Genomics 103 (2014) 177–182

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Review

Is this the real time for genomics?

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ARTICLE INFO

Article history:
Received 15 November 2013
Accepted 11 February 2014
Available online 19 February 2014

Keywords:
Genome-based tests
Next-generation sequencing
Real-time microarray
Lab-on-Chip
Point-of-Care
Diagnostics

ABSTRACT

In the last decades, molecular biology has moved from gene-by-gene analysis to more complex studies using a genome-wide scale. Thanks to high-throughput genomic technologies, such as microarrays and next-generation sequencing, a huge amount of information has been generated, expanding our knowledge on the genetic basis of various diseases. Although some of this information could be transferred to clinical diagnostics, the technologies available are not suitable for this purpose. In this review, we will discuss the drawbacks associated with the use of traditional DNA microarrays in diagnostics, pointing out emerging platforms that could overcome these obstacles and offer a more reproducible, qualitative and quantitative multigenic analysis. New miniaturized and automated devices, called Lab-on-Chip, begin to integrate PCR and microarray on the same platform, offering integrated sample-to-result systems. The introduction of this kind of innovative devices may facilitate the transition of genome-based tests into clinical routine.

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1. Introduction

The genomic era started with the completion of the Human Genome Project in 2001, opening new interesting challenges from biological research to medicine applications. During this period, we have witnessed the astonishingly fast development of high-throughput technologies, including hybridization and sequence-based ones, which allowed the transition from studies involving single genes to those employing a more extended genomic approach. This is generating a plethora of

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http://dx.doi.org/10.1016/j.ygeno.2014.02.003

data that are collected in public databases such as GenBank for DNA/RNA and protein sequences, and OMIM (Online Mendelian Inheritance in Man) for gene and genetic phenotypes. These public resources, daily updated, currently contain 46,608 human gene sequences and 4023 phenotypes with a known molecular basis [1–5]. Some of this information could be already transferred to clinical diagnostics, but the technologies available are not adequate for this purpose. The actual challenge is the use of genome-based technologies in clinical practice.

In the next paragraph, we will briefly discuss the two most important technologies used in genomic screening analysis.

2. High-throughput technologies in genomics: DNA microarrays and next-generation sequencing

DNA microarrays and next-generation sequencing (NGS) are the two most important technologies for high-throughput genomic analysis [6]. During the past 20 years, DNA microarray technology has been developed and consolidated as a routine tool in research laboratories and is now transitioning to the clinic. While we are witnessing this transition, NGS is catching up [7,8]. Over the past 8 years, a number of NGS technologies have emerged that enable the sequencing of large amounts of DNA in parallel and at significantly lower costs than conventional methods. NGS technologies are suitable to different applications, such as whole or targeted genome sequencing, and RNA sequencing (RNA-seq). Due to cost reduction, the latter application may soon replace DNA microarrays in transcriptome profiling analysis [9]. However, the transition of NGS into clinical practice is slowed up by non-automated experimental procedures and lack of efficient and user-friendly methods to store, process and analyze the large amount of data produced [10].

Despite the great potential of traditional DNA microarrays and NGS, a number of issues need to be tackled to implement these technologies in clinical diagnostics. In this review we will analyze emerging DNA microarray platforms that may offer immediate opportunities to implement genomic tests in clinical medicine. Thanks to several characteristics, these sample-to-result systems are available for real-time detection and offer a more reproducible, qualitative and quantitative multigenic analysis.

3. DNA microarray technology: state of the art

DNA microarrays technologies are based on the ability of DNA to find and spontaneously bind its complementary sequence in a highly specific, rapid and reversible manner [11]. Over the years, this technology has been applied to genome analysis in distinct medical fields allowing the association of polygenic alterations to specific pathologies [12–22]. Obtained DNA microarray data are collected in public repositories, such as Gene Expression Omnibus (GEO), which contains also NGS and other forms of high-throughput functional genomic data [23]. Although the collected information could be relevant from a medical perspective, they are not easily accessible from a clinical practice standpoint and only few of them have already been transferred to the bed-side.

To date, a small number of microarray-based tests have been cleared for diagnostics. The main reason is related to the complexity of this technology, which is suitable to research laboratories but not to diagnostic ones. Below we will briefly describe the first diagnostic, prognostic or pharmacogenetic tests based on DNA microarray technology.

