Chapter 2

# High-Throughput Platforms in Real-Time PCR and Applications

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**Abstract**

The miniaturization of reactions by designing nanoliter-scale PCR platforms, as Taqman® OpenArray®, Dynamic Array™, or SmartChip, has been a big step forward in real-time PCR. Each platform has some particular characteristics that differentiate them. These nanoliter-scale PCR platforms enable substantial savings in the amount of reagents and sample because the reaction volumes are at nanoliter levels. In addition, it is possible to perform thousands of reactions in a few hours. Therefore, high-throughput real-time PCR platforms result in promising systems that are capable of processing a large number of samples simultaneously and also to perform a large number of assays per sample. All of this can be translated in the amazing applicability of this technology in all kinds of analytical fields, such as medical research, animal science, and food safety, among others.

**Keywords:** real-time PCR, high-throughput, SNPs, microRNA, gene expression

## 1. Introduction

In the decade of the nineties, real-time PCR became popular and meant an important step in molecular biology because of the great advantages over conventional PCR biology and its quantitative abilities. This revolution in PCR methods was achieved, thanks to the use of intercalating fluorophores, specific labeling probes with fluorescence emission, as well as equipment with the ability to detect this fluorescence during the reaction [1]. In this way,

measurement of gene expression, absolute quantification of pathogens, and genotyping analysis became routine analysis in life science research.

Until now, the limiting factor for real-time PCR compared to other molecular techniques was the number of tests that could be performed simultaneously. For example, hybridization techniques using microarrays, as those designed by Illumina® and Affimetrix®, allow the analysis of thousands of genetic markers in one single array [2, 3]. The realization of this type of screening by real-time PCR in standard formats (384 and 96 microwell plate) is infeasible due to both operational and economic reasons. In this context, in the few last years, some companies started to develop real-time PCR methods using a smaller reaction volume. As a beginning, some options for sample miniaturization such as the TaqMan® array microfluidic cards designed by Applied Biosystems® or the arrays designed by Qiagen® became available in the market. These formats use small volume that minimizes sample and reagent consumption. Their use is nowadays very popular in the field of gene expression, where it is possible to find predesigned array cards for certain diseases and metabolic pathways [4, 5]. They have also applications in other fields as microRNA screening or detection of foodborne pathogens [6, 7]. However, this improvement in the number of assays and samples that can be analyzed at the same time is still far away from the capabilities of microarray techniques.

The development of real-time high-throughput platforms was a breakthrough that changed the perception about real-time PCR and their possibilities. From the very beginning, the use of nanoliter reaction volumes and the possibility of custom designs converted this technology into an attractive tool for research purposes. Thus, this nanoliter-scale PCR became very common in fields such as medicine and pharmacogenomics research, allowing the inclusion of large number of assays and samples in one run. All the previous features have positioned real-time PCR in direct competition with microarrays and next-generation sequencing, also because it is a more sensitive and specific technique.

## 2. Principles and available high-throughput real-time PCR platforms

Currently, there are three commercially available high-throughput real-time PCR platforms: Dynamic Array™ chip (Fluidigm®, South San Francisco, CA, USA) TaqMan® OpenArray® (Applied Biosystems®, Carlsbad, CA, USA), and SmartChip (Wafergen Bio-systems Inc., Fremont, CA, USA). The operating principle of the three high-throughput platforms is the use of a nano-scale approach with thousands of reactions in a single run, reducing the sample and reagents consumption to the minimum. However, there are very important differences depending on the platform used.

### 2.1. Dynamic array

Microfluidic Dynamic Array™ with the integrated fluidic circuit (IFC) along with Biomark™ System from Fluidigm was the first real-time high-throughput platform available in the market [8]. The design of Dynamic Array™ is based on an integrated network of channels, chambers and valves that automatically combine the reactions. Thus, the dynamic plate is

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formed mainly by three components. On the one side, there are separate sample inlets, one for each sample. On the other side, there are separate primer-probe inlets, one for each probe. The IFC is placed in the middle, and it is here where the reaction chambers are located. This mechanism uses manual pipetting to load samples and primers-probes, and avoids the use of robotic liquid-handling to set up microwell plates. However, this kind of plates needs another instrument (IFC controller) controlled by a software to pressure load the assay components in the wells of the IFC in a process of 55 min. These wells, called reaction chambers, are formed by two containment valves and one interface valve. Thus, pressure is applied to the fluids contained in sample and detector inlets simultaneously. In this way, the fluids are transported into reaction chamber and fluid lines, respectively. The interface valve prevents sample and detector fluids from mixing. Then, the two containment valves are closed and interface valve is opened, pushing the detector fluid into the reaction chamber for mixing with the sample. Once the components are properly mixed, the interface valve is closed again and the chip is ready for cycling [9]. The real-time PCR cycling is performed in the Biomark™ System. At the end of each cycle, the chip is imaged and at the end of the whole process, the software generates PCR curves and a heat map, where each square represents a reaction chamber and the color indicates the CT value.

| IFC | 48.48 Dynamic Array IFC | 96.96 Dynamic Array IFC | 192.24 Dynamic Array IFC | FLEXsix IFC |
|---|---|---|---|---|
| Application | Genotyping/gene expression | Genotyping/gene expression | Genotyping/gene expression | Genotyping/gene expression |
| Pipetting steps | 96 | 192 | 216 | Variable |
| Assay inlets | 48 | 96 | 24 | 6×12 |
| Sample inlets | 48 | 96 | 192 | 6×12 |
| Reactions chambers | 2304 | 9616 | 4608 | 864 |
| Reaction volume (nl) | 9 | 7 | 8 | 9 |

**Table 1.** Dynamic array chips commercially available and their principal characteristics.

The company has a web tool [10] to customize assays for genotyping and gene expression with the specific conditions in which works, Dynamic Array™. There are different designs of Dynamic Array™ chips (**Table 1**) with reaction volumes ranging from 7 to 9 nl, depending on the array [11]. Of all the available nanoliter-scale platforms, Dynamic Array™ uses the smallest reaction volumes, while the other platforms use volumes of 33 and 100 nl. FLEXsix IFC is the model with the least number of reaction chambers (864). However, it can be considered as a model to select targets and to optimize assay. It has 6 partitions that allow 12 performing samples and 12 assays per sample in independent runs. The next designs in terms of number of chambers are 48.48 Dynamic Array™ and 96.96 Dynamic Array™ that analyze 48 and 96 samples and assays per sample, respectively. These three models can be used both for genotyping and gene expression assays. The last model was called 192.24 Dynamic Array™ and it

allows performing 24 assays per sample and 192 samples. As an example of their great processing capability and taking into account the whole workflow, the 96.96 Dynamic Array™ enables 9216 reactions in less than 4 h and it is capable to generate 36,000 data points per person and day. Despite the clear potential of these four designs available to the customer, Dynamic Array™ systems lack intermediate formats or even bigger dynamic chips that would allow the realization of a greater number of assays per sample.

