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History

Fifty Years of Molecular (DNA/RNA) Diagnostics

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As the journal *Clinical Chemistry* celebrates its 50th birthday and enters its 51st year, we reflect on recent advances in nucleic acid diagnostics. Although the structure of DNA was also described ~50 years ago, only 20 years have past since a practical method of amplifying DNA for the clinical laboratory appeared, well known today as the PCR. Since the advent of PCR, growth in nucleic acid diagnostics has been extraordinary and is the fastest growing segment of many clinical laboratories. *Clinical Chemistry* is now subtitled *The International Journal of Molecular Diagnostics and Laboratory Medicine* in recognition of the importance of DNA/RNA diagnostics.

In this review, we offer personal perspectives on five key advances in molecular diagnostics by some of the pioneers in the field. Russ Higuchi reviews real-time PCR, which is an elegant solution for nucleic acid quantification in research and the clinical laboratory. Carl Wittwer describes DNA melting analysis as a simple and surprisingly powerful tool that can be seamlessly appended to PCR. Tom Gingeras reviews the increasing power of array technology, with feature density rivaling microelectronics miniaturization. Progress on integrated devices that combine cell separation, sample preparation, amplification, and analysis is described by Larry Kricka. Finally, the field of circulating nucleic acids is reviewed by Dennis Lo, with a myriad of clinical applications enabled by a new understanding of the biology of extracellular DNA and RNA.

Many early advances in molecular diagnostics have been highlighted in *Clinical Chemistry*. With DNA/RNA diagnostics becoming ever more important in the 21st century, the Journal serves as an advanced window into the future of molecular clinical chemistry.

Real-Time PCR

—Russ Higuchi

When late in 1986 I joined Cetus, the birthplace of PCR, "closed-tube" PCR using a thermostable DNA polymerase had just been achieved *(1, 2)*. Before this, the thermolabile polymerase had to be added at each cycle. Tom White, our vice-president in charge of Research and Development, told me that what was needed now was some way to visualize the amplification product, also without opening the tube. Such closed-tube detection was termed "homogenous".

A few years later I was working with Gavin Dollinger on his idea of "tagging" things that needed to be traced (e.g., explosives, money, and pharmaceuticals) with specific, amplifiable DNA sequences *(3)*. We noticed that very high molecular weight DNA was occasionally generated in PCRs from such tagged substances. If we could reproducibly promote such large DNA, Gavin reasoned, it might be detected directly in the tube by light scatter. We set out to use biotinylated primers and streptavidin in the PCR to try to catalyze the formation of long branched chains of amplicons. We succeeded only in forming precipitates. To determine whether DNA was in the precipitates, we added ethidium bromide to the completed PCRs and held the tubes up to ultraviolet (UV)[7] light. The precipitates were fluorescent, but the fluorescence was not specific to the presence of double-stranded DNA (dsDNA). Bob Griffith, who was helping us with these experiments, was directed to try some different

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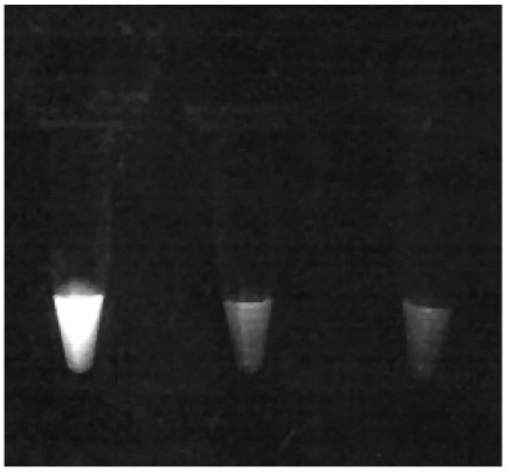
[7] Nonstandard abbreviations: UV, ultraviolet; dsDNA, double-stranded DNA; CCD, charge-coupled device; C_t, threshold cycle; T_m, melting temperature; SNP, single-nucleotide polymorphism; AZT, zidovudine; μTAS, miniaturized total analytical system; and EBV, Epstein–Barr virus.

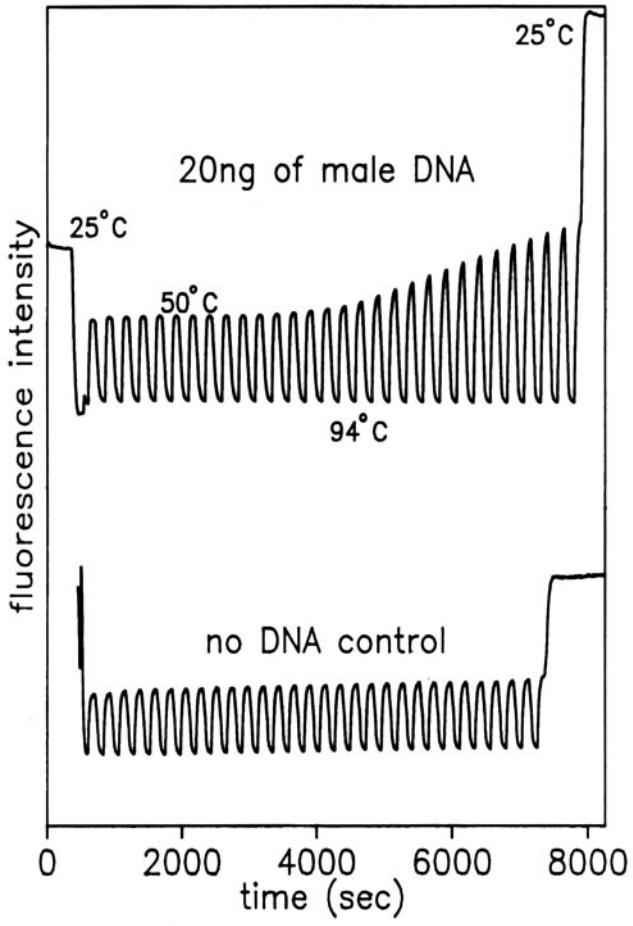
conditions. He showed me the gel with the expected DNA band from the control without streptavidin and mentioned that, without thinking, he had added the ethidium bromide at the beginning of the PCR.