The *MammaPrint* test by Agendia, the first in vitro diagnostic multivariate index assay (IVDMIA) to be cleared by the US Food and Drug Administration (FDA) in 2007, is an individualized metastasis risk assessment test with prognostic value for breast cancer patients with stage 1 or 2. It analyzes the expression of 70 genes and stratifies patients into two distinct groups: low risk or high risk of distant recurrence [19,24–26].

The *Pathwork Tissue of Origin* Test by Pathwork Diagnostics is a microarray-based gene expression assay for improving classification of clinicopathologically ambiguous tumors. It can detect the expression level of 1550 genes in each tumor sample of unknown or poorly differentiated origin (primary or metastatic) and to determine the similarity to 15 tissue types belonging to known tumors [25,27,28]. This test gained FDA clearance in 2008 for frozen samples and in 2010 for formalin-fixed and paraffin-embedded samples.

The *AmpliChip CYP450* by Roche, cleared by the FDA in 2004, is a pharmacogenetic test based on Affymetrix microarray technology that analyzes allelic variations in two highly polymorphic cytochrome P450 genes (CYP2D6 and CYP2C19), whose encoded enzymes regulate the metabolism of drugs from a variety of classes. By predicting altered drug metabolism, it is possible to prevent harmful drug interactions and to ensure the optimal use of drugs [25,29–31].

Despite the undeniable advantages of these tests, their applications in clinical settings are still limited by the use of traditional microarray technology at few highly specialized laboratories. In the next section, we will analyze in detail the advantages and limits of traditional microarray technology.

4. DNA microarray technology: advantages and limits

The main benefits of DNA microarray technology are *high-throughput analysis*, *miniaturization* and *safety* [32]. The *high-throughput analysis* allows parallelism through a direct comparison between thousands of probes spotted on the microarray and their complementary targets. This advantage has been reached thanks to *miniaturization* of the array surface, leading also to a significant improvement in terms of decrease of reaction volumes, increase of sample concentration and acceleration of hybridization kinetic. *Safety* derives from the use of fluorochrome labeling methods, which avoid handling by the operator of radioactive or toxic compounds during the experimental process.

Despite the undisputed advantages of DNA microarray technology, there are many limiting factors that hinder their routine use in diagnostics. Some of these, such as the lack of *accuracy* and *reproducibility* [33–37], depend on the experimental phases. Although a detailed description of these limiting factors goes beyond the focus of this review, we will briefly describe them below.

- *Sample preparation and labeling*. In this phase, the sample quality and quantity have a crucial role since its partial or total degradation can affect the entire experimental outcome.
- *Hybridization and post-hybridization washing*. The specific binding between probes and their complementary targets mainly depends on the stringency of washing buffers and temperature. Small changes in these phases, which are not fully automated, may produce non-specific interactions.
- *Image acquisition*. Image acquisition can be influenced by scanning parameters, such as intensity and signal resolution, which are used to increase the sensitivity and reduce the noise. The resulting image has to be further submitted to quality assessment and pre-processing data analysis steps (such as grid overlay) that could greatly affect the final results.

Another important aspect affecting accuracy and reproducibility of DNA microarrays is the *low dynamic range* of detection. Part of the signals detected by traditional DNA microarrays fall in a window with a linear dynamic range, whereas those near to background or saturating levels are not.

The complexity of traditional DNA microarray resides not only in its experimental procedures and image acquisition, but also in analysis and interpretation of obtained data that require dedicated software and bioinformatics staff. Almost all experimental and analytical phases depend on the operator, and the *lack of automation* further reduces accuracy and reproducibility. Additional limits of traditional DNA microarray technology include the *requirement of highly skilled personnel*, *high costs*, and *prolonged procedures* (generally 48 h).

The limiting factors summarized above represent the most important constraints when a microarray test has to be cleared for in vitro clinical diagnostic use by regulatory authorities [25,26,38,39]. In the next sections, we will discuss how research and technology progresses are trying to overcome these limits.

5. New DNA microarray platforms

In order to overcome some of the limiting factors associated to microarray-based tests in clinical practice, industries are starting to commercialize miniaturized devices, called Lab-on-Chips (LOCs), incorporating both disposable microelectronic and microfluidic components. The latter allows manipulating the small volume fluids, loading the chip and filling the reaction chambers without the risk of bubble formation or leaks [40–43]. These devices permit the integration of all, or almost all, experimental phases in a single chip, simplifying the entire analytical process and drastically reducing the handling by the operator, thus allowing the execution of the test in a short time. Additional advantages of LOCs include decrease of reagent and sample volumes, possibility to integrate a PCR chamber, rapid analysis, low-cost and minimal risk of sample contamination. The reduction of human error, together with the standardization and automation of all process operations, increase accuracy and reproducibility of the results obtained. Thanks to these characteristics, some LOCs are evolving towards Point-of-Care (POC) devices, offering the opportunity to move genome-based analysis to the bed-side. Moreover, POCs can be easily transported to remote locations decreasing the need of large and specialized diagnostics facilities [40,44,45].

In the next paragraphs, we will discuss four novel DNA microarray platforms that include an associated or integrated PCR, highlighting their technical features as indicated by their manufacturers.

5.1. PCR-microarray LOCs

The detection limit of traditional DNA microarrays imposes the amplification of extracted DNA/RNA. PCR, therefore, is often used for amplification and labeling of samples before their hybridization onto traditional DNA microarrays. In the new devices, PCR is now being integrated, on the same DNA microarray platform. These PCR-microarray LOCs combine the advantage of microfluidics with the opportunity to perform a fast and small volume low-density multigenic analysis (hybridization and detection), on the same device. The development of these technologies has reached such a level that enables the production of diagnostic tests, enabling the detection of several infectious disease (such as viral influenza, sexually transmitted diseases, and poverty related disease), pharmacogenomics and genotyping (such as SNP) analysis [40,44,46].

In the next sections, we will discuss one hybrid and three integrated PCR-microarray platforms.

5.1.1. INFINITY System

An example of a hybrid (not integrated) platform is the *INFINITY System* by AutoGenomics [47–49]. This platform associates a multiplex PCR with automated microarray hybridization, by using BioFilmChip microarrays that can be configured with hundreds of biomarkers. Labeling, hybridization, scanning and data analysis steps have been automated and are performed within the Infinity Analyzer, whereas sample extraction, purification and PCR amplification are performed separately using reagents and instrumentation provided by the same company. Although hybrid, this platform is versatile enough to allow diagnostic tests for a wide range of disease signatures in the areas of women's health, cancer, viral influenza, as well as pharmacogenomics. Some of these tests are already FDA cleared and CE marked while others are under review [47,48].

5.1.2. Rheonix CARD

The *Rheonix CARD platform* by REONIX is a fully automated microfluidic platform based on disposable plastic cards [44,50]. The laminated polystyrene Rheonix CARD is a cartridge that is able to manipulate reagents internally, with its active fluidic network of pumps, valves and channels. The Rheonix CARD is inserted onto the Rheonix EncompassMDx instrument that includes: liquid handler, thermal cycler, hybridization heaters, barcode reader, imager and PC. For each assay, the phases of cell lysis, nucleic acid purification, multiplex PCR, labeling, hybridization and microarray detection are performed under software control, producing a sample-to-result LOC. Only one step requires the operator presence: the initial introduction of untreated specimens directly onto the disposable card. Rheonix CARD assays for different clinical purposes (from infectious disease to pharmacogenomics) have been developed but are not yet cleared by the FDA [48,50].

5.1.3. Verigene System

The *Verigene System* by Nanosphere is an automated platform based on disposable test cartridges. The platform consists of two modules, the Verigene Processor (fully automated) and the Verigene Reader, which are able to drive the different experimental phases on the multi-chambered fluidic cartridges. When an untreated specimen is loaded onto the cartridge by the user, it undergoes to nucleic acid extraction and purification (with magnetic microparticles), PCR amplification (optional), hybridization and washing within the Verigene processing unit [51]. In particular, the array hybridization is carried out using nanoparticle-conjugated oligonucleotides (~200) of Nanosphere's property (Gold nanoparticles hybridization technology) [52]. After hybridization, the cartridge is removed from the processor unit and only the microarray glass slide is inserted into the Verigene Reader, where signals are detected and analyzed [51]. Verigene tests have been approved by the FDA for different purposes of clinical relevance [51,53–55].