One of the main advantages of Dynamic Array™ platforms compared to their direct competitors is the flexibility derived from the inexistence of preprinted assays. Dynamic Array™ has the possibility to choose the analyses that are going to be performed in each run. Although this flexibility is a clear advantage, the platform has problems of compatibility with TaqMan® assays. While in OpenArray® platform the same company designs and preprints the assays, in Dynamic Array™ another company provides the primers and probes, resulting in the subsequent problems of compatibility. These problems have been observed already in some intercomparative studies. For instance, Fedick et al. [12] used Dynamic Array™ and OpenArray® platforms for genotyping members of the Ashkenazi Jewish community. They observed problems of compatibility with the design of TaqMan® probes for genotypic analysis with Dynamic Array™. This incompatibility reduced the sensibility and the specificity of the assay in comparison with OpenArray®. Also, Farr et al. [13] detected problems of compatibility between RT primers and TaqMan® assays when Dynamic Array™ was employed for microRNA screening. These problems of compatibility could be solved simply by implementing a meticulous design of custom primers, which are adapted to Dynamic Array™ conditions.

### 2.2. TaqMan® OpenArray®

TaqMan® OpenArray® (Applied Biosystems) was the second high-throughput platforms released in the market. In the year 2006, Morrison et al. [14] published the Biotrove Inc (MA, USA) design of a through-hole array. It was composed of 3072 holes of 33 nl reaction volume in 48 groups of 64 holes each one. These plates have a hydrophobic exterior surface and hydrophilic interior (the inside surface of the 33 nl holes). This intelligent design allows fluid deposited on the hydrophobic surface to move into the hydrophobic holes. These arrays require the use of the OpenArray® NT autoloader for loading plates and the OpenArray® NT cycler for running the array [15]. In 2009, this technology was acquired by Applied Biosystems®, which now is a part of Thermo Fisher Scientific (MA, USA). For this reason, presently the OpenArray® technology works with OpenArray® AccuFill™ System for loading samples and the QuantStudioTM 12K Flex real-time PCR system that allows running four OpenArray® plates at the same time. The main applications described for this instrument up to now are gene expression, genotyping, microRNA screening, or drug metabolism analysis.

The first difference with the Dynamic Array™ is that in the OpenArray® design, the primers and probes are preloaded in the holes by the company. As a result, the user only needs to load the samples and the PCR mix onto the plate. However, the primers and probes cannot be changed while with Dynamic Array™ it is possible to choose which primers are used in the reaction if we have a library of them. On the other hand, the number of plate designs available for the customer with OpenArray® technology is higher.

The first step in an experiment with OpenArray® is to decide the right plate design that is convenient for the number of samples and assays per sample we want to perform. The division of the plate in 48 subarrays of 64 holes each allows variable designs, which are different for gene expression and genotyping assays (**Table 2**). The customers place the order for the desired design through an application on the web page of Thermo Fisher Scientific. It is possible to find millions of predesign TaqMan® assays in the database of the company, all of them successfully tested by the manufacturer. In addition to them, the costumer can order his custom assays by loading the target sequence in the web page and the company would design then the primers and the probes according to the real-time PCR conditions in which OpenArray® technology works. It is also possible to obtain some predesigned OpenArray® plates commercially available for specific genotyping and gene expression purposes. For example, there are OpenArray® plates for human identification and for expression of inflammation, cell-stem, or cancer-related genes [16]. Also, there is an OpenArray® plate for screening of microRNA in human samples [17].

| Format | Gene expression | | Genotyping | | |
|---|---|---|---|---|---|
| | Number of assays | Number of samples | Format | Number of assays | Number of samples |
| 18 | 18 × 3* | 48 | 16 | 16 | 144 |
| 56 | 56 | 48 | 32 | 32 | 96 |
| 112 | 112 | 24 | 64 | 64 | 48 |
| 168 | 168 | 16 | 128 | 128 | 24 |
| 224 | 224 | 12 | 192 | 192 | 16 |
| | | | 256 | 256 | 12 |

* In format 18×3 the assays are performed in triplicate.

**Table 2.** OpenArray® formats for genotyping and gene expression available for the costumers.

The two most widely used approaches to generate fluorescence in real-time PCRs are SYBR® Green and labeled probes. SYBR® Green is a chemical reagent that is introduced into the secondary structure of the DNA double helix. Consequently, the more the PCR products formed the more the fluorescence emitted. This molecule is commonly used in gene expression, mainly due to their affordable price. SYBR® Green allows the obtainment of a larger number of tests at a relatively low price. With regard to labeled probes, these nucleotide sequences were designed with the purpose of increasing the specificity of quantitative PCR. These probes bind to the middle of the target sequence, which is going to be amplified. They are labeled in the 5′ end of their structure with a fluorophore that will be released when the target section is amplified, resulting in fluorescence emission during amplification. Labeled probes are usually preferred in genotyping assays because using two labeled probes with two different fluorophores in the same reaction is possible to differentiate the two alleles. In the early development

of OpenArray®, most of the experiments were performed using SYBR® Green [14, 15, 18] instead of TaqMan® Probes. However, nowadays this technology works mostly with TaqMan® probes both for genotyping and gene expression, because they increase the specificity of the assays and data processing is easier.

A characteristic trait of OpenArray® plates is that they need to be loaded with the help of OpenArray® AccuFill™ System, while Dynamic Arrays™ are loaded manually. This does not mean a saving in equipment, because Dynamic Arrays™ needs another apparatus to transfer the samples and the primers to the reaction chambers. Moreover, this transfer process takes 55 min, while loading plates with OpenArray® AccuFill™ System only takes a few minutes. With OpenArray® AccuFill™ the process is very simple. First, the samples are mixed with the Master Mix in a 384-well plate. The order of the samples on this plate depends of the OpenArray® format that is going to be used. Afterward, the tip system of the OpenArray® AccuFill™ equipment transfers each sample to the corresponding subarray, showing a specific movement according the chosen array format. The sample is deposited in the hydrophobic surfaces of the array and the liquid is naturally transferred to the hydrophilic 33 nl holes. Once the array is filled, it has to be properly sealed and it is ready for the real-time PCR analysis. One of the great advantages of the OpenArray® system is that is possible to run four plates at the same time. Since the whole real-time PCR process takes about 4 h, in just one run 12,288 data points can be obtained.