I had two reactions. One was surprise that the PCR had worked at all in the presence of ethidium bromide, a known DNA polymerase inhibitor. Indeed, PCR would have been inhibited if the concentration of ethidium bromide had been somewhat higher. The second was to realize that because PCR creates much more dsDNA than is put into it, there should be more ethidium bromide fluorescence created as well. Such fluorescence would be detectable without opening the tube.

The PCRs were repeated without streptavidin. When held up to a UV light, the tube containing the amplification target glowed brightly compared with negative controls (Fig. 1A). Although this "endpoint" reading of PCR was of itself very useful, we thought that taking fluorescence readings on a cycle-by-cycle basis might provide a quantitative assay with a wide dynamic range *(4)*. The fewer PCR cycles it took to detect an increase in fluorescence, the more copies of the target DNA sequence there were to begin with. To demonstrate this "real-time" reading of the PCR (which we later called "kinetic"), we attached one end of a bidirectional fiber optic cable to the open top of a PCR tube in a thermocycler and the other to a fluorescence spectrophotometer. With excitation at 500 nm, a fluorescence trace at 600 nm was recorded (Fig. 1B). The ups and downs in fluorescence that corresponded to the downs and ups in temperature from thermocycling were seen, as well as the net gain in fluorescence at low temperature attributable to the accumulation of dsDNA PCR product.

To assess the quantitative performance of PCR in this mode, it soon became clear that we needed a way of reading multiple amplifications in parallel. One way, ultimately adopted in the first commercial real-time PCR instrument, was to use multiple fiber optic cables (one per





A B

Fig. 1. Real-time visualization of PCR.

(*A*), three completed PCRs in microfuge tubes illuminated by UV light. All tubes contained ethidium bromide. The first tube on the *left* had target sequence cognate to the primers; the two others did not. (*B*), fluorescence traces from ethidium-bromide-containing PCRs taken with a fiber optic cable and a spectrophotometer. The *upper trace* is from a PCR begun with 20 ng of human male DNA and primers specific to a Y-chromosome sequence. The *lower trace* is from a control PCR with the primers but without the target DNA added. Fig. 1B was reprinted with permission from *Biotechnology* 1992;10:413–7.

tube) and a rapid multiplexing system for sequentially exciting and reading the fluorescence from each tube. I thought that a simpler way would be to use digital imaging; Bob Watson and I therefore implemented such a system using a charge-coupled device (CCD) camera from a home-built, digital gel-documentation system *(5, 6)*. The camera was set up in a darkroom looking down over the exposed tops of the closed PCR tubes set into the thermocycler block. UV lights were used to illuminate the tubes in the block, and at every annealing/extension phase of the PCR an image was "grabbed". At every well position in the image, the pixel values were summed. The cycle-by-cycle plot of these sums generated "growth curves" for each PCR.

The raw growth curves were clearly not useful (Fig. 2A) without some processing. It made sense to normalize PCRs relative to each other based on their early cycle fluorescence readings because the beginning inherent fluorescence should be the same for each tube. This made the growth curves of replicate PCRs nearly identical. A global, threshold fluorescence value could then be set (initially we referred to this as the "AFL", or arbitrary fluorescence level). The number of cycles it took to cross this threshold, which can be interpolated to fractions of cycles, is inversely and linearly related to the logarithm of the initial number of target molecules (Fig. 2B and C). By comparing these cycle numbers, commonly referred to as cycles to threshold (C_ts), with those from a reference set of PCRs with known initial numbers of target molecules (as a calibration curve), the starting target number in each "unknown" can be inferred.

Early on I realized that the slope of the calibration curve was related to the per-cycle efficiency of PCR replication. One hundred percent efficiency is a perfect doubling per cycle; thus, one cycle would be required to make up for a twofold dilution of starting template. With a lower efficiency, more than one cycle would be required. The slope of the calibration curve determines the number of cycles required to make up for the dilution. For 10-fold dilutions, the relationship between this slope and PCR efficiency can be expressed as:

$$(10^{-1/\text{slope}} - 1) \times 100\% = \text{per-cycle efficiency}$$

I put this calculation into an early version of our spreadsheet template for analyzing quantitative real-time PCR data. Today it can be found in most data analysis software used with real-time PCR instruments. Such predetermined estimates of PCR efficiency can quantify, in one run, different target sequences in the absence of calibration curves for each *(7)*.