5.1.4. In-Check Lab-on-Chip

STMicroelectronics has developed the *In-Check Lab-on-Chip* platform for fast, automated and qualitative nucleic acid analysis (shown in Fig. 1, panels a–e) [45,56–58]. This platform is made up of a silicon-based LOC, an independent modular Temperature Control System (TCS) to control thermal process, an Optical Reader (OR) to acquire fluorescence microarray signals and software for image analysis. The silicon chip integrates microfluidic handling, a miniaturized multiplex PCR chamber and a low-density microarray, containing up to 400 spots [59]. The microarray and PCR modules are fluidically connected and are both thermally driven by a TCS module. After hybridization, the microarray is read by the OR in a few seconds. This product is in continuous development and has been recently implemented with nucleic acids extraction methods, in order to obtain a fully integrated system sample-to-result [60]. Its application to diagnostics has already begun with the customization of infectious diseases tests that are cleared for in vitro diagnostics [46].

5.2. PCR and microarray integration: an opportunity for real-time detection

More than fifteen years ago, we witnessed the transition from endpoint to real-time detection PCR [61]. In the real-time PCR configuration, the fluorescence emission is measured during the exponential growth phase where increase of fluorescent signal is directly proportional to the number of amplicons generated. Another improvement of real-time PCR configuration was the wide dynamic range of detection of starting target molecules, which increases up to eight orders of magnitude compared with traditional PCR. Thanks to these advantages, the real-time PCR has easily moved to clinical practice, allowing tests for a wide range of applications, from SNPs detection to copy number variations [62,63].

In a traditional DNA microarray experiment, the measurement of fluorescence levels is performed when the equilibrium phase of hybridization is reached. This *steady-state* phase produces a limited dynamic range of detection. The tremendous technological advances in LOC devices are now offering the opportunity for a real-time configuration of DNA microarrays [64–67]. Their ability to acquire signals in real-time

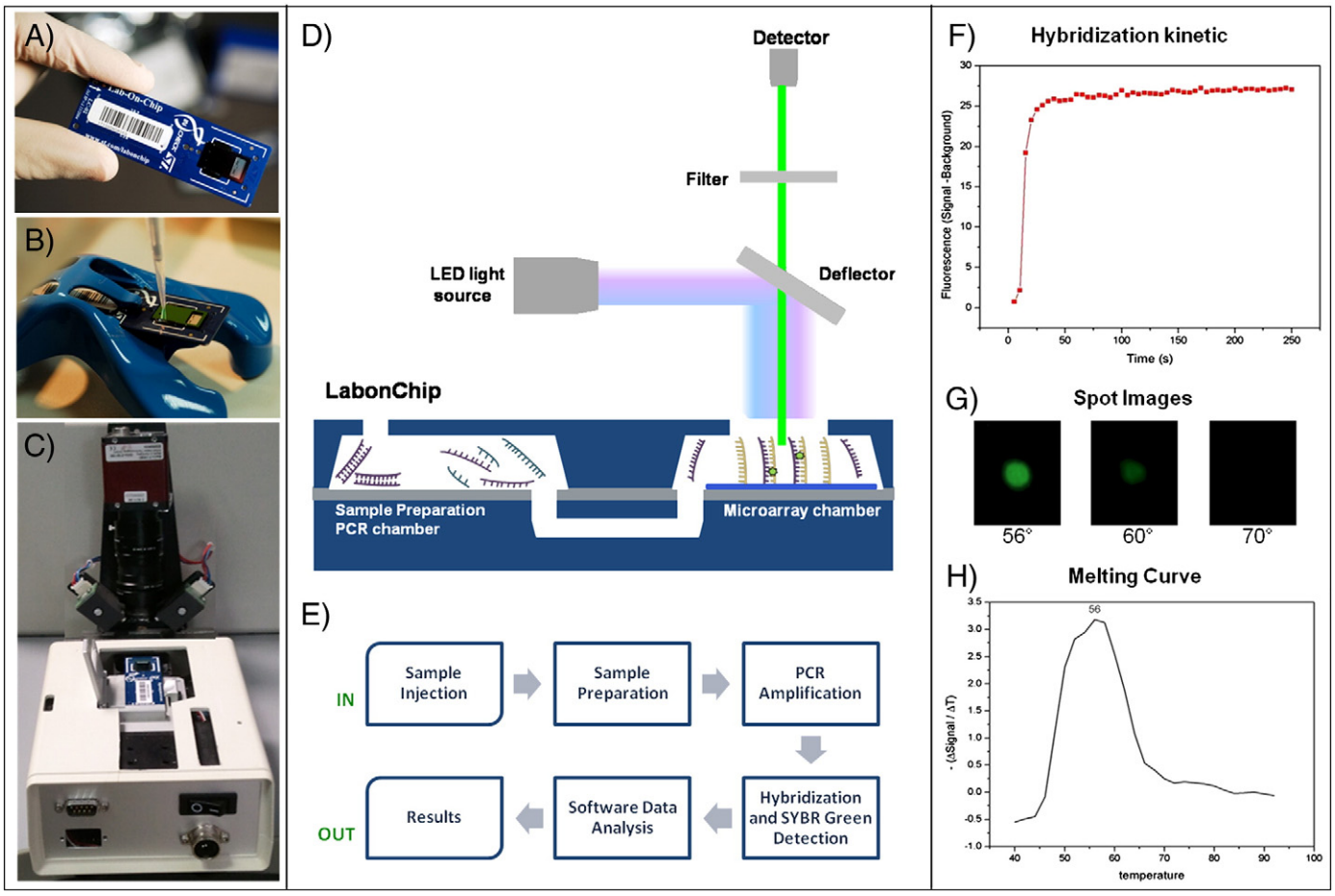

Fig. 1. Real-time detection on the In-Check LOC platform. (A) Silicon based device mounted on a 1 × 3 printed circuit board (PCB) support. (B) Sample loading phase on biochip holding by a clamp support. (C) Fluorescence detection phase by using an Optical Reading system. (D) A cross section of the platform highlighting the main internal units of the silicon biochip and those of the OR system. (E) Diagram showing the In-Check platform real-time workflow. (F) Hybridization kinetic — SYBR Green fluorescence signal at different hybridization times. The graph shows how hybridization reaches the steady-state level in a few seconds. (G) Microarray images of the same spot at three different hybridization temperatures (in °C). (H) Melting curve showing fluorescence as function of temperature. The curve shows a peak at 56 °C corresponding to the melting temperature of the hybridization products.

[68] allows an increase in the dynamic range detection and the possibility to perform absolute quantification of amplicons cycle by cycle. In a real-time configuration, the kinetics of hybridization can also be monitored and used to distinguish specific from aspecific products and reduce the time of execution of the test.

In Fig. 1 (panels d–h) we show an example of real-time microarray detection performed on the *In Check Lab-on-Chip* platform. The hybridization level was detected by the OR system using SYBR Green, a molecule that emits fluorescence when it binds to double-stranded DNA. As shown in the hybridization curve obtained (h), the hybridization process takes only a few seconds and can be monitored step by step. The readout is instantaneous as opposed to the off-line time-consuming scanning procedure that requires both lengthy hybridization and washing-steps. Finally, by performing a melting curve (panels g, h) it is possible to control the specificity of hybridization signals. The presence of a mismatch would produce a shifted melting curve.

Thanks to LOCs miniaturization, PCR-Microarray integration and real-time detection, it is now possible to control the rapid process of hybridization and increase the accuracy and reproducibility of microarray technology.

6. Conclusion

The genomic approach is opening new horizons in understanding diseases, leading to the development of new diagnostic tools, but their translation to clinical practice is still at the beginning. As detailed in this review, a real progress may soon be reached with the advent of PCR-microarray platforms combining multigenic analysis with real-time detection. Their sample-to-result characteristic and simple use will enable them to bridge the technical gap between research and clinics. The miniaturization, integration and automation of these tools increase accuracy and reproducibility, making them more suitable for routine use. With these advances, genome-based tests have the potential to become a standard tool for mainstream diagnostics, in order to monitor disease onset and progression, facilitate individualized patient therapy and, ultimately, improve patient outcomes.

Acknowledgment

Authors gratefully acknowledge Cristina Calì, Alfia Corsino, Maria Patrizia D'Angelo and Francesco Marino for their administrative and technical support. This work was supported in part by the Italian Ministry of Education, Universities and Research through grant CTN01_00177_817708.

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