The huge amount of data originated during an OpenArray® experiment resulted in the need for specific software that would enable to handle the results. For this, there are two options to process OpenArray® data. One is the software that controls the equipment and the other option is using the analytical platform available on the net. Thermo Fisher Scientific has created a Cloud where the user can upload the experiment files and analyze them with no need of installing anything on the computer. The Cloud also offers the possibility of sharing the files with another person, so that several people from different sites can work on the same files.

TaqMan® OpenArray® plates have a characteristic that may be considered as an advantage and a disadvantage at the same time, the preprinted primers. When custom plates are ordered, the company designs the primers and probes for the target sequence according the OpenArray® working conditions, and preprints them in each hole of the plate. The advantage is that the company performs quality control assays, but the problem is that the minimum order in the custom mode is 10 plates per order. Thus, if the researchers are not satisfied with the results obtained for some assays or even if they want to change the design, they have to order another 10 plates, with the subsequent economic costs.

### 2.3. SmartChip

In 2010, the last high-throughput platform was released to the market under the commercial name of SmartChip real-time PCR system (WaferGen Biosystems, CA, USA). This technology is based on a chip of 5184 individual wells. Within the three available technologies described until now, this is the one that uses the largest volume of reaction: 100 nl per reaction [95]. SmartChip has some characteristics that make it stand out from Dynamic Array™ and OpenArray®. A clear advantage of SmartChip systems is that they allow profiling of more

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than 1000 genes in a single run and in quadruplicate. These platforms would therefore enable the user to perform a screening of genes closer to the amplitude of microarrays technologies than those obtained with the other two systems. However, the number of samples that can be analyzed in this case is only one per run. In fact, the company describes the SmartChip technology in three basic steps: (1) discovery: analyzing hundreds to thousands of genes, (2) validation: analyzing tens to hundreds of genes and (3) screening: analyzing tens of genes as informative genes of a disease signature. In addition to the top model >1000 genes format of SmartChip, the company offers also customized formats for specific hypothesis-based purposes. These formats permit the inclusion of 384 to 12 samples in a single chip (**Table 3**).

| Assay configuration | Samples analyzed at the same time |
|---|---|
| 1243 | 1 (in quadruplicate) |
| 12 | 384 |
| 24 | 216 |
| 36 | 144 |
| 48 | 108 |
| 54 | 96 |
| 72 | 72 |
| 80 | 64 |
| 96 | 54 |
| 120 | 42 |
| 144 | 36 |
| 216 | 24 |
| 248 | 20 |
| 296 | 16 |
| 384 | 12 |

**Table 3.** Possible configuration of SmartChip panel for custom analysis.

The company offers three ready-to-use predesigned panels: the SmartChip Human Oncology Panel with 968 gene-specific assays in quadruplicate, the SmartChip Human microRNA Panel with 778 microRNA-specific assays in quadruplicate, and a SmartChip Bacterial Vaginosis Panel that includes 19 pathogens common in woman vaginosis. For custom assays, it is necessary to send the sequences to the company service. With this information, they design the primers according to the specific conditions of the SmartChip real-time PCR and preload them into the chip. Until now, this technology has been applied mainly in gene expression studies, working only with SYBR® Green chemistry.

The SmartChip complete system is composed of a cycler and a dispenser, with the corresponding required software. For loading or dispensing the samples on the chip, the user has two options. The SmartChip nanodispenser, to dispense only one sample in the entire chip, and designed specifically for the >1000 panels, and the SmartChip multisample nanodispenser, created for loading up to 384 samples on a single chip. In both options, the loading time is less than 10 min. Afterward, the chips are run in a SmartChip cycler, in a process that lasts 2 h. The obtained data would be analyzed with the SmartChip qPCR software.

## 3. Application fields of high-throughput real-time PCR platforms

### 3.1. Genotyping

#### *3.1.1. Basic concepts of genotyping*

One of the most promising applications of high-throughput real-time PCR in science is genotyping. This technique is based on the analysis of single nucleotide polymorphisms (SNPs) of the DNA. SNPs are single base pairs in a specific position of genomic DNA where different sequence alternatives, the alleles, exist in normal individuals in the population and the minor allele has an abundance of 1% at least [19]. The DNA is composed of four different nucleotides: adenine, guanine, thymine, and cytosine. Thus, in theory the SNPs could be tetra-allelic but in practice, this is a rare situation and normally SNPs are bi-allelic. As such, this type of genetic polymorphism is very common in genomic DNA of mammals. For example, around 1.4 million of single-nucleotide polymorphisms have been identified in the initial sequencing of the human genome, of which 60,000 are located in the coding region of genes [20]. The importance of SNPs is huge because these nucleotide modifications can be silent but they can also be responsible for the predisposition to certain diseases or be the direct cause of them. For instance, there are some studies that have related the presence of certain SNPs in individuals with diabetes, obesity, hypertension, or even cancer [21–24].

The development of next-generation sequencing (NGS) and microarrays have allowed the discovery and analysis of hundreds of thousands of SNPs [25]. However, broad screening approach has also a few drawbacks. For example, to study the presence of certain SNPs associated with a disease in a population the number of SNPs that are going to be analyzed is as important as the number of samples that can be processed simultaneously. This is exactly where real-time PCR gains importance over other technologies. In this context, there are two common techniques to detect single nucleotide polymorphisms with real-time PCR. The first option is using SYBR® Green and melting curve analysis. This method is based on detecting small differences in PCR melting (dissociation) curves after real-time PCR cycles. These differences in the dissociation curve allow to detect the differences in a single nucleotide [26]. The other option is based on the use of probes, applying two probes with different 5′-end labeling in the same reaction. Thereby, if both the fluorophores are detected it means that the sample is a heterozygote for the target DNA region. On the contrary, if the equipment only detects one of the fluorophores the sample is a homozygote. The major problem of selecting

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real-time PCR for genotyping analysis is the high costs involved in analyzing a wide range of samples and SNPs, mainly due to prices of probes and master mix. High-throughput real-time PCR platforms made PCR an ideal tool to analyze SNPs, thanks to their nanoscale working capacity and the consequent saving of reagents and samples. One of the research areas that have more directly benefited from this technology is human medicine. The previous fact is based on the possibility of including a large number of samples and assays in one day of work. This can be translated into the tracking of a large number of SNPs per sample, in order to assess their influence on the development of certain diseases.

### *3.1.2. High-throughput real-time PCR in genotyping*

In 2011, Chan et al. [27] evaluated the use of the Fluidigm 48.48 Dynamic Array biochip for genotyping purposes in clinical setting, using peripheral blood and buccal wash samples, obtaining promising results. PCR has found its place also in cancer research and it has been widely used already. In a recent study, Henríquez-Hernández et al. [28] compared the classical method of PCR restriction fragment length polymorphism (RFLP) and the high-throughput automated assay Biotrove OpenArray(®) NT cycler, using them for genotyping 118 patients with cancer. Their results suggest that the modern PCR technology is more viable and reproducible than the traditional RFLP method. They concluded that OpenArray® technology can be considered as a robust, simple handling, and user-friendly tool for genotyping purposes in the field of oncology. Henríquez-Hernández et al. [29] also used the potential of this tool to evaluate the influence of the SNPs present in a population with prostate cancer in radiation-induction toxicity. In a different study, Henríquez-Hernández [30] evaluated the influence in prostate cancer of genetic variations in genes involved in testosterone metabolism and found one polymorphism characteristic of Spanish patients. Another example is the research performed by Julin et al. [31] using TaqMan® OpenArray® genotyping assay to correlate the leukocyte telomere length and the overall risk of aggressive prostate cancer. In a similar approach, Zhao et al. [32] used genotyping assay with OpenArray® technology to evaluate the influence of SNPs of 16 antioxidant genes in the increased risk of glioma.

Apart from oncology, this technology has demonstrated its applicability in other fields of medicine. For instance, some authors have reported the influence of SNPs in the risk of developing Type 2 diabetes [33], their relationship with male fertility, or even the existence of a link between polymorphisms and smoking in male fertility [34, 35]. In a very recent work of Araujo and coworkers [36], the association of 39 genes and the etiology of nonsyndromic cleft lip and palate in Brazilian population was evaluated. In the field of dermatology, Villarreal Martínez et al. [37] found an association between six SNPs and the risk of developing psoriasis. Apart from the clear role of high-throughput real-time PCR in experimental science, another possibility of this technique in human medicine is its application in molecular diagnostic testing in a hypothesis-based manner. For instance, Arrojo Martins et al. [38] used OpenArray® plates for screening mutations involved in nonsyndromic deafness. Dionisio Tavares Niewiadonski et al. [39] applied the same approach for genetic testing of blood donors to assess the genotype frequencies of nucleotide-polymorphisms (SNPs) associated with venous thrombosis, hyperhomocysteinemia and hereditary hemochromatosis. High-throughput platforms

have also managed to find their way in pharmacogenomics research. They are very useful in this kind of studies because of the possibility of testing a wide range of SNPs with implications in drug metabolism in a fast and cheap way. In this sense, Iskakova et al. [40] studied SNPs involved in the absorption, distribution, metabolism, and excretion of multiple drugs in Kazakhs. Also, it makes possible the development of personalized treatments according to individual genetic characteristics.

Another promising application of this nanoliter-scale PCR in combination with SNPs is individual identification and paternity test. Until a few years ago, short tandem repeats (STRs) were the most common genetic markers used for individual identification, paternity, and kinship testing [41]. STR consists of a unit of 2–13 nucleotides repeated hundreds of times in a row on the DNA strand. STR analysis measures the exact number of repeating units. This fact makes STRs more informative than SNPs in genetic analysis because SNPs remain only in a nucleotide variation [42]. However, this lack of information can be solved by simply including more SNPs in the assay. As an indicative example, a number of 2–2.25 SNPs give the same information than 1 STRs [43]. In comparison to STR assays, high-throughput real-time platforms make feasible the use of SNPs for individual and paternity analysis, both for humans and animals, because of its relatively affordable price, the number of samples that can be analyzed in a run and the easier analysis of the obtained results. In a similar context, Pomeroy et al. [44] developed an OpenArray® to be used for forensic human DNA applications, with only seven SNPs for individual identification. In the same year, Wang et al. [45] showed the possibilities of other nanoliter-scale platform, the Fluidigm 48.48 Dynamic array, for genotyping purposes in human. In an interesting work by Kerr et al. [46], OpenArray® panel with 32 SNPs was used to create a pedigree of the Scottish by the analysis of 10,000 blood samples. It is also possible to find commercial arrays for human identification. In this sense, Thermo Fisher Scientific has predesigned OpenArray® for human individual identification with 32 and 64 SNPs.

Individual identification is also important in food safety to ensure traceability according to Commission Regulation (EC) 178/2002 [47]. Molecular genetic analysis and more precisely SNPs are a useful tool for this purpose. Capoferri et al. [48] used 16 SNPs to verify traceability of the meat production chain with successful results. However, this study used a 96-well plate real-time PCR assay with the consequent high consumption of reagents and had the limitation of not being capable of analyzing a large number of samples at the same time. High-throughput real-time PCR platforms solved these problems, as demonstrated by Fernández et al. [49] and Pozzi et al. [50] who developed a OpenArray® panel with 32 SNPs that allow individual and cattle rustling identification. For an analytical target of 32 SNPs, this nanoscale technology permits to analyze 96 samples per array. Besides, QuantStudio™ 12 K flex is capable of running four OpenArray® plates at the same time, in a process of only 4 h. Thus, in a working day of 8 h, 768 samples can be analyzed. This number reflects the enormous possibilities of high-through real-time PCR.

Many other areas of life science research, such as animal and plant sciences, can benefit as well from the diverse advantages of nanoliter-scale platforms. Recently, Catanese et al. [51] developed a panel of 96 SNPs with the aim of genotyping anchovy populations. It is notewor-

thy that they selected these best 96 markers from a panel of 424 SNPs using an OpenArray® platform, and then they selected Fluidigm 96.96 Dynamic Array™ for routine analysis due to economic reasons. One of the important points in agriculture is the breeding of crops with certain characteristics of productive interest. In different studies, a panel of 192 SNPs was used for the study of genetic diversity in sugar beet, to study the vigor of the genotypes, and to assess the resistance of these plants to pathogens [52–55].

To sum up, the existing literature clearly indicates the great potential of high-throughput real-time platforms for genotyping. In the future, it could become routine technique, for example, in assessing individual patients in order to test their predisposition to certain diseases through the use of predetermined SNP panels. These panels would allow an early change of habits, reducing the possibility of suffering from certain diseases such as Type 2 diabetes or obesity. In this way, nutrigenetics has become an important research field, studying how the different genetic variants of people influence their metabolism of nutrients, diet, and the diseases associated therewith [96]. The development and commercialization of SNP panels for which their influence in nutrition-related diseases has been demonstrated would be a big step forward in personalized nutrition.

### 3.2. Transcriptomics

#### *3.2.1. Basic concepts in transcriptomics*

Transcriptomics, in its many forms, refers to the study of the expression profiles of genes through the complete set of RNA transcripts (transcriptome). This revolutionary ``omic'' technology comprises different techniques as microarrays, RNA sequencing, and real-time PCR. Until now, only microarrays and RNA-NGS allowed genome-wide surveys. However, these two methods have some drawbacks. Microarrays can only detect sequences homologous to the one that is on the array, and RNA-NGS is costly and the analysis of results required highly specialized technicians. Also, RNA-NGS is perfect for blind studies but not sufficiently adequate for targeted analysis. Real-time PCR is a highly quantitative and sensitive technique suitable for different transcriptomics aims. However, 384- and 96-well plates only allow the analysis of a small number of transcripts and samples at the same time. In this context, high-throughput real-time PCR platforms represented a step forward and placed real-time PCR closer to microarrays and RNA-NGS.

#### *3.2.2. High-throughput real-time PCR in mRNA-based transcriptomics*

The most classic part of transcriptomics is the study of messenger RNA (mRNA). It has great interest in life sciences because mRNA is the intermediate step between the genes and the proteins. In this sense, the analysis of the modifications in gene expression levels through mRNA is an important tool which gives us an idea of the influence of internal and external factors in gene expression. For the quantification of mRNA levels by real-time PCR, a previous conversion of mRNA to cDNA, the template of PCR, is needed. This quantification could absolute or relative to a control gene. Although probes are more specific than SYBR® Green, this last chemistry is normally the option chosen for gene expression, mainly due to economic

reasons. The first studies evaluating the possibilities of high-throughput real time PCR platforms were performed for gene expression assays by Morrison et al. [14], who evaluated the possibilities of OpenArray® technology. Although nowadays OpenArray® technology works with TaqMan® probes, the first study used SYBR® Green chemistry. These researchers reported already the need of increasing sample concentration through volume miniaturization, in order to maintain a constant number of target molecules in the reaction. In 2008, Spurgeon et al. [9] evaluated the 48.48 Dynamic Array™ chip for gene expression purposes. Thanks to their studies, a preamplification step of cDNA samples was added for these dynamic platforms before real-time PCR analysis, with the aim of maintaining a high level of target molecules in the reaction. However, this preamplification reaction means that a new step needs to be included in the experimental high-throughput workflow, increasing the total processing time and costs.

As in the case of genotyping applications, in transcriptomics human medicine was the first field benefited with the development of nanoliter-scale real-time PCR. In 2012, Chen et al. [56] used a SmartChip real-time PCR platform to evaluate gene expression in colon cancer, while Javelaud et al. [57] used OpenArray® technology to evaluate the expression of hundreds of genes in melanoma cell-lines. An interesting application was carried out by Li et al. [58] using OpenArray® to quantify gene expression from degraded RNA of formalin-fixed paraffin-embedded tumors. They concluded that this platform could be optimized for gene expression in this kind of preserved samples, opening an interesting avenue of research in cancer. In breast cancer, Song et al. [59] evaluated the expression of 1243 mRNA in breast tissue using SmartChip, showing the potential of this technology to analyze the transcriptome in a similar level of microarrays. However, the SmartChip of 1243 only allows the analysis of one sample in quadruplicate (**Table 3**). The results obtained with OpenArray® plates led the team of Ciarloni et al. [60] to validate a panel of 29 genes with interest in colorectal cancer, starting with an initial panel of 667 candidate genes. In a similar way, Kim et al. [61] validated a panel of eight promising genes in patients with metastatic renal cell carcinoma from an initial panel of 424 genes. Other examples of the application of high-throughput platforms in medicine studies include the work from Patel et al. [62], who employed an OpenArray® panel of 631 genes to evaluate gene expression in human embryonic and induced pluripotent stem cells. Koh et al. [63] employed Fluidigm 48.48 Dynamic Array™ for monitoring the tissue-specific global gene expression in humans thought circulating cell-free RNA in the blood. Thus, this real-time method could be employed for transcriptome analysis of humans and evaluate their global health without the need for invasive tissue sampling.

The work carried out by Forreryd et al. in 2014 [64] deserves special mention. They assessed different high-throughput gene expression platforms to predict skin sensitization without the use of animals. The study used Fluidigm® 96.96 Dynamic Array™ and OpenArray® with nCounter®. The OpenArray® system demonstrated the easier protocol and the less time required for analysis, obtaining the results in 3 h, in contrast to the 7 h of Fluidigm® 96.96 Dynamic Array™ and the 22 h of nCounter®. However, Fluidigm® 96.96 Dynamic Array™ had a superior sensitivity due to the additional cDNA preamplification step implemented prior to PCR and it was also the cheapest option. Likewise, the authors suggested the importance

of selecting appropriate reference genes because one of the two reference genes employed in the study did not amplified well. The final authors' conclusions reflected the great potential of nonarray-based platforms for reducing assay costs and increasing sample throughput. Finally, the results of this comparative study highlight the importance of establishing clear priorities in terms of sensibility, analytical time, and costs when acquiring a high-throughput platform.

Besides medicine, other research fields have also benefited from the capacities of high-throughput real-time PCR. For instance, de Boer et al. [65] used Fluidigm 48.48 Dynamic Array™ to evaluate the potential of gene expression as a biomarker of chemical contamination, using a species of arthropod often employed for ecotoxicological testing. They concluded that the combination of these analytical platforms and multivariate analysis could be a valuable tool in ecotoxicology, combining high throughput capacity with analytical sensitivity. In a similar approach, Harty et al. [66] employed a Fluidigm 96.96 Dynamic Array™ to evaluate expression profiles of adhesion G protein coupled receptor in zebrafish and their possibilities as models in human medicine. In another animal model, Tosches et al. [97] employed a Fluidigm 48.48 Dynamic Array™ to evaluate the pathway of melatonin using a zooplankton. These platforms also found their applicability in animal production as demonstrated by Robic et al. [67], who evaluated the influence of an SNP in the transcripts of quantitative trait loci (QTL), responsible for the accumulation of androsterone in boar fat. In aquaculture, Bonacic et al. [68] employed OpenArray® plates to evaluate gene expression in lipid metabolism with diets rich on omega-3 and omega-6 in fish. Other applications include immunological studies and virology. The work from Rosa et al. [69] reported the usefulness of Fluidigm 96.96 Dynamic Array™ to evaluate different transcriptional patterns in the cattle tick *Rhipicephalus microplus* in response to microbial challenge. Their findings demonstrated that certain pathogens cause downregulation of immune-related genes, favoring their survival and vector colonization. In parallel, Tierney et al. [70] used a Fluidigm 48.48 Dynamic Array™ to analyze the transcript patterns of Epstein-Barr virus with the aim of know more about the first phases of infection.

### *3.2.3. High-throughput real-time PCR in microRNA-based transcriptomics*

The discovery of microRNAs (miRNAs) has changed the previous concepts of gene expression regulation. These small noncoding RNA molecules with a length between 21 and 25 nucleotides and they are found in plants, animals, and some viruses [71]. In human, miRNA are found in plasma, urine, cerebrospinal fluid, and saliva and have an important function as post-transcriptional regulators of gene expression. Their different levels have been associated with a wide range of human diseases [72] and have potential as biomarkers [73]. There are many technologies for the measurement of miRNAs such as microarrays, NGS, and real-time PCR, being the last one and the most sensitive and reproducible method. Also, the releasing of high-throughput real-time PCR platforms have allowed a reduction of costs and minimization of the time required for detection of broad miRNA signatures.

Some studies have demonstrated the applicability of nanoliter-scale PCR in the analysis of microRNA. Jang et al. [74] made an interplatform comparative study using conventional real-time PCR, microarrays and Fluidigm 48.48/96.96 Dynamic Array™, concluding that these

nanoscale platforms could be used to develop cost-effective and customized assays, with rapid turnaround for profiling and validating of miRNA expression. Farr et al. [13] performed a similar study comparing the usefulness of high-throughput platforms for validation of a circulation microRNA signature in diabetic retinopathy. An interesting point of this work is the direct comparison of the two principal high-throughput real-time PCR platforms commercially available: OpenArray® and Dynamic Array™. Authors found that OpenArray® system was the most reproducible platform with less inter- and intrarun variations and had a more user-friendly software for further analyses. One of the main problems of Dynamic Array™ is the replicate variability, caused by their low-volume assay. OpenArray® use the double of the volume used in Dynamic Array™, and in this sense researchers found that variability increased in parallel with volume reductions.

This technology was rapidly introduced in human medicine because of their potential uses in this field. Keller et al. [75] evaluated the expression profiles of 863 microRNAs in 454 blood samples using SmartChip real-time PCR system and found a disease association with microRNAs profiles. Their potential as biomarkers was shown by Mooney et al. [76], who found minimal variations in miRNA profiles of healthy volunteers according to sex and sample timing using OpenArray® plates. Given their value as biological markers, the field of oncology research soon adopted this methodology to screen the microRNA profiles of this disease. Thus, Hudson et al. [77] used the commercially available TaqMan® OpenArray® Human MicroRNA Panel and found overexpression of miR-10a and miR-375 in medullary thyroid carcinoma. Using the same panel, others researchers achieve great results for lung cancer detection [78], for rectal cancer [79], and brain metastasis in mutant lung cancers [80]. With Fluidigm 96.96 Dynamic Array™, Kara et al. [81] discovered the downregulation of 18 miRNAs in patients with colorectal cancer. Research on other human diseases has also used miRNA molecules as biomarkers. The microRNA profiles have been related to human late-onset Fuchs' dystrophy [82], Alzheimer [83] or Crohn disease [84]. It is remarkable that most of these studies used the TaqMan® OpenArray® Human microRNA Panel.

The results of the studies cited before show the great importance of miRNA in human diseases and how their variation could be a good biomarker for diagnostic purposes. Most of these studies used the TaqMan® OpenArray® Human microRNA Panel for the screening of microRNA, showing its potential. However, this panel only allows the analysis of three samples simultaneously, eliminating one of the mains advantages of high-throughput real-time PCR platforms. So, the next step would be the validation of panels with fewer microRNA targets focused on specific diseases, giving the possibility of introducing more samples at the same time. Apart from diagnostic of diseases, microRNA also has potential in other fields. Benson et al. [85] observed that rifampin treatment modifies the microRNA profiles. Thus, it is possible that the administration of determinate substances causes a variation of miRNA profile in humans allowing their use as a new-generation anti-doping biomarkers [86]. This knowledge can be transferred to food safety and by employing miRNA as biomarkers in the administration of banned substances in animal production, thus developing, for instance, a panel of miRNA for each drug [71].

### 3.3. Detection of pathogens and antimicrobial and virulence genes

One of the great applications of high-throughput real-time PCR platforms is the detection of pathogen for medical and food safety purposes. The development of real-time PCR was a major breakthrough in microbiological analyses. The classical microbiology used large incubation times with the need of multistep procedures for the detection of some bacteria. Real-time PCR avoids the waiting time caused for this incubation. Likewise, real-time PCR allows quantification of genes with great importance, such as resistance or virulence genes.

The introduction of the technology described in this chapter allows performing a large number of assays in a large number of samples. These achievements have a great importance in hospital and food safety microbiology labs. In 2008, Stedtfeld et al. [18] developed an OpenArray® plate to detect human pathogens based on the detection and quantification of virulence factors of these pathogens. Another step forward in microbiological analysis was the OpenArray® panel developed by Gonzalez et al. [87], which allow the identification, including virulotype, and subtypes of O157 and non-O157 enterohemorrhagic *Escherichia coli* with a panel of 28 genes in a single anaylisis. All of this will make the characterization of Enterohemorrhagic *Escherichia coli* possible in less than 4 h, avoiding the use of large number of conventional PCR assays, saving time and money. In a similar study, Dhoubhadel et al. [88] used the Fluidigm 48.48 Dynamic Array™ for molecular serotyping and serotype-specific quantification of *Streptococcus pneumoniae*. Researchers found a good correlation between conventional serotyping and serotype by nanofluidic PCR system. The use of this high-throughput technology brings great saving of time but it also has the drawback of needing a large number of samples for the analysis. In this context, it is important to mention that sometimes microbiology labs do not have a large number of samples for a specific assay.

In the work of Ishi et al. [89], Dynamic Array™ platform and TaqMan® Probes were used for the simultaneous quantification of food and waterborne pathogens as *Salmonella* Typhimurium, *Listeria monocitogenes, Vibrio parahaemolyticus, Clostridium perfringens* or even viruses. One of the advantages of use Dynamic Array™ for pathogen detection is the possibility of changing which microorganism to detect according to the current needs. The existing results show the possibility of routine monitoring of multiple pathogens in large number of food and water samples. Grigorenko et al. [90] developed an OpenArray® panel to detect bloodborne pathogens as viruses, bacteria, or parasites in humans. The aim was to detect these pathogens in blood donors. With this technology they were able to detect at least 10 cell/ml blood of *Trypanosoma cruzi* and 10,000 cell/ml blood of *Escherichia coli*, a detection limit that seems too high for *Escherichia coli*.

On certain occasions, instead of detecting pathogenic microorganism, it is interesting to detect virulence and resistance genes. With regard to this, Looft et al. [91] used OpenArray® to evaluate the change in resistance genes in swine intestinal microbiome in pigs fed with feed supplemented with antibiotics. Tseng et al. [92] evaluated the virulence gene content of Shiga Toxin-Producing *Escherichia coli* from finishing swine using Fluidigm 96.96 Dynamic Array™. The antibiotic resistoma in sewage sludge composting was evaluated by Su et al. [93] using the Smart-Chip real-time PCR system and a chip containing almost 300 antibiotic resistance genes. Similarly, Xu et al. [94] screened 285 antibiotic resistance genes in drinking water

treatment plants and distribution systems. These studies give an idea of the potential of these platforms for wide screening, as it is possible to evaluate most of the important antibiotic resistance genes in one run. All of this permits to establish a relationship between this resistoma and other factors such as microbial community or water treatments.

## 4. Conclusions

The development of high-throughput real-time PCR platforms was a big step in real-time PCR. There was an important development from the 384 reactions that could be performed at the same time in conventional systems to the 12,288 reactions that can be performed in one run in nanoliter-scale platforms. However, this nanoliter real-time PCR has yet some drawbacks in comparison to other techniques. While in NGS it is possible to analyze unknown sequences, with real-time it is necessary to know beforehand the target sequence to design the primers and the probes. While in microarray technology it is possible to analyze thousands of sequences at the same time, the highest target with these nanoliter platforms is 1200 different sequences. However high-throughput real-time PCR platforms have great advantages, achieving operational improvements that the other platforms are not capable of reaching. They have a great facility for processing large number of samples, the sample consumption is less than in other platforms and the time necessary to get the results is at most 7 h with easy protocols. Also, the analysis of the results is relatively easy and the price is relatively cheap. But without any doubt the great advantage of these platforms is their specificity and their sensibility, which makes them the gold standard in quantification analysis. Although, fast mode is common in real-time PCR, high-throughput platforms do not work in this mode. Therefore, the introduction of fast mode would be another big step because it would increase the number of assays that can be performed in one day. Another clear challenge is to increase the number of assays and samples that can be analyzed at the same time. Even though one platform can perform 1200 assays in one run, this is only for one sample. Being capable of performing this number of assays for a large number of samples would place real-time PCR at the same level than others platforms for genotyping and transcriptomics analyses. The future of these platforms goes through the design of plates for routine applications in medicine, nutrition, or food safety. However, in food safety and microbiology this technique has been under used until now. Thus, the development of plates that could analyze the microbiome of multiple types of samples could be a great advance approaching this technique to NGS for microbiome analysis.

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## References

[1] Stevens J, Heid CA, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res. 1996;6:986–994.

[2] Zhou Y, Utsunomiya Y, Xu L, Hay EHA, Bickhart D, Carvalheiro R, et al. Genome-wide CNV analysis reveals variants associated with growth traits in *Bos indicus*. BMC Genomics. 2016;17:419.

[3] Salomon Torres R, Villa Angulo R, Villa Angulo C. Analysis of copy number variations in Mexican Holstein cattle using axiom genome-wide Bos 1 array. Genom Data. 2016;7:97–100.

[4] Onken M, Worley L, Tuscan M, Harbour JW. An accurate, clinically feasible multi-gene expression assay for predicting metastasis in uveal melanoma. J Mol Diagn. 2010;12:461–468.

[5] del Pozo CH, Maria Calvo R, Vesperinas Garcia G, Gomez Ambrosi J, Fruehbeck G, Calvo R, et al. Expression profile in omental and subcutaneous adipose tissue from lean and obese subjects. Repression of lipolytic and lipogenic genes. Obes Surg. 2011;21:633–643.

[6] Ishii S, Segawa T, Okabe S. Simultaneous quantification of multiple food- and waterborne pathogens by use of microfluidic quantitative PCR. Appl Environ Microbiol. 2013;79:2891–2898.

[7] Hu W, Park C. Measuring microRNA expression in mouse hematopoietic stem cells. Methods Mol Biol. 2014;1185:121–140.

[8] Korenkova V, Scott J, Novosadova V, Jindrichova M, Langerova L, Korenková V, et al. Pre-amplification in the context of high-throughput qPCR gene expression experiment. BMC Mol Biol. 2015;16:5.

[9] Spurgeon S, Jones R, Ramakrishnan R, Seoighe C. High throughput gene expression measurement with real time PCR in a microfluidic dynamic array. PLoS One. 2008;3:e1662.

[10] Fluidigm [Internet]. 2016. Available from: https://www.fluidigm.com/assays [Accessed: 2016/07/18].

[11] Fluidigm [Internet]. 2016. Available from: https://www.fluidigm.com/ifcs [Accessed: 2016/07/18].

[12] Fedick A, Su J, Jalas C, Northrop L, Devkota B, Ekstein J, et al. High-throughput carrier screening using TaqMan allelic discrimination. PLoS One. 2013;8:e59722.

[13] Farr R, Januszewski A, Joglekar M, Liang H, McAulley A, Hewitt A, et al. A comparative analysis of high-throughput platforms for validation of a circulating microRNA signature in diabetic retinopathy. Sci Rep. 2015;5:10375.

[14] Morrison T, Hurley J, Garcia J, Yoder K, Katz A, Roberts D, et al. Nanoliter high throughput quantitative PCR. Nucleic Acids Res. 2006;34:e123.

[15] van Doorn R, Szemes M, Bonants P, Kowalchuk G, Salles J, Ortenberg E, et al. Quantitative multiplex detection of plant pathogens using a novel ligation probe-based system coupled with universal, high-throughput real-time PCR on OpenArrays™. BMC Genomics. 2007;8:276.

[16] Thermo Fisher Scientific [Internet]. 2016. Available from: https://www.thermofisher.com/es/es/home/life-science/pcr/real-time-pcr/real-time-openarray/open-array-panel.html [Accessed: 2016/07/18].

[17] Thermo Fisher Scientific [Internet]. 2016. Available from: https://www.thermofisher.com/order/catalog/product/4461104 [Accessed: 2016/07/18].

[18] Stedtfeld R, Baushke S, Tourlousse D, Miller S, Stedtfeld T, Gulari E, et al. Development and experimental validation of a predictive threshold cycle equation for quantification of virulence and marker genes by high-throughput nanoliter-volume PCR on the OpenArray platform. Appl Environ Microbiol. 2008;74:3831–3838.

[19] Brookes A. The essence of SNPs. Gene. 1999;234:177–186.

[20] Sachidanandam R, Weissman D, Schmidt S, Kakol J, Stein L, Marth G, et al. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature. 2001;409:928–933.

[21] Hara K, Boutin P, Mori Y, Tobe K, Dina C, Yasuda K, et al. Genetic variation in the gene encoding adiponectin is associated with an increased risk of type 2 diabetes in the Japanese population. Diabetes. 2002;51:536–540.

[22] Iwashima Y, Katsuya T, Ishikawa K, Ouchi N, Ohishi M, Sugimoto K, et al. Hypoadiponectinemia is an independent risk factor for hypertension. Hypertension. 2004;43:1318–1323.

[23] Menzaghi C, Ercolino T, Di Paola R, Berg A, Warram J, Scherer P, et al. A haplotype at the adiponectin locus is associated with obesity and other features of the insulin resistance syndrome. Diabetes. 2002;51:2306–2312.

[24] Goode E, Ulrich C, Potter J. Polymorphisms in DNA repair genes and associations with cancer risk. Cancer Epidemiol Biomarkers Prev. 2002;11:1513–1530.

[25] Biesecker L, Spinner N. A genomic view of mosaicism and human disease. Nat Rev Genet. 2013;14:307–320.

[26] Simko I. High-resolution DNA melting analysis in plant research. Trends Plant Sci. 2016;21:528–537.

[27] Chan M, Loh T, Law H, Yoon C, Than S, Chua J, et al. Evaluation of nanofluidics technology for high-throughput SNP genotyping in a clinical setting. J Mol Diagn. 2011;13:305–312.

[28] Henríquez-Hernández LA, Valenciano A, Lloret M, Riveros Perez A, Herrera Ramos E, Lara PC. High-throughput genotyping system as a robust and useful tool in oncology: experience from a single institution. Biologicals. 2013;41:424–429.

[29] Henríquez-Hernández LA, Valenciano A, Foro Arnalot P, Alvarez-Cubero MJ, Manuel Cozar J, Suárez-Novo JF, et al. Polymorphisms in DNA-repair genes in a cohort of prostate cancer patients from different areas in Spain: heterogeneity between populations as a confounding factor in association studies. PLoS One. 2013;8:e69735.

[30] Henríquez-Hernández LA, Valenciano A, Foro Arnalot P, Alvarez Cubero M, Suárez-Novo JF, Castells-Esteve M, et al. Genetic variations in genes involved in testosterone metabolism are associated with prostate cancer progression: a Spanish multicenter study. Urol Oncol. 2015;33:331.e1–331.e7.

[31] Julin B, Shui I, Heaphy CM, Joshu CE, Meeker AK, Giovannucci E, et al. Circulating leukocyte telomere length and risk of overall and aggressive prostate cancer. Br J Cancer. 2015;112:769–776.

[32] Zhao P, Zhao L, Zou P, Lu A, Liu N, Yan W, et al. Genetic oxidative stress variants and glioma risk in a Chinese population: a hospital-based case–control study. BMC Cancer. 2012;12:617.

[33] Lu F, Qian Y, Li H, Dong M, Lin Y, Du J, et al. Genetic variants on chromosome 6p21.1 and 6p22.3 are associated with type 2 diabetes risk: a case–control study in Han Chinese. J Hum Genet. 2012;57:320–325.

[34] Ji G, Yan L, Liu W, Huang C, Gu A, Wang X. Polymorphisms in double-strand breaks repair genes are associated with impaired fertility in Chinese population. Reproduction. 2013;145:463–470.

[35] Ji G, Yan L, Liu W, Qu J, Gu A. OGG1 Ser326Cys polymorphism interacts with cigarette smoking to increase oxidative DNA damage in human sperm and the risk of male infertility. Toxicol Lett. 2013;218:144–149.

[36] Secolin R, Felix T, de Souza L, Barros Fontes M, de Araujo T, Félix T, et al. A multicentric association study between 39 genes and nonsyndromic cleft lip and palate in a Brazilian population. J Craniomaxillofac Surg. 2016;44:16–20.

[37] Villarreal Martinez A, Gallardo Blanco H, Cerda Flores R, Torres Munoz I, Gomez Flores M, et al. Candidate gene polymorphisms and risk of psoriasis: a pilot study. Exp Ther Med. 2016;11:1217–1222.

[38] Arrojo Martins F, Ramos P, Svidnicki, Maria Carolina Costa Melo, Castilho A, Martins FT, Sartorato E. Optimization of simultaneous screening of the main mutations involved in non-syndromic deafness using the TaqMan® OpenArray™ genotyping platform. BMC Med Genet. 2013;14:112.

[39] Dionisio Tavares Niewiadonski V, Dos Santos Bianchi, Juliana Vieira, de Almeida-Neto C, Gaