined by the interaction between the terminal bases of incoming oligonucleotides with the already hybridized oligonucleotide. In their model experiments, Stomakhin and his coworkers [Stomakhin et al., 2000] analyzed a 28-nucleotide long sequence. Ten gel pads ($1 \times 1 \times 0.02$ mm) were placed in a linear arrangement on an electrically conductive silicon chip. They contained immobilized staggered 8-mers complementary to the queried sequence. After the primary hybridization of the 28-mer to the array, a secondary hybridization was carried out with a mixture of 10 5-mers that extended the complementary segments. Bound 5-mers could be analyzed directly on the silicon chip after extraction.

Specific 5-mers contiguously stacked to 8-mers and hybridized to the target DNA were identified. In some cases, a second, weaker peak could be observed corresponding to a second 5-mer contiguously stacked to the first one. Control experiments confirmed the high specificity of the CSH. It appeared that stacking interaction between C and T is weaker than others; and that two GC-rich 5-mers can bind to target DNA by stacking to each other, even without an adjacent bound 8-mer.

Additional pilot experiments demonstrated the potential resolution of CSH in combination with MALDI MS. A single nucleotide polymorphism was created in the model 28-mer and analyzed in homozygous and heterozygous situations using large sets of 5-mers mismatched at different positions (Fig. 1B). Contiguous stacking with complementary, and only complementary, 5-mers clearly discriminated polymorphic samples, including a G-T mismatch, which is usually the hardest to identify by conventional hybridization. Different 5-mers can be labeled with mass tags of about 100 Da; this makes them better discernable by spectroscopy without compromising their hybridization ability.

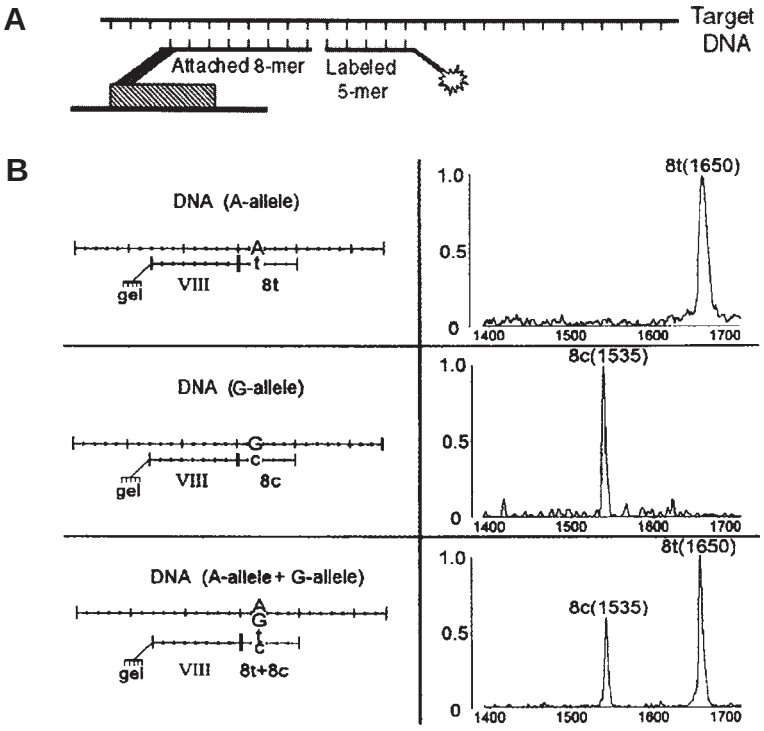

FIGURE 1. **Contiguous stacking hybridization. A: General scheme in which target DNA is hybridized with an immobilized 8-mer and a fluorescently labeled complementary 5-mer as shown. The hybridization of the 5-mer under chosen conditions is possible only as the result of stacking interaction with the adjacent 8-mer. B: Detection of model SNP in target DNA using CSH with subsequent identification of the hybridized 5-mer by MALDI MS. On the left: scheme of hybridizations; on the right: mass-spectrum of the bound 5-mers. From top down: two homozygous target samples and their heterozygous mixture. Modified from Stomachin et al. [2000].**

DETECTION OF POLYMORPHISMS: HYBRIDIZATION

Generally, the goal of hybridization on microchips is the discrimination between perfect and mismatched duplexes. The theory of hybridization of nucleic acid to gel-immobilized oligonucleotides has been developed [Livshits and Mirzabekov, 1996]. The efficiency of the discrimination depends on the position of the mismatch within the probe, the length of the probe, its AT-content, and reaction conditions. Central mismatches are easier to detect. The shorter the probes, the higher the discrimination potential between matched and mismatched duplexes. At the same time, overall duplex stability decreases for shorter probes, and eventually becomes too low for routine use. As a result, the choice of diagnostic oligonucleotides is a trade-off between discriminatory power, on one hand, and reasonable hybridization conditions and signal/noise ratio, on the other. Reliable discrimination of perfect duplexes formed by 15 to 20-mers with a single mismatch at a central position can be usually achieved [Drobyshev et al., 1997; Mikhailovich et al., 2001].

Another important consideration is the dependence of T_m on the concentration of the test sample. The higher its concentration, the more thermodynamically favorable is the binding, and the higher the T_m. At saturating concentration of the test probe, no discrimination between matched and mismatched duplexes is possible at low temperature, and it is necessary to approach T_m to discriminate. On the other hand, at low concentration of the test probe the discrimination is possible at low temperature, but cannot be achieved at higher temperature—because of probe dissociation and weakening of signal. Again, the optimization of the test probe concentration is a trade-off between discriminatory potential and sensitivity.

The concentration of the immobilized species does not affect the melting temperature, though it does affect the kinetic of association–dissociation of the duplexes (Khomiakova et al., unpublished). This statement is valid as long as the

quantity of the target sample significantly exceeds the quantity of the immobilized probe, which is always true because of the relatively large volume of the hybridization solution. The reasoning above assumes that the hybridization is carried out at thermodynamic equilibrium. In practice, kinetic factors can enhance the discrimination even when it is unfavorable thermodynamically. For instance, extensive washes after the completion of hybridization reduce the mismatched signal dramatically because of faster dissociation of imperfect duplexes.

In addition, we found that the dissociation of duplexes under non-equilibrium conditions depends on the concentration of immobilized probes. In practical terms, this means that non-equilibrium dissociation temperature, defined as the temperature at which the initial hybridization signal decreases 10-fold, can be manipulated by changing the amount of the oligonucleotide in the gel pad. We took advantage of this fact by developing an algorithm to equalize apparent dissociation temperatures of AT-rich and GC-rich duplexes. The former probes are loaded at higher concentration than the latter, facilitating the discrimination on the same chip in similar hybridization conditions [Livshits and Mirzabekov, 1996].

Fotin et al. [1998] compared the thermodynamics of duplexes in gel and in solution and found that, although they were qualitatively similar, melting temperatures of the same duplexes in gel were almost 20°C lower than in solution. We ascribed this difference to high concentration of amide groups in gel matrix, similar to the destabilizing effect of urea. Our latest generation chips employ different gel chemistry [Arenkov et al., 2000], and the difference in melting temperatures in these gels does not exceed 5–6°C.

The length of the linkers between the gel and the oligonucleotides appeared to have no significant effect on duplex stability. The role of the linker in hybridization on solid surface was thoroughly studied by Shchepinov et al. [1997], who concluded that variations in the linker length result in more than 100-fold difference in the efficiency of hybridization. The optimal length has been found to be roughly 30 to 60 atoms and was greatly affected by the chemical nature of the linker. Although the effect of linker length on the hybridization of gel-immobilized oligonucleotides has not been studied in detail, we suggest that it is minimized by the flexibility of the gel matrix itself. In addition, the chemistry of the gels is optimal for efficient hybridization according to the findings of Shchepinov and his coworkers [Shchepinov et al., 1997]: they are highly amphiphilic and not charged.

The choice of oligonucleotides for interrogation is crucial, especially for discrimination of single nucleotide mismatches. The process of selection can be exemplified by the work of Guschin et al. [1997] and Mikhailovich et al. [2000, 2001] on the development of a microchip for the identification of microorganisms and their mutations. The probe sequences were selected using original software that tested each potential probe against all available 16S rRNA and mutated sequences by a function that estimated relative duplex stability according to the number and position of mismatches. If the 16S rRNA of any microorganism outside the genus of interest or a known mutated sequence formed stable duplexes with any of the potential probes, this oligonucleotide was excluded from the set. Other considerations are similar to the selection of PCR primers: convenient length and, accordingly, melting temperature; average GC-content; and no internal complementarity that could promote secondary structure and reassociation in solution.

Identification of Viruses

Viruses in clinical or industrial settings are often identified by hybridization on microchips. One important application of this approach is the detection of orthopoxviruses.

Although eradication of smallpox virus was declared in 1980, the virus itself and a related group of orthopoxviruses (variola, monkeypox, camelpox, cowpox, and others) remain a source of concern. First, stocks of the smallpox virus have been preserved for experimental use and can, in principle, escape. Second, dwindling acquired immunity in the world population may result in infection by related viruses. Third, smallpox and related viruses can be used as biological warfare. With this in view, we developed a chip able to identify several major orthopoxviruses [Lapa et al., 2001]. Oligonucleotide probes hybridize to a species-specific segment of the gene CrmB encoding the viral analog of TNF-receptor. The 14- to 16-nucleotide-long probes with the variable position located near

the middle were designed in such way that their melting temperatures for perfect duplexes fall within the range of 3–4°C. The resulting microchip contains 15 oligonucleotide probes and enables one to identify six viral species by taking a glance at the fluorescent pattern. Importantly, the hybridization on the microchip could be performed within a rather broad range of temperatures, thus further enhancing its applicability in field conditions.

Identification of Nitrifying Bacteria

Biochip technology may be especially important for the identification of those bacteria that do not propagate easily in the lab. For this reason, a chip has been designed to identify members of different groups of nitrifying bacteria [Guschin et al., 1997]. Oligonucleotides were chosen to recognize segments of small (16S) ribosomal RNA from several groups of nitrifying bacteria, as well as segments common to all bacteria, common to almost all living organisms, and an anti-sense control. Nine oligonucleotides ranging from 15 to 20 nucleotides in length were immobilized in polyacrylamide pads. RNA samples obtained in vitro after PCR-amplification of ribosomal RNA provided better discrimination than the amplified DNA samples or non-amplified RNA isolated from bacteria, although all of these probes worked. RNA samples from "unknown" bacteria were labeled with fluorescein and mixed with RNA from *E. coli* labeled with tetramethylrhodamine as an internal control for two-color detection.

Identification of Thermophilic Prokaryotes and Archea

In a similar work, gel-based chip technology was successfully used for the identification of thermophilic bacteria and archea in samples from Siberian high-temperature petroleum reservoirs [Boulygina et al., 2000]. In this case, oligonucleotide probes were selected from the sequences of small ribosomal 16S-RNA genes based on the following criteria: all of them were 20-bases long, had average GC-content, and had maximum level of specificity when compared to a complete set of known 16S-sequences. The diagnostic chip consisted of 40 pads to identify 10 genera. Segments of 16S genes were amplified by PCR with universal primers, and then RNA-probes were generated by in vitro transcription from an attached promoter, fragmented, and labeled with TR. Identification of microorganisms by hybridization to the chip was in good agreement with other methods (microbiological isolation and/or sequencing of DNA).

Detection of Antibiotic Resistance

We invested significant effort in the development of chip-based diagnostics of antibiotic resistance in the tuberculosis pathogen *Mycobacterium tuberculosis*. Using conventional bacteriological methods, it takes six to eight weeks to determine whether newly diagnosed cases are antibiotic-resistant. DNA-based diagnostics provides a quick alternative to traditional methods.

The most frequent rifampicin-resistant mutations Rif^R in the *rpoB* gene encoding β-subunit of RNA-polymerase are well known [Musser, 1995]. We developed customized chips consisting of 42 oligonucleotide probes to identify 30 clinically important mutations [Mikhailovich et al., 2001]. DNA for these tests was PCR-amplified and then fluorescently labeled. The hybridization was performed in the presence of 1 M guanidine thiocyanate to bring down the optimal temperature of the process to 37°C. In total, 90 pure mycobacterial cultures and 28 clinical samples, all confirmed Rif^R, were tested. One sample displayed no mutations either tested on the microchip, or after direct sequencing of the *rpoB* gene. It is known that in a small percentage of Rif^R mutants *rpoB* gene has no mutations. Otherwise, there was full concordance between the results of hybridization on the chip and conventional susceptibility test. In addition, direct sequencing of 19 samples confirmed the identity of mutations.

Beta-thalassemia Mutations

Beta-thalassemia is a group of genetic hematological diseases associated with various mutations in globin genes (see HBB, MIM# 141900). Drobyshev et al. [1997] applied a gel-based chip for the analysis of some of these mutations. The chip carried an immobilized set of 10-mers representing a selection of known point mutations. The 10-mers occupied all possible positions relative to mutations. Nineteen-mer oligonucleotides corresponding to wild-type and mutant sequences were used as model probes. The actual probes were generated by PCR-amplification of potentially mutant fragments of genomic

DNA using primers carrying an RNA-polymerase promoter. Then an RNA-probe was synthesized in vitro and labeled with fluorescent tags. Melting curves were determined in non-equilibrium conditions for all duplexes formed in gel pads after hybridization. The shorter the probe, the faster it reached equilibrium; thus the melting of 19-mers was close to equilibrium curves under the conditions used.

While 19-mer oligodeoxynucleotides reliably identified point mutations located in positions 3 through 8 within the decamers, the results obtained with riboprobes were more complex: only mutations in the middle of the 10-mers (positions 4, 5, 6) provided sufficient discriminating power. The signal strength was five times higher and hybridization time decreased from several hours to tens of minutes with randomly fragmented riboprobes as compared to intact 75- or 133-nucleotide-long probes, due to the difference in diffusion rate.

Muopioid Receptor

Clinical studies suggest that human mu-opioid receptor (MOR or OPRM1; MIM# 600018) gene may contribute to the heritable component of addiction or sensitivity to addiction. In particular, two point mutations in MOR, 17 C>T and 118 A>G, occur at high allelic frequencies and affect the properties of the receptor in vitro. We developed custom reusable chips to query these polymorphisms by hybridization and by in-gel single nucleotide extension, and verified the outcomes by sequencing [LaForge et al., 2000]. For hybridization, the base call oligonucleotides were 11 to 12 bases long with the mutant nucleotide in the middle. The hybridization samples were prepared by PCR-amplification of the segments of interest, in vitro transcription of RNA, its fragmentation, and fluorescent labeling with TR.

All 35 samples analyzed gave perfect concordance between the results of direct sequencing and hybridization to the chip. Interestingly, the ratio of signal intensity for matched and mismatched samples depended on the allelic composition of the sample. The difference was 10-fold for 118A homozygote, three-fold for 118G homozygote, and fluorescence of corresponding pads was nearly equal for heterozygotes.

For extension analysis, a set of four 26-mers with all possible nucleotides at their 3′-end was prepared and immobilized in gel pads to query the 17 C>T mutation. They were hybridized with the corresponding PCR product and extended using fluorescein-labeled dideoxynucleotides. Again, there was full concordance between this minisequencing procedure and prior sequencing data.

Chromosomal Rearrangements

Hematological malignancies are often accompanied by specific rearrangements of chromosomes. More than 50 frequently occurring translocations are known, and many of them have significant importance in defining clinical prognosis and therapeutic strategy. Traditionally, they were identified by cytogenetic analysis, a labor-intensive procedure of limited reliability. Lately, RT-PCR followed by Southern hybridization of the products became the method of choice for the diagnostics of rearrangements. Taking into account the large number of known rearrangements and rather tedious procedures involved, it is highly desirable to develop microchip-based technology for the diagnostics. As the first step toward this goal, we developed gel-based microarrays able to identify four translocations by hybridization to a limited set of immobilized oligonucleotides (Nasedkina et al., 2002).

The immobilized probes were designed to span the rearrangements chosen for pilot experiments. They included three known variants of *BCR-ABL* fusion [t(9;22)], three variants of *MLL-AF4* fusion [t(4;11)], two transcripts resulting from *PML-RAR* alpha fusion [t(15;17)], and two types of *TEL-AML1* fusion [t(12;21)]. Several cell lines with known rearrangements were used in preliminary experiments. Gel pads carrying an oligonucleotide complementary to wild-type *c-ABL* transcript served as positive controls. Diagnostic oligonucleotides were 25 nucleotide long and used in pairs to complement both sense and anti-sense strands of the rearranged RNA. Surprisingly, the intensity of signals differed for complementary oligonucleotides. Additional experiments suggested that this might be caused by preferential amplification of one strand during PCR.

The probes were synthesized by nested RT-PCR procedure. In 20 cases out of 21 clinical samples analyzed, results were in full concordance with the conventional analysis. The ratio

of fluorescence intensity of positive signals to negative controls varied from three to 20 for different probes, i.e., was always sufficient for unequivocal interpretation.

The experiments could be performed either separately for each translocation, or by multiplex PCR for all studied translocations in a single tube. The former approach gave somewhat better results.

DETECTION OF POLYMORPHISMS USING ENZYMATIC REACTIONS

Amplification Over and Within a Chip

Components of PCR amplification mix can be delivered to gel pads by diffusion, and the specificity of the reaction in each individual pad can be defined by the immobilized primer. In one variation of the amplification on a chip, the reaction is carried out simultaneously inside the pads and in the reaction mix covering the pad [Strizhkov et al., 2000]. The pads contain immobilized forward primers, and the mix contains TR-labeled reverse primers. The addition of free forward primers to the mix increases the efficiency of amplification. This setup enabled us to monitor the kinetic of the reaction in real time.

A number of variations of the on-chip amplification protocol were tested. The diffusion rate of the product is an important limiting factor in the efficiency of PCR. It is advantageous to carry out initial amplification of relatively long fragments that remain mostly in solution, and then to use nested primers, one of which is anchored inside the gel pad, to generate a shorter, fast-diffusing product. The whole analysis, including hybridization, can be performed on 10^2 to 10^3 molecules of DNA in two hr. The accuracy of the reaction was further corroborated by the hybridization of the immobilized products with internal fluorescent probes and by the analysis of melting curves of the hybrids. The efficiency of amplification calculated by averaging the logarithmic part of the amplification curve is 1.89 per cycle for both 103-bp- and 248-bp-long fragments, i.e., close to theoretical maximum.

This method can be used for the detection of SNPs. In this case, a discriminating primer with allele-specific 3′-end should be present, and Stoffel-fragment of Taq-polymerase should be used instead of the intact enzyme to avoid proofreading activity during the amplification. The ratio of positive-to-negative signal in our experience exceeds five, and the best discrimination can be achieved by comparing real-time amplification curves [Strizhkov et al., 2000]. Porous gel pads provide higher intensity and more even distribution of the fluorescent signal throughout the gel pad as compared with the standard 5% gel pads that remain fluorescently labeled mostly along the edges due to the limited diffusion of the amplification products.

PCR With Detachable Primers

The most promising approach to integrated analysis of PCR products is on-chip amplification of target DNA. The major obstacle to this procedure is that amplification is inhibited if both forward and reverse PCR primers are tethered to the gel. We use two different protocols to overcome this limitation.

In the first, we carry out the PCR amplification simultaneously in solution over the microchip in the presence of both unbound primers and within the gel pads containing one immobilized and one soluble diffused primer. This method was successfully applied to identify Rif^R mutations in *M. tuberculosis* and to discriminate between Shiga- and Shiga-like toxin genes [Strizhkov et al., 2000]. To carry out the former task, it sufficed to analyze a single 81-bp-long amplicon since all 39 known mutations are localized within this region [Musser, 1995]. DNA from clinical samples of sputum was amplified to yield a 123-bp fragment. This fragment was annealed to a set of 16 immobilized primers corresponding to mutations most frequently found in the Moscow region. A number of other primers were added as controls, and the annealed product was extended and amplified using nonproofreading Stoffel fragment. Real-time recording of the stability of duplexes was especially informative for unambiguous identification of mutations. The whole procedure is inexpensive, sensitive enough to analyze 10^3 cells, and takes 1.5 hr.

Using the same approach, Strizhkov et al. [2000] studied Shiga and Shiga-like toxin genes of *Shigella dysenteriae* and *Escherichia coli*, which are often responsible for deadly gastrointestinal infections. These genes differ by four point mutations. Primers were designed to identify these mutations, immobilized in gel pads, and in-gel

PCR amplification was carried out to discriminate the mutant forms.

Rapid identification of toxin genes of *Bacillus anthracis* acquired special importance after recent bioterrorist attacks. Biological action of *B. anthracis* is determined by the presence of two genes, lethal factor (*lef*) and protective antigen (*pag4*). The test pads of the diagnostic microchip contained immobilized forward primers. The real-time monitoring of the reaction demonstrated exponential accumulation of products after 23 to 25 cycles of on-chip PCR with no signal in the control pads [Gryadunov et al., 2001].

Allele-Specific Single Base Extension

SNPs and other polymorphisms can be revealed by a combination of PCR amplification in a tube with on-chip single base extension. In this protocol, single base extension analysis is carried out inside the gel pad using thermostable DNA polymerase. In principle, two approaches to the discrimination of a single 3′-terminal match/mismatch are possible (Fig. 2).

In the first, multibase protocol, the 3′-end of the primer precedes the polymorphic nucleotide; then it is necessary to determine which of the four possible ddNTPs could be incorporated at the end of the primer after its annealing to target DNA. To perform this procedure, either all four ddNTPs must be differently labeled (Fig. 2A), or four separate reactions on four microchips must be performed (Fig. 2B).

In the second, multiprimer protocol, four different primers must be prepared differing at their 3′-ends only. After annealing to target DNA, a labeled mix of all four ddNTPs is added; only the matching primer can be extended. In this case, the reaction can be carried out on a single microchip (Fig. 2C).

The protocols outlined above, multiprimer

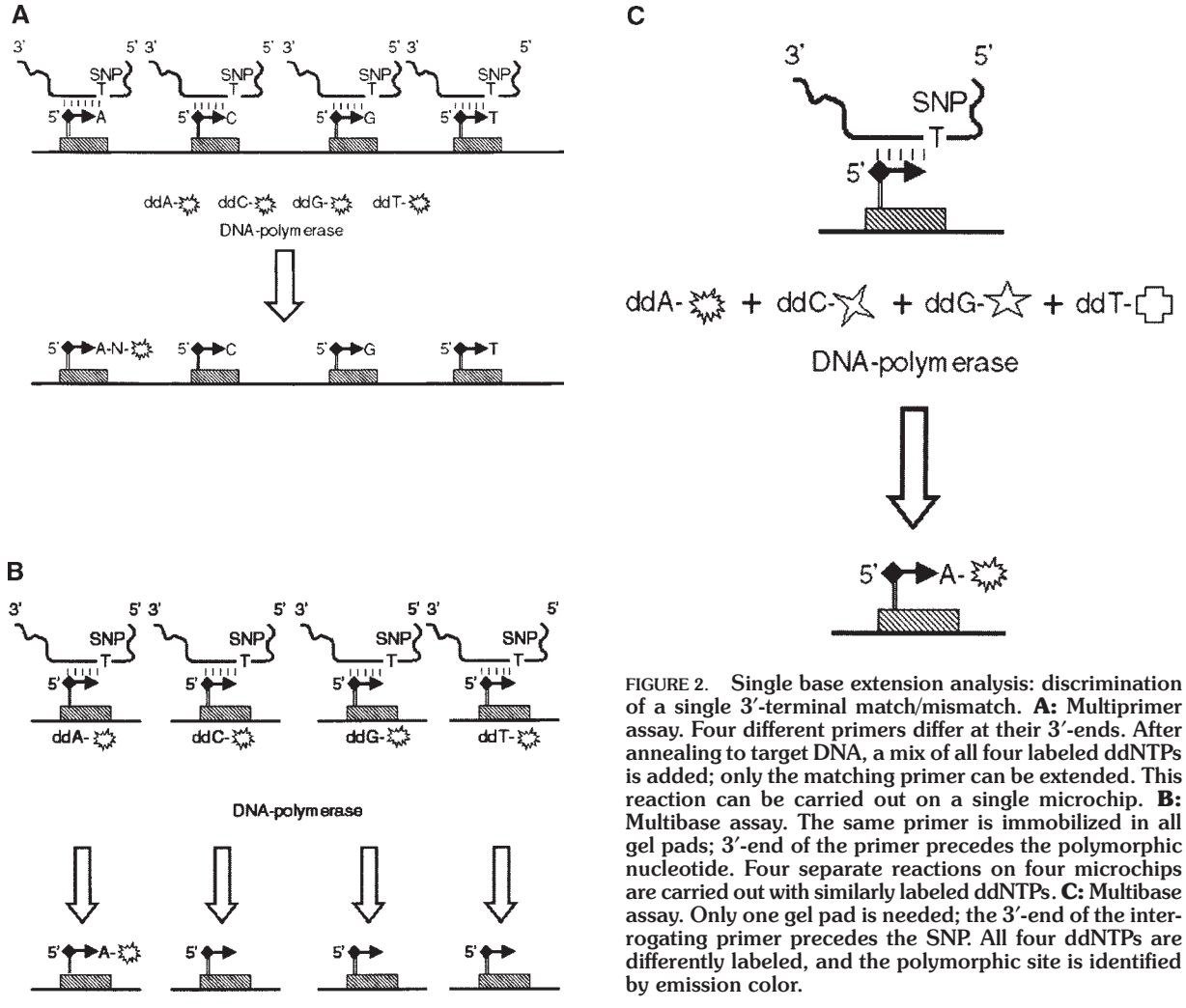

FIGURE 2. **Single base extension analysis: discrimination of a single 3′-terminal match/mismatch. A: Multiprimer assay. Four different primers differ at their 3′-ends. After annealing to target DNA, a mix of all four labeled ddNTPs is added; only the matching primer can be extended. This reaction can be carried out on a single microchip. B: Multibase assay. The same primer is immobilized in all gel pads; 3′-end of the primer precedes the polymorphic nucleotide. Four separate reactions on four microchips are carried out with similarly labeled ddNTPs. C: Multibase assay. Only one gel pad is needed; the 3′-end of the interrogating primer precedes the SNP. All four ddNTPs are differently labeled, and the polymorphic site is identified by emission color.**

and multibase extension, enabled us to detect nucleotide polymorphisms in beta-thalassemia patients and in Anthrax toxin genes [Dubiley et al., 1999]. In both protocols single base extension reactions are performed isothermally, at elevated temperatures. In these conditions, the duplex between the target DNA and immobilized primer undergoes repetitive association and dissociation, resulting in the amplification of the signal within individual gel pads up to 10-fold.

The conditions of the reaction were optimized for ThermoSequenase (Amersham, Piscataway, NJ). Primers from 17 to 20 nucleotides long were tested. Although optimal temperature proved to be the same for all of them (66°C), the signal was stronger for longer primers. The discrimination of the terminal nucleotides by both methods was better than 50-fold as measured by the ratio of fluorescent signals from gel pads containing matched vs. mismatched single-stranded probes. Most importantly, double-stranded samples of target DNA could be confidently discriminated as well, although the corresponding fluorescent signals are approximately five times weaker due to extensive reassociation of the strands. Comparison of multibase and multiprimer assays performed with the same DNA samples showed similar discrimination efficiency.

ThermoSequenase is sensitive not only to mismatches located at the very 3′-end of the primer, but also to mismatches within the preceding five or so base pairs. Therefore, discrimination of two or more SNPs located within a few positions from each other requires special precautions in designing the interrogating oligonucleotides. One possibility is to use oligonucleotides with universal bases at those positions that are not being called, another is to design non-overlapping oligonucleotides to call adjacent SNPs in the opposite DNA strands.

The efficiency of the IMAGE chip technology platform using enzymatic detection of polymorphisms is illustrated in Figure 3. After multiplex PCR amplification in the solution covering the chips, species of mycobacteria and a point mutation in *M. tuberculosis* were identified by in-gel primer extension.

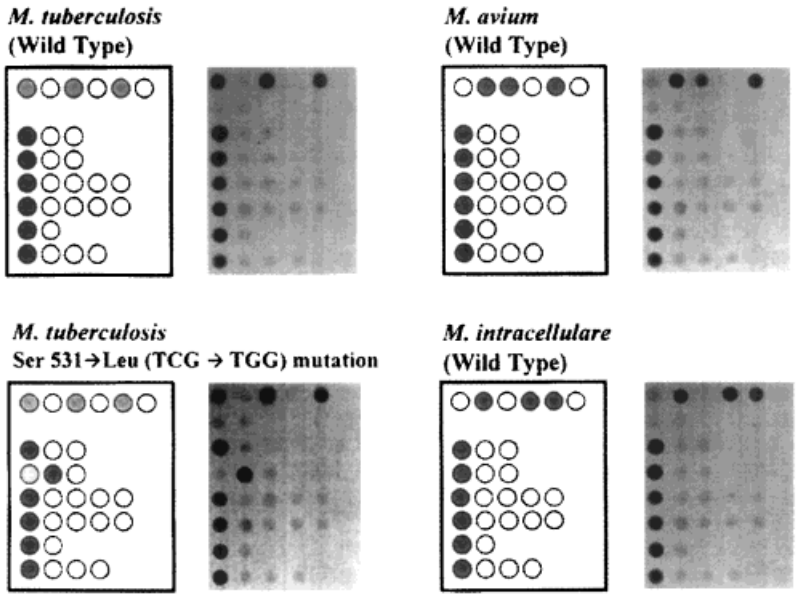

FIGURE 3. **Identification of mycobacteria using PCR amplification on IMAGE chips. The diameter of the gel pads on these test chips is 250 μM. It can be scaled down to 80 μM by using smaller pins. The top row consists of six probes that discriminate SNPs in 16S gene of different species, so that the combination of positive signals identifies the species unambiguously. The second row from the top consists of blank (control) gel pads. Each of the lower rows identifies a mutation in the rpoB gene responsible for rifampicin resistance. The leftmost gel pad in each of these rows corresponds to the wild-type sequence, while other pads contain specific probes for known mutations of a given nucleotide. Therefore, with a wild-type sample, the strongest signal in each row is the leftmost; if a mutation is present, one of the other pads is brighter than the first. A scheme next to each IMAGE chip depicts the expected result. Variation in signal intensity is the result of uniform hybridization conditions for a whole array of probes with different kinetics of association and dissociation. (Courtesy Dimitry Gryadunov, with modifications.)**

Allele-Specific Extension Using Detachable and Dormant Primers

Tillib et al. [2001] further integrated the amplification and analysis of the amplified products. The procedure is based on use of detachable primers tethered to the gel through the 5′-terminal spacer containing a triribonucleotide rUrUrC-group introduced during synthesis.

At the first step of the assay, a genomic DNA sample is fragmented to 200- to 300-nucleotide-long pieces, denatured, and diffused into the gel pads containing immobilized primers. After hybridization, the unbound DNA is washed away, while enriched target DNA remains in the gel. The gel is then equilibrated with basic components of PCR mix, briefly soaked in RNase solution, and covered with mineral oil to separate individual pads. The digestion of the spacer with ribonuclease A releases one of the primers from the gel matrix, while the second primer remains attached to the gel.

After 30 PCR cycles, on-chip amplified DNA is denatured, and all unattached DNA and released primers are washed off the gel pads. Finally, the amplification products—extended immobilized primers—are interrogated by hybridization with 5′-fluorescently labeled oligonucleotide probes. The difference in intensity between the positive signal and negative control is better than 10-fold.

Further development of this approach employs two detachable primers (forward and reverse), and a third dormant primer immobilized in the same pad (Fig. 4). The 3′-terminal nucleotide of the inner primer D is phosphorylated, which renders the primer inactive during the first amplification step. After the detachment of the first two primers and amplification, the chip is briefly treated with phosphatase, and allele-specific extension of the activated primer becomes possible. It can be used to identify a SNP in target DNA located at the 3′ end of the D-primer.

In principle, the match/mismatch at the end of the D-primer could be discriminated by single nucleotide extension with a fluorescent derivative (as shown in Fig. 2). In practice, however, this technique gives a relatively high level of false signals, and we applied the somewhat more cumbersome but more reliable approach illustrated in Figure 4. The D-primer is used for full-length extension of the annealed product. Then the pad

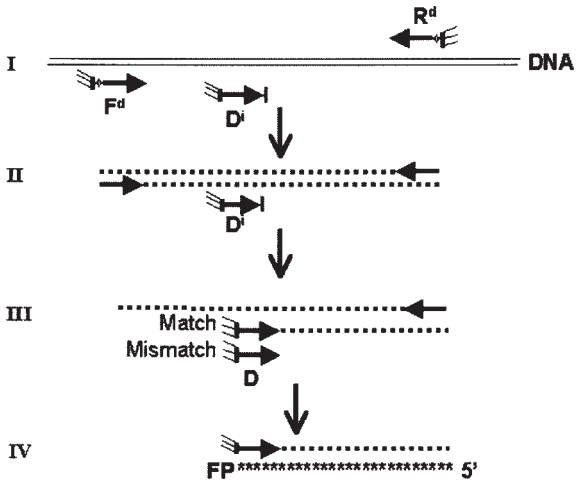

FIGURE 4. **Detachable and 3′-blocked primers. I: Sets of three primers are immobilized within each gel pad and fragmented denatured genomic DNA is hybridized with the microchip. Each primer set includes two detachable primers, forward (F^d) and reverse (R^d), and an internal dormant primer (D^i). The 3′-terminal nucleotide of the inactive dormant primer D^i in each gel pad corresponds to a specific SNP. II: The microchip is covered with oil, and both detachable primers F^d and R^d are released by treatment with ribonuclease A; PCR amplification is then carried out. III: Amplified DNA is hybridized with immobilized inner dormant primer (D^i). Dormant primers are activated by dephosphorylation, and the second round of PCR (or an extension) is carried out. Only primers D that match hybridized DNA at the 3′-terminal position are efficiently extended. IV: The yield of the extension reaction is assessed by hybridization to a fluorescently labeled region-specific probe FP. (Courtesy Sergei Tillib.)**

is cleared from all unbound materials and the extended product, anchored to the gel through D-primer, is revealed by hybridization to a fluorescent probe.

This advanced procedure was applied to analyze mutations in three genes of *M. tuberculosis*, *rpoB*, *kat*G and *rpsL*, responsible for the rifampicin, isoniazid, and streptomycin resistance, respectively. The ratio of the fluorescence intensities for allele-specific extensions of terminally matched and mismatched primers was high enough to determine the presence or the absence of the mutations unambiguously by simple visual comparison. About 10^5 *M. tuberculosis* DNA molecules can be reliably analyzed by this method. After some refinement, this technique should be sensitive enough to reveal human SNPs.

The major disadvantage of on-chip modification of immobilized oligonucleotides is its irreversible nature. Single-use microchips can drive the costs to prohibitive level, especially in mass applications.

DETECTION OF POLYMORPHISMS AND PROOFREADING USING GENERIC MICROCHIPS

By definition, generic microchips contain all possible oligonucleotides of a certain length. Initially, they were intended for sequencing by hybridization (SBH). Despite significant efforts, the technology of SBH has not been adapted for large-scale projects primarily because of inefficient discrimination between perfect and imperfect duplexes, secondary structure in the hybridizing samples of nucleic acids, and the presence of short tandem repeats. The length of sequences that can be decoded on arrays of short oligonucleotides is constrained by these limitations. In addition, the increase of length of oligonucleotides in a complete array results in linear increase in the length of sequences that can be decoded, while the number of probes in the array grows exponentially. At the same time, generic microchips can be successfully used for proofreading and small-scale sequencing, including identification of SNPs in the fragments of interest.

Our generic chip consists of a set of 4,096 gel pads with all possible hexamers [Drobyshev et al., 1999]. Hexameric sequences melt at low temperature, inconvenient for routine use. Therefore, they are extended at both ends with a universal base 5-nitroindole or with an equimolar mixture of four nucleotides, thus resulting in the formation of eight-base-pair-long duplexes [Fotin et al., 1998].

The use of the generic chip enables one to measure simultaneously the thermodynamics of the hybridization of all possible perfect and mismatched duplexes for a given hexamer by monitoring their equilibrium melting curves [Fotin et al., 1998]. The data for perfect and mismatched duplexes that were obtained using the microchip method correlated with data obtained in solution.

Extension of the ends of immobilized oligonucleotides with degenerated bases significantly diminishes the differences in melting curves of the AT- and GC-rich duplexes and improve discrimination of perfect duplexes from those containing poorly recognized terminal mismatches.

The applicability of generic chips for small-scale sequencing and proofreading has been investigated by Chechetkin et al. [2000]. After hybridization of target fluorescently labeled single-strand DNA to the generic chip, melting curves were measured in parallel for all individual gel pads. It turned out that the non-equilibrium melting is as informative as the equilibrium process, but takes much less time.

An integrated data processing scheme was developed to analyze the data. At the preprocessing stage, melting curves for individual duplexes were unified by introducing a modifying function based on their nucleotide composition. This operation improved discrimination between perfect and imperfect duplexes. At the next stage, computations were performed to maximize the indicator function that assesses the results of tiling of the deduced sequence with oligonucleotides that form perfect duplexes. The whole reconstruction of an unknown sequence requires limited computer power, but has intrinsic limitations. First, the sequence of interest must have fixed, determined ends: because of the nature of the process, tiling of the ends is insufficient for sequencing. Second, the sequence should not have repeats equal in length or longer than the oligonucleotides on the chip. In theory, the repeated sequence will produce brighter fluorescence in the corresponding pad because of increased binding; however, the reliability of experimental measurements and their calibration are not currently sufficient to make this feature meaningful.

The effectiveness of the procedure has been demonstrated by the correct sequencing of 10 out of 13 randomly chosen DNA fragments of 31, 51, and 70 nucleotides (Fig. 5). We envision that further improvements of the technique will make it feasible and efficient in specific applications: preliminary characterization of large sets of short sequences; identification of SNPs; and especially, single nucleotide deletions and insertions.

Proudnikov et al. [2000] applied hybridization on generic chips to detection of point mutations in viruses. Oral poliovirus vaccine (OPV) sometimes contains neurovirulent mutants that must be monitored to ensure the safety of the vaccine. It is usually done by expensive, long, and complicated tests on large groups of monkeys. Major mutations responsible for neurovirulence are known, and corresponding segments of viral nucleic acid amplified by PCR can be used to identify these mutations. In our work, asymmetric PCR was performed on two strains of viruses and their mutants. The products (92 and 82 bp long) were

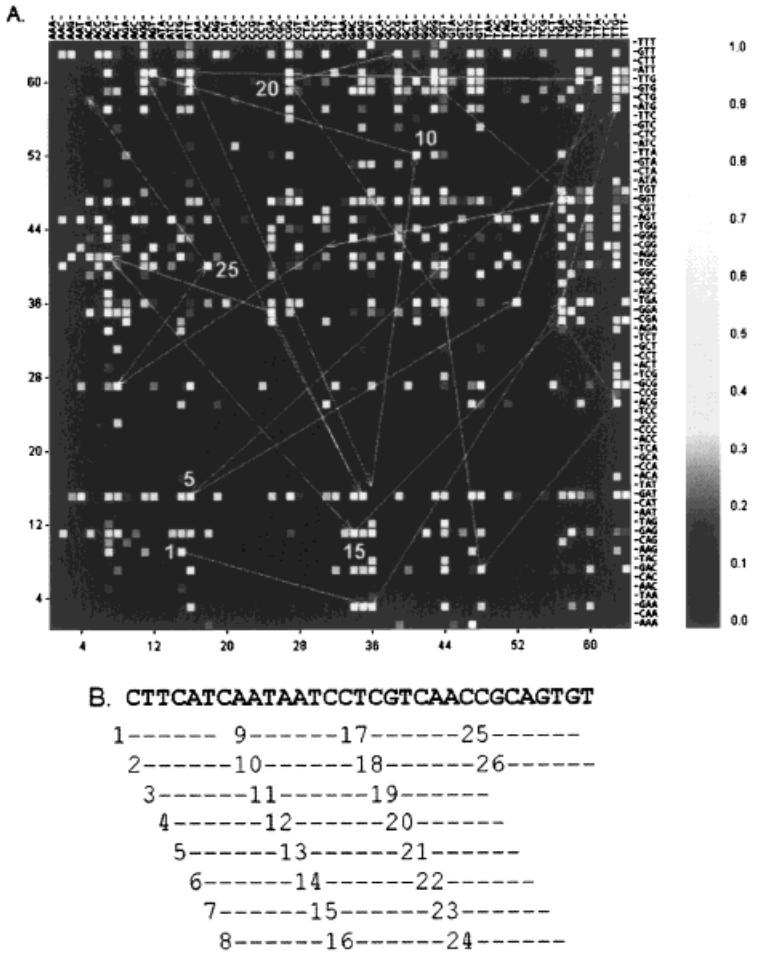

FIGURE 5. **Hybridization of target fluorescently labeled DNA with generic hexamer chip. A: The computer-generated distribution of the integral fluorescence intensities corresponding to the hybridization with the target sequence 5′ - CTTCATCAATAATCCTCGTCAACCGCAGTGT-3′. B: Scheme of tiling target sequence by hexamers. The pads associated with consecutive tiling hexamers are connected on the chip by lines with arrows. (Courtesy V.R. Chechetkin and A.Y. Turygin.)**

fragmented, labeled with TR, and hybridized successively to the same generic chip. To imitate clinical preparations, mutant viruses were mixed at different ratios with wild-type virus.

After hybridization at 5°C and a wash, melting curves were recorded for each individual pad using an automated workstation. Hybridization was assessed quantitatively as an integral area under the melting curve and emulated as a computer image of the chip [Drobyshev et al., 1999]. To facilitate the visualization of perfect duplexes, the signals were rearranged by special computer software in such a way that each perfect duplex was surrounded by its terminally mismatched homologs, yielding fluorescence signals. Visually, positive signals could be observed as cross-like sets of fluorescence pads surrounding the bright pad of the perfect duplex, and all true polymorphisms are found at the intersections of rows and columns of several positive signals of perfect duplexes in one of the samples.

The best sensitivity for the analysis of the mixtures of viruses is achieved when the mutation is located in the middle of the corresponding hexamer, position 3 or 4. For these, the method reliably determines 10% of mutated virus in the preparation. After initial screening of mutations, the procedure can be simplified and accelerated, because the temperature can be optimized for hybridization at constant temperature instead of a complete set of melting curves. However, discrimination of different mismatches still requires different optimal temperatures and usually cannot be performed by one-step hybridization.

CONTIGUOUS STACKING HYBRIDIZATION (CSH)

To overcome the need of excessively large and complex arrays for SBH, we proposed to use CSH [Lysov et al., 1988; Khrapko et al., 1991] as described above and illustrated in Figure 1. In this approach, the initial hybridization of the target DNA with the generic chip is followed by an additional round of hybridization with labeled relatively short oligonucleotides. These secondary oligonucleotides form extended duplexes with the target DNA stabilized by high energy of stacking interactions between the terminal bases of the immobilized oligonucleotide and incoming oligonucleotide. For example, melting temperature of a pentamer duplex can be increased by 5 to 15°C when it is stacked to an 8-mer duplex and forms a contiguous 13-mer duplex with broken phosphoidiester bond in one strand [Khrapko et al., 1991; Parinov et al., 1996; Vasiliskov et al., 2001]. The process of secondary hybridization can be repeated several times over. In theory, CSH increases the sequencing potential of a generic array: if the length of the immobilized oligonucleotide is L bases and that of the incoming oligonucleotide is l bases, the resolution power of this experimental setup approaches an equivalent of an array of probes $(L+l)$ in length [Lysov et al., 1988].

Vasiliskov et al. [2001] used CSH to investigate the energy of stacking interactions and their effect on match/mismatch discrimination. The energy of stacking turned out to be surprisingly high, sometimes exceeding the energy of hydrogen bonding. Among different interfaces, interactions with adenine as one of the members are the strongest. Besides, both overlapping and missing bases suppress CSH dramatically. The demonstration of high selectivity of CSH in this work is important for further improvement of minisequencing on generic chips as well as refinement of CSH-based applications of MALDI MS. Stomakhin et al. [2000] tested the potential of this approach on a set of model oligonucleotides (see above).

Dubiley et al. [1997] demonstrated how CSH could be used to detect and count short repeats. Test oligonucleotides were mixed with the target DNA and bound within the gel pads by CSH with the immobilized probe; then the latter was ligated with the test oligonucleotide. Successive rounds of stacking hybridization and ligations were used to determine the number of short repeats in a target sequence, a problem that cannot be resolved by straightforward hybridization to generic arrays. Eventually, five tetranucleotide repeats in a model sequence were counted by stacking hybridization to non-overlapping pentamers.

CONCLUSIONS, PERSPECTIVES, AND TECHNICAL GOALS

Acrylamide gel pad matrices offer several advantages over other solid support-based microassays. The three-dimensional immobilization provides high capacity and complete spatial isolation of the probes. Hybridization and enzymatic reactions of immobilized compounds are similar to the same reactions in solution. Processes in gel pads can be recorded in real time. When covered with mineral oil, gel pads are completely isolated from each other, and the IMAGE chip becomes a sub-nanoliter titer plate.

TABLE 1. **Comparison of Different Approaches to Detection of DNA Polymorphisms Using Gel-based Microarrays**

Approach	Advantages	Disadvantages
Hybridization	Arrays are easy to customize and manufacture	Requires substantial work to optimize probes and hybridization conditions
	Generate simple, easy-to-interpret patterns	Microarrays are designed for specific tasks
	Affordable	
	Easy to adapt to field conditions	
	Reusable	
Enzymatic analysis	High sensitivity	Microarrays usually cannot be reused
	High discrimination power	The procedure may be complex and expensive
Generic chips	Universal design suitable for mass production	Requires sophisticated equipment to monitor melting and analyze results
	Broad range of applications	Limited accuracy of sequencing
	Suitable for small-scale sequencing	Requires prior knowledge of end sequences
Contiguous stacking hybridization (CSH)	Potentially high-throughput technique	Requries expensive equipment
	Can be used with a limited set of 5-mers in a wide variety of applications	Microarrays usually cannot be reused

Hybridization in gel pads provides improved discrimination between perfect and mismatch duplexes. As the result, identification of SNPs or other polymorphisms on IMAGE chips is possible with base call sets of only a few oligonucleotides. This obviates the need for tiled arrays of numerous overlapping oligonucleotides and complex interpretation of the results obtained on these arrays [Chee et al., 1996].

Short oligonucleotides offer higher discrimination power. However, their association constant during hybridization is relatively low, and fluorescent signal may be weak. Since the density and, therefore, the total amount of oligonucleotides anchored on glass or plastic surface is limited, the use of short probes (8–10 nucleotide long) on two-dimensional microchips is not feasible. The possibility of using shorter oligonucleotides as such or in contiguous stacking hybridization is another advantage of gel-based chips.

Detection of DNA-polymorphisms using gel-based microarrays in combination with various approaches is summarized in Table 1.

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