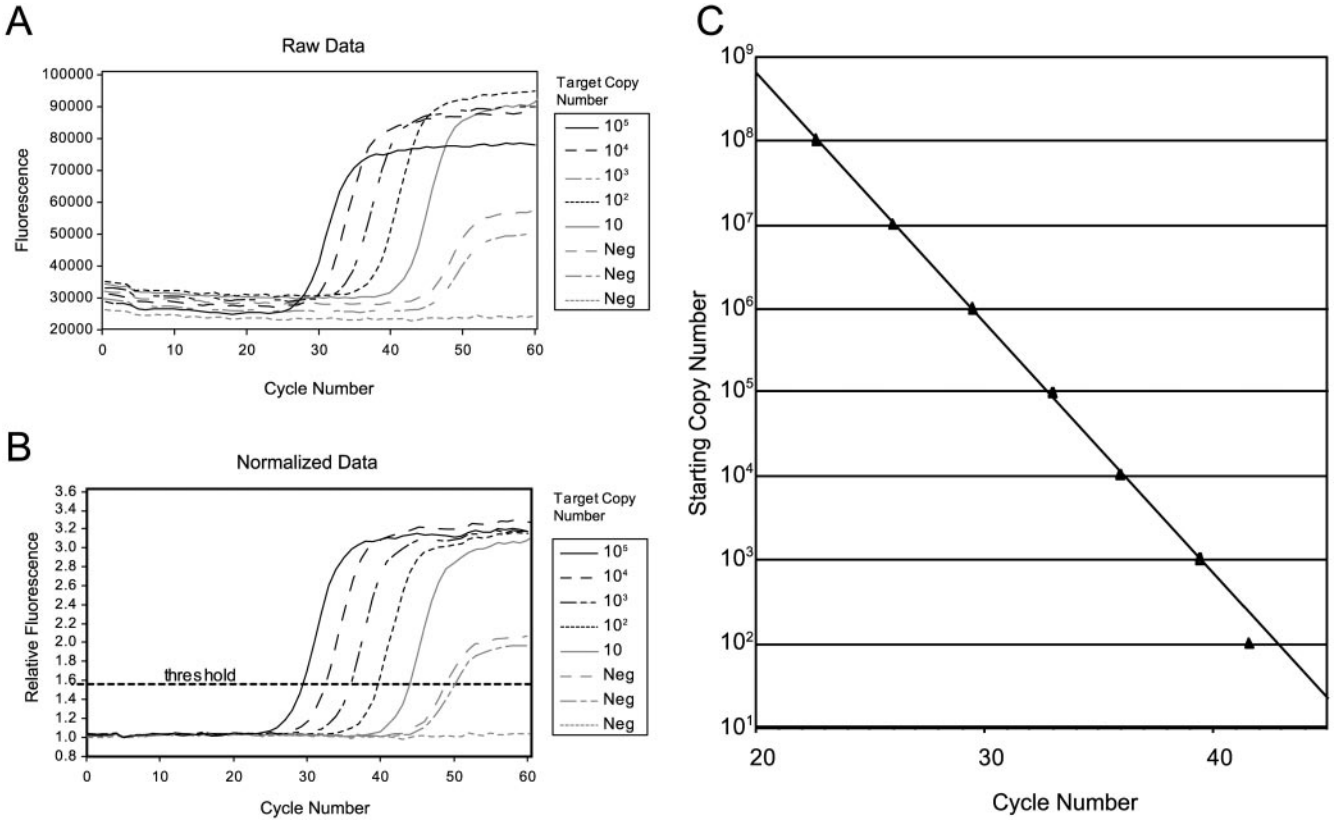


Fig. 2. Real-time PCR for HIV RNA.

(*A*), fluorescence output from a CCD camera showing growth curves from the indicated starting copy numbers of HIV RNA template. (*B*), the data normalized as described in the text. The threshold fluorescence from which C_ts (see text) are derived is indicated. (*C*), calibration curve based on linear regression of the log starting copy number and the growth curve C_ts. This figure was adapted, with permission, from *Biotechnology* 1993;11:1026–30.

Although real-time PCR with dyes, in particular SYBR® Green I, are now well accepted, at first it was difficult to overcome concerns about nonspecific amplification. Thus, the commercialization of real-time PCR depended on the subsequent development of sequence-specific, probe-based homogeneous detection systems, in particular the dual-fluorescently labeled TaqMan® probes *(8, 9)*. With improvements in PCR specificity, such as that from "hot-start" polymerases *(10)*, as well as the ability to validate results by use of Carl Wittwer's melting curve analysis (see next section), dye-alone methods, which are inherently simple and inexpensive, have gained in popularity.

For the future, would it not be nice to be able to combine the high "bandwidth" (in terms of numbers of targets) of microarray-based nucleic acid quantification with the sensitivity of real-time PCR? Only the most highly expressed mRNAs are generally detectable on microarrays: many interesting genes with low expression (transcription factors, for example) remain unaccounted for in many experiments *(11)*. There have been some attempts at a "microarray of PCRs", but as far as I know, none has led to a practical system allowing both the individual "addressing" of primers and delivery of precise amounts of sample to the micro-PCRs. The problems do not seem intractable, however, and perhaps a solution will be available soon.

Melting Curve Analysis

—Carl Wittwer

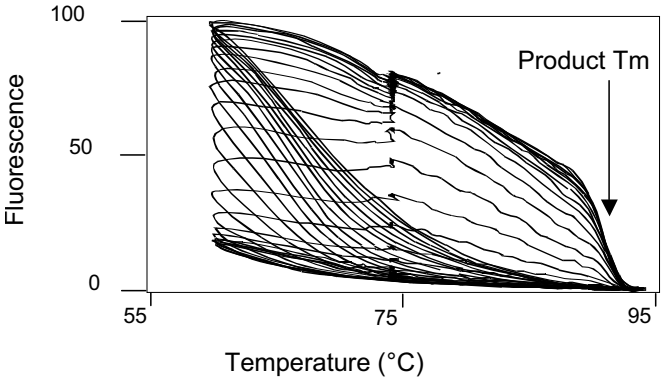
Melting of the double helix to separate random coils is a fundamental property of DNA. The melting profile of a DNA duplex depends on the surrounding buffer (particularly the salt concentration), the GC content, the length, and the sequence of the duplex. Conventional DNA melting is measured by UV absorbance, often takes hours to perform, and requires large amounts of DNA. In contrast, monitoring of DNA melting by fluorescence requires only a few minutes, and adequate amounts of DNA are conveniently provided by PCR. When performed in the same tube as PCR amplification, melting analysis provides a homogeneous, closed-tube system for product identification. With high-resolution DNA melting, sequence alterations can be easily genotyped and entire amplicons scanned for variations.

We first observed DNA melting during PCR by accident. Funded by an NIH small business grant, we were adapting flow cytometry optics to monitor rapid-cycle PCR in glass capillary tubes *(12)*. As a measure of total double-stranded product, we chose SYBR Green I, a new dye being marketed as an ethidium bromide replacement for DNA detection in gels. SYBR Green I was brighter than ethidium bromide, and its spectrum is similar to that of fluorescein, so that one instrument channel could be used for either product or probe analysis.

For probe-based detection, we synthesized oligonucleotides with fluorescein and Cy5 amidites that were commercially available at the time, hoping to use fluorescence resonance energy transfer for signal generation. Our initial attempts to monitor PCR by hydrolysis of dual-labeled probes failed; therefore, instead of separating two fluorophores by hydrolysis, we labeled two adjacent probes, one with fluorescein and one with Cy5, so that the fluorophores were brought together by hybridization. In retrospect, this was serendipitous because hybridization fluorescence directly monitors probe melting, whereas probe hydrolysis does not.

Real-time PCR for quantification is usually performed by acquiring fluorescence once each cycle. However, instead of looking only once each cycle, we acquired fluorescence continuously throughout PCR, including the temperature transitions. By plotting fluorescence vs temperature, we generated curious spiral curves (Fig. 3). Each loop of the spiral was generated from one PCR temperature cycle. With SYBR Green I, the sharp decrease in fluorescence just below the PCR denaturation temperature corresponded to the PCR product melting tempera-

A. SYBR Green I



B. Hybridization Probes

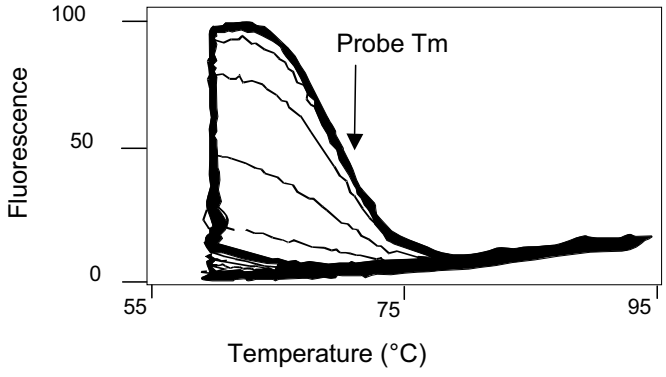


Fig. 3. Continuous monitoring of rapid-cycle PCR.

(*A*), SYBR Green I monitoring of PCR *(12)*. Thirty cycles were completed in 15 min. (*B*), adjacent hybridization probe monitoring of PCR products. The approximate T_ms of PCR product (*A*) and hybridization probes (*B*) are indicated. Reprinted with permission from *Methods* 2001;25:430–2.

ture (T_m). With adjacent hybridization probes, the major fluorescence decrease corresponded to the melting temperature of the less stable probe.

We reasoned that sequence variants under hybridization probes should make the duplexes less stable and enable genotyping by melting profiles. Although originally observed during PCR, melting curve generation after PCR has become most popular. Unlike a static "dot blot" at a single temperature, an entire melting profile is obtained across the region of temperature transition. The first example of the use of this technique was published in *Clinical Chemistry* in 1997 for factor V Leiden *(13)*. Factor V Leiden testing by melting analysis was cleared by the United States Food and Drug Administration (FDA) in 2003 and is one of the few FDA-cleared tests in clinical genetics. The method has become a favorite for clinical laboratories because many alleles can be identified with a single probe set. Rather than needing additional probes and colors for each allele analyzed, alleles are differentiated by T_m instead of fluorescent color *(14)*. The number of alleles that can be differentiated depends on the resolution of the instrument and analysis software.

Probe melting analysis was further simplified when we noticed (again by accident) that genotyping was still possible with only one probe instead of two *(15)*. The fluorescence of labeled oligonucleotides either increases or decreases with duplex formation, depending on the fluorophore and the sequences of the probe and target. In retrospect, this is not surprising because of the drastic change in environment of the fluorescent label with duplex formation.

Continuing along the trend of simplification, melting curve genotyping with unlabeled probes was reported recently *(16)*. Whereas previous methods required either one or two oligonucleotides with covalently labeled fluorophores, the new method requires only an unlabeled probe and a saturating DNA dye. The time and cost reductions for assay development are liberating.

As probe melting techniques have become simpler over time, melting analysis of PCR products has become more powerful. Initially, product melting with SYBR Green I was a low-resolution method to identify different products *(17)*. Whereas gels separated products by size, melting analysis separated products by T_m. The main advantage of melting analysis was that products were not exposed to the environment as possible future contaminants. However, many different products had approximately the same T_m, limiting the resolution of the method with conventional real-time instruments.

Recently, high-resolution melting techniques for analysis of PCR products have appeared with the goal of maximizing the amount of information that can be extracted from this simple process. With high-resolution melting, a large majority of single-nucleotide polymorphisms (SNPs) can be genotyped without probes *(18)*, requiring only two flanking primers in the presence of a saturating DNA dye. Homozygotes are distinguished by T_m shifts, and heterozygotes produce telltale heteroduplex transitions that alter the shape of the melting curve. SNP genotyping in products >500 bp in size is possible *(19)*.

Because heteroduplexes alter the shape of the melting transition, high-resolution melting analysis is a convenient method for heterozygote screening. The reported sensitivity and specificity for detecting single-base heterozygotes are 97% and 99%, respectively, in products up to 1000 bp *(20)*. In contrast to all other mutation-scanning techniques that require separation steps after PCR, high-resolution melting analysis is closed-tube, requires no processing after PCR, and can be performed in 1–2 min. The required instrumentation is relatively inexpensive compared with conventional real-time instruments, which are neither required nor desirable because of limited resolution.

With only a saturating DNA dye and high-resolution melting analysis, both product and probe melting profiles can be obtained during the same temperature ramp after PCR. Probe melting is observed at low temperatures and product melting at high temperatures (Fig. 4). The product transition can be used either to verify the probe genotype or to simultaneously scan the product for heteroduplexes. This ability to both scan for any sequence change and genotype common sequence alterations in the same reaction may drastically reduce the need for sequencing of complex genes. Because of its simplicity, melting analysis is an attractive method in research and clinical diagnostics.

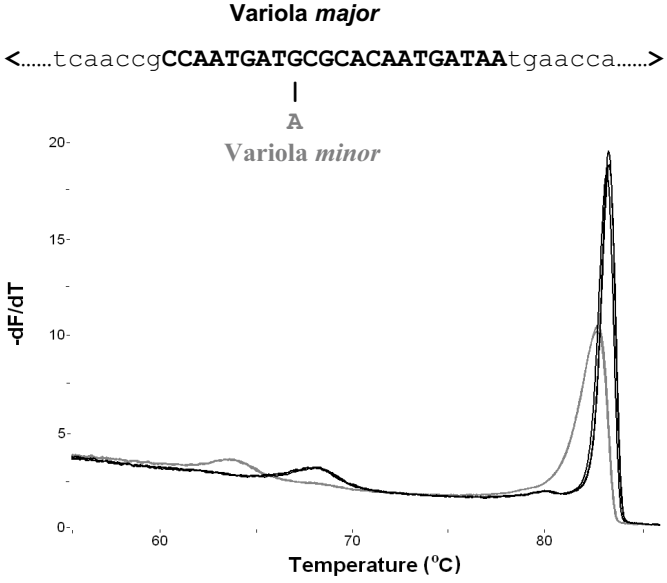


Fig. 4. Variola strain typing doubly confirmed by unlabeled probe *(16)* and PCR product *(19)* melting profiles.

Variola major (smallpox) and Variola minor species differed by a single G-to-A base change under the probe. The unlabeled probe (*bold sequence*) showed melting transitions of 68 °C (perfect match) or 63 °C (G:T mismatch), depending on the strain. In concert with the probe typing, the product T_m of the G strain was slightly higher than that of the A strain, a difference easily discerned with high-resolution techniques. Figure courtesy of Robert Pryor, Department of Pathology, University of Utah (Salt Lake City, UT).

Array Technology
—Tom Gingeras

The 1991 publication by Fodor et al. *(21)* in *Science* was striking on several levels: Photolithography was used as a general method to carry out the parallel synthesis of thousands of peptides, and the utility of high-density peptide arrays as tools for interrogating the interactions of important proteins (e.g., antigens:antibodies and agonists: receptors) was clearly demonstrated. Although the last figure in the report showed a three-dimensional representation of a fluorescent oligonucleotide array checkerboard, it was the last paragraph of the article that soundly caught my attention. In an eloquent piece of understatement, Fodor et al. *(21)* concluded by stating: "Oligonucleotide arrays produced by light-directed synthesis could be used to detect complementary sequences in DNA and RNA. Such arrays would be valuable in gene mapping, fingerprinting, diagnostics and nucleic acid sequencing." I recognized this concept as a possible way to solve difficult research challenges, and it remained subconsciously in my memory for 2 more years before emerging as a tool in practical applications.

For several years before 1993, we had been working on identifying pathogenic organisms, including HIV-1 RNA, directly in patients' blood. It was important to determine whether the organisms possessed mutations conveying resistance to therapeutic treatments. HIV-1 resistance to zidovudine (AZT), the only antiviral drug available at the time, was a critical challenge. Resistance to AZT could be determined by detection of mutations in the polymerase (*pol*) gene at a combination of four amino acid sites. However, with the introduction of alternative antiviral drugs, it was apparent that genotyping more of the *pol* gene would be necessary to manage the therapeutic treatment of HIV-1-infected patients. To understand the cellular tropism and epidemiology of the HIV-1 virus, considerable portions of the genome needed to be sequenced. This additional sequence information would allow clinicians to evaluate the prognosis and therapeutic course of patients. However, sequencing of patient-derived RNA retroviruses as part of routine patient monitoring was impractical in 1993.

A fortuitous conversation with Steve Fodor in late 1993 pointed me back to his 1991 publication. I suggested that an array might be designed to analyze the sequence of large portions of the HIV-1 genome as a high-throughput diagnostic tool for patient monitoring. Steve indicated that this would be challenging but agreed that it was worth trying. Thus, we set out to design and synthesize the first research array tool as a possible precursor to a full diagnostic application. As a result, separate high-density oligonucleotide arrays that resequenced the *pol* and protease genes of HIV-1 were the first commercial research tools made by Affymetrix *(22)*.

Since 1996, the design, synthesis, and applications of high-density oligonucleotide arrays have evolved dramatically *(23, 24)*. The protease resequencing array contained 12 224 oligonucleotide probes. Each of the probes was synthesized on the array surface as an array "feature", measuring ~120 × 120 μm. Today, high-density arrays containing 6.4×10^6 oligonucleotide probes are routinely constructed with 5 × 5 μm features. This 520-fold increase in probe density has occurred through a steady evolution of array technologies. Interestingly, each increase in probe density has been preceded and motivated by a novel basic or clinical diagnostic application.

For example, contiguous regions of a genome can be interrogated at a uniform resolution by use of probes in a tiling pattern. In doing so, both genotyping and fingerprint information can be collected about the organism. This tiling strategy required more than the 12 224 probes used in the HIV-1 resequencing application. By decreasing the feature size to 50 × 50 μm, a fourfold increase in probe density was achieved, and such arrays were used to simultaneously detect drug resistance mutations in *Mycobacterium tuberculosis* and to speciate 121 isolates from nine other *Mycobacterium* species *(25)*. Even higher density tiling arrays for interrogation of the entire *M. tuberculosis* genome were made by again reducing the synthesis area for each probe. Such arrays were used to track individual clinical *M. tuberculosis* isolates by identifying isolate-specific genome deletions *(26)*.

Recently, tiling arrays with 14 × 14 μm features have been used to interrogate whole human chromosomes. Analyses of chromosomes 21 and 22 by such arrays on 10 cancer cell lines indicate that there are many more RNA transcripts and promoter regulatory regions than are accounted for by current genome annotations *(27, 28)*. Most of these transcripts possess little coding capacity and therefore may represent a novel class of regulatory noncoding transcripts that may be important in some human diseases *(29)*.

It seems certain that important basic and clinical research questions will continue to drive the development of array technologies toward greater and greater information content. Fig. 5 illustrates the relationship between the number of oligonucleotide probes needed to interrogate different genomes at 1 bp resolution using two probes per interrogation and the required size of the features. Thus, it will require 6×10^9 probes synthesized using ~0.3 × 0.3 μm features to interrogate every base of the human genome on a single array. Although this degree of resolution is not achievable with current photolithographic methods, the nonrepetitive portions of the human genome can be interrogated on 14 arrays with 5 × 5 μm features and a mean spacing of 35 bp. Such arrays are being used today in new applications such as genome-wide studies of transcription sites, binding of transcriptional regulatory factors, origins of replication, chromatin modification, and DNA methylation. In turn, the application of these basic research studies to the demanding world of clinical diagnostics will require yet more innovations of array technology.

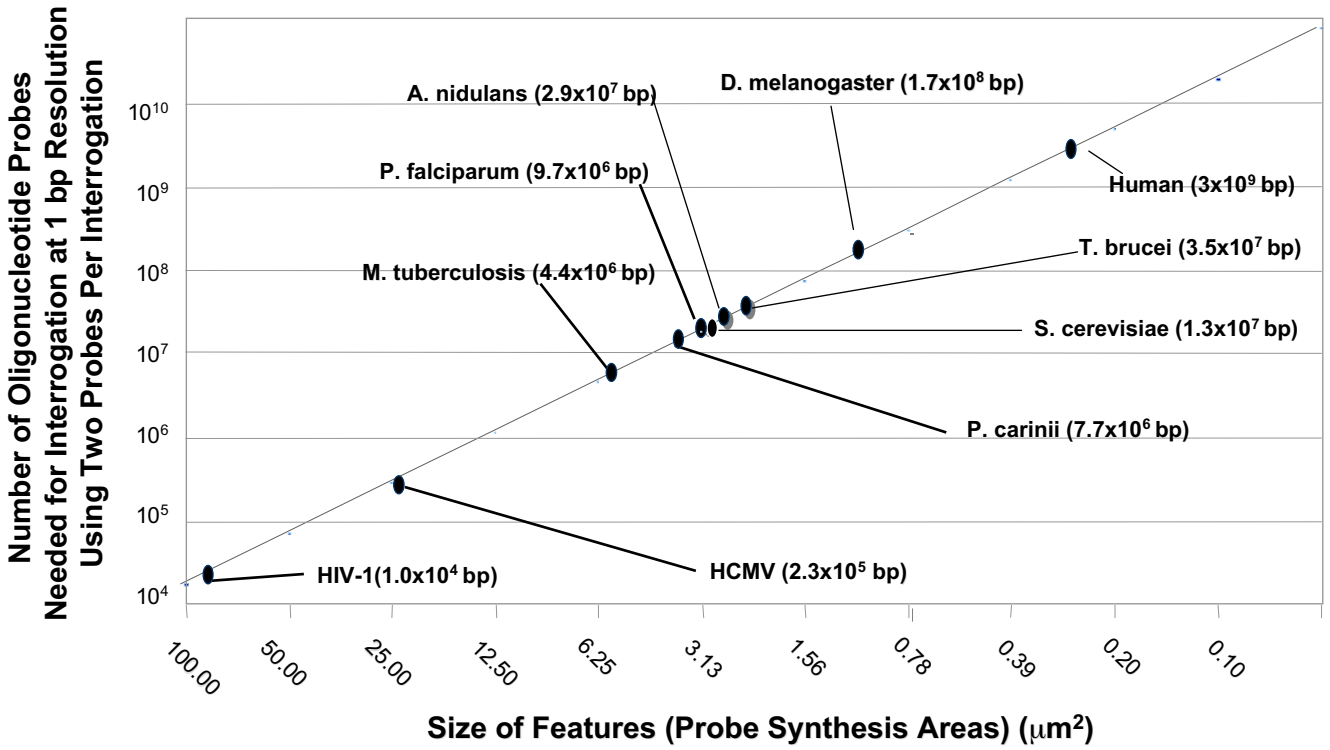


Fig. 5. Graph illustrating the relationship between feature size and the number of oligonucleotide probes required for interrogation of a whole genome at 1 bp resolution on a single microarray.

The tiling strategy uses two probes per interrogation. Larger genomes, such as human and *Drosophila melanogaster*, require higher-density oligonucleotide microarrays with reduced feature sizes. For example, it would require 6×10^9 probes synthesized with ~0.3×0.3 μm features to interrogate every nonrepeat base of the human genome (1.5×10^9 bp) on a single array. Similarly, the smaller *Plasmodium falciparum* genome (9.7×10^6 bp) can be interrogated by use of 1.9×10^7 probes synthesized with ~2.5×2.5 μm features. *HCMV*, human cytomegalovirus.

Integration of Molecular Diagnostics on a Microchip
—Larry Kricka

Clinical chemists have always seized any new technology that could improve the quality of their analytical work and at the same time significantly improve efficiency. Prime examples are the early adoption of computers to handle the growing volume of laboratory data quickly and reliably and the installation of automation to cope with the mounting workload. It thus is no surprise that clinical chemists have watched with particular interest the rapid developments in microtechnology and nanotechnology and the evolution of the miniaturized total analytical system (μTAS) approach to analysis.

A μTAS, or lab-on-a-chip, is a miniaturized device that combines all of the steps in an analytical process in a series of interconnected microchannels and microchambers on one small (usually $<1 \times 1$ cm) planar device *(30)*. These devices are made from silicon and glass by use of fabrication techniques adopted from the microelectronics industry, such as photolithography and reactive ion etching, or from plastics by embossing techniques. The application of this type of technology to microanalytical devices can be traced back to the pioneering work of Terry et al. *(31, 32)* at Stanford, who fabricated a gas–liquid chromatograph on a 5-cm diameter silicon wafer in the early 1970s. Work on analytical microchips subsequently waned, but interest was reawakened in the early 1980s *(33–35)*. This renewed interest in microchip research and development of μTAS devices stemmed from a new appreciation of the numerous advantages of microchips. These include economy of scale, multiplexed analysis (a small microchip can accommodate many test sites), improved reliability, portability, and integration of multistep analytical procedures on a single device.

The emerging importance of genetic testing and its manifest complexity quickly identified it as a type of test that would particularly benefit from the μTAS approach. A genetic test involves several sequential steps, including sample preparation (e.g., cell isolation and DNA purification), amplification of target sequence (e.g., PCR), and detection and quantification of amplified products (e.g., hybridization and electrophoresis). Initially, individual steps in the procedure were adapted to a microchip format, most importantly PCR, a process central to most if not all genetic tests *(36, 37)*.

Peter Wilding and I began our work on microchips in the mid-1980s. We were greatly encouraged by Jay Zemel in the Engineering School at the University of Pennsylvania (PENN), who was engaged on fluidic studies in silicon microchannels. We quickly recognized the broad range of

potential applications for microchip devices in clinical analysis and began projects designed to explore the utility of microchips in immunoassays, genetic testing, semen analysis, and in vitro fertilization. A frustrating aspect of the initial phase of our work was securing grant support. The NIH thought the work too basic and the National Science Foundation thought it too clinical, and each agency referred us to the other for support. We had filed invention disclosures with the university (PENN); we therefore logically embarked on a venture capital route to fund our work. This led to a start-up company, Chemcore, that ultimately merged with Caliper Technologies. We were fortunate in the early days to have access to the microfabrication facilities in the University of Pennsylvania School of Engineering, and it was this group that made our very first chips. Subsequently, as the project grew, we began to subcontract our prototype production to commercial silicon foundries. In the 1980s, most silicon foundries were used to fabricating the ever smaller features on microelectronic chips, and they were always amazed by our requirement for the larger-sized features of our chip designs ("you want to make it how big?!"). Subsequently, we developed in-house expertise in microfabrication, using facilities at the Cornell NanoScale Science & Technology Facility at Cornell University.

Our early PCR microchips consisted of simple channels or chambers etched in silicon and capped with glass *(38–40)* (Fig. 6). These chips were effective for PCR, although miniaturization did present some problems, mainly related to the surface chemistry of the materials used to construct the microchips. Subsequently, other individual steps were miniaturized. For example, micromachined filters within microchambers in a glass-capped silicon chip were effective for leukocyte isolation from whole blood as a prelude to analysis of genomic targets

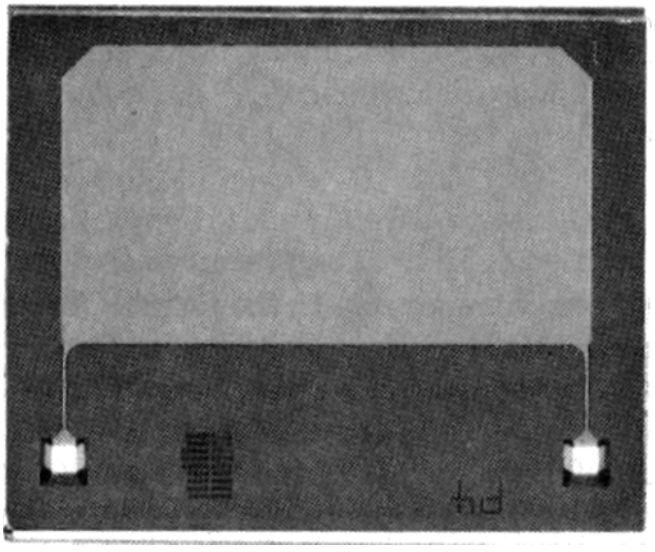


Fig. 6. Single-chamber PCRChip etched to a depth of 80 μm.

Chamber volume, ~9 μL; overall size, 17 × 15 mm. Reprinted with permission from *Clin Chem* 1994;40:1815–8.

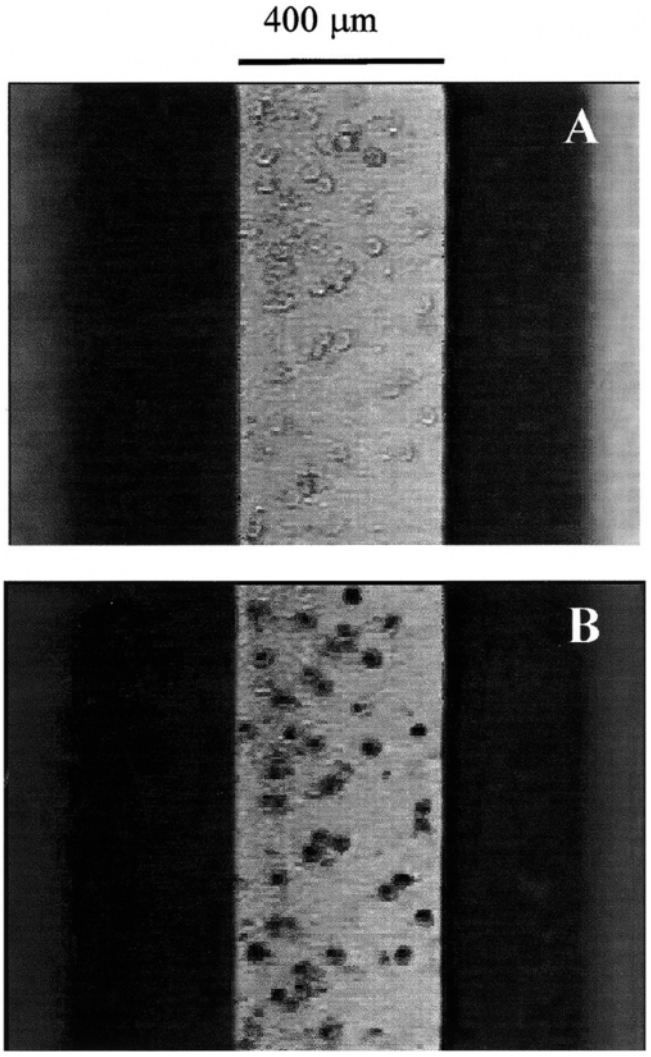


Fig. 7. Microscopic view from the top of an integrated cell isolation-PCR microchip mounted on the Plexiglas microchip module.

(*A*), leukocytes isolated by the 400-μm wide weir-type filter. (*B*), new methylene blue-stained leukocytes isolated by the weir-type filter. Reprinted with permission from *Genome Res* 2001;11:405–12.

(41) (Fig. 7). The final step in many genetic tests is PCR amplicon detection. This too can be easily accomplished in a microchip by use of capillary electrophoresis.

The next stage toward the development of μTAS systems was integration of two of the steps in the process, such as cell isolation and PCR or amplification and capillary electrophoresis, on a single microchip. Finally, workers in several laboratories have combined all steps to achieve complete μTAS devices, with on-chip integration of sample preparation, PCR, and real-time detection of the amplicon *(42–49)*. An even greater degree of integration has been achieved for DNA sequencing on a microfluidic chip, entailing integration of the steps of clone isolation, amplification, Sanger extension, purification, and finally electrophoretic analysis *(50)*.

The natural progress of a successful new technology is the transition from idea to working prototype, then to a

phase of exhaustive evaluation in a routine laboratory environment, and finally to a commercial product approved by the appropriate regulatory agency. For analytical microchips, the final two steps are not completed. A family of microchips for DNA and RNA analysis by capillary electrophoresis as well as various types of DNA hybridization chips have been commercialized for research applications, but a commercial μTAS system for clinical use is still eagerly awaited by the clinical chemistry community.

Circulating Nucleic Acids

—Dennis Lo

With the notable exception of virology, the clinical material used in the analysis of nucleic acids is usually cellular. However, much recent interest has focused on the biology and diagnostic applications of circulating cell-free nucleic acids found in the noncellular fraction, i.e., plasma, of humans.

My team's involvement in this field began in 1996 after 7 years of trying to develop noninvasive prenatal methods to detect fetal nucleated cells in the maternal circulation *(51)*. The rarity of these cells made the analysis of their genetic material rather elusive *(52)*. In the autumn of 1996, we noticed two articles in *Nature Medicine* reporting the detection of tumor-derived microsatellite alterations in DNA isolated from the plasma and serum of patients with cancer *(53, 54)*. The similarity between the placenta, which contains fetal genetic material, and a malignant tumor made us wonder whether the fetus would also release its DNA into the plasma and serum of the pregnant mother. Some workers had previously referred to the placenta as being "pseudomalignant" *(55)*, and it is unusual to see a tumor as big as a newborn baby!

At that time we did not know the best method to extract DNA from human plasma and serum; we therefore simply used boiled plasma and serum directly in PCR. Surprisingly, even this relatively crude extraction allowed us to demonstrate, for the first time, the presence of cell-free fetal DNA in the plasma and serum of pregnant women *(56)*. With the advent of real-time quantitative PCR, we were able to measure the absolute and relative concentrations of such circulating fetal DNA in maternal plasma *(57)*. The mean fractional concentration of circulating fetal DNA was 3–5%, suggesting that noninvasive prenatal diagnosis using fetal DNA in maternal plasma should be relatively robust, a prediction that has turned out to be true. Circulating fetal DNA has been used to assess fetal RhD status *(58)*, the prenatal investigation of sex-linked diseases *(59)*, and the prenatal exclusion of β-thalassemia *(60)*. Recently, the introduction of mass spectrometry for nucleic acid analysis has further enhanced the robustness of detecting single nucleotide differences between the mother and the fetus through maternal plasma analysis *(61)*. Furthermore, quantitative aberrations in circulating fetal DNA have also been detected in a variety of disorders, such as preeclampsia *(62)*. The development of a gender- and polymorphism-independent fetal DNA marker should expand the general applicability of such observations.

The success of detecting circulating fetal DNA in maternal plasma has prompted us to look for circulating fetal RNA. This phenomenon was finally demonstrated in 2000 *(63)*, with subsequent experiments pinpointing the placenta as the predominant source of such RNA *(64)* (Fig. 8). Circulating RNA is surprisingly stable, possibly through its association with particulate matter *(65)*. Plasma RNA analysis, perhaps in combination with microarrays, greatly increases the number of markers that can be used for prenatal monitoring *(66)*.

In addition to prenatal diagnosis, the application of circulating nucleic acids to cancer detection and monitoring has also rapidly progressed over the last few years. To date, microsatellite alterations, oncogene mutations, oncogene amplifications, epigenetic changes, and mitochondrial DNA mutations have been detected in the plasma and serum of cancer patients *(67)*. In particular, the detection of Epstein–Barr virus (EBV) DNA in the plasma and serum of patients with nasopharyngeal carcinoma and other EBV-associated malignancies has undergone rapid development. Circulating EBV DNA has been found to be a sensitive tool for the detection, monitoring, and prognostication of such cancers *(68–70)*.

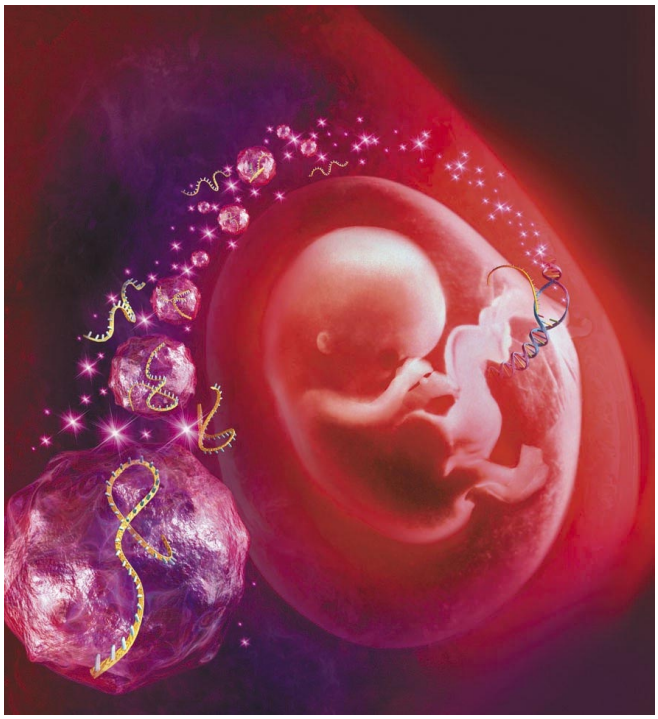


Fig. 8. Diagram illustrating the release of placenta-expressed mRNA into the maternal circulation.

Such mRNA molecules are thought to be protected from degradation by association with particulate matter. Figure courtesy of Rossa Chiu (Department of Chemical Pathology, The Chinese University of Hong Kong, Hong Kong Special Administrative Region, China) and Sam Shum (Sam Freelance Services, Hong Kong).

It is envisioned that many other applications of circulating nucleic acids will be forthcoming over the next few years. Emerging applications include prognostic stratification of acute medical emergencies *(71)* and transplantation monitoring *(72, 73)*. Much still must be learned about the characterization of such circulating nucleic acids *(74, 75)* and the functional implications of such molecules *(76)*.

Aspects of microchip analysis are licensed by the University of Pennsylvania to Caliper LifeSciences. L.J.K. has a financial interest as a named inventor on these patents. Dennis Lo holds patents or patent applications on certain applications of circulating nucleic acids in maternal plasma. Aspects of these technologies have been licensed to BTG plc and Plasmagene Biosciences. Dennis Lo is a shareholder of Plasmagene Biosciences. Aspects of melting analysis and rapid PCR are licensed by the University of Utah Research Foundation to Idaho Technology, and from Idaho Technology to Roche Applied Science. C.T.W. has a financial interest as a named inventor on these patents and holds equity interest in Idaho Technology. T.R.G. is an employee of Affymetrix, a company that offers array products. R.H. is an employee of Roche Molecular Systems, a company that offers real-time PCR products.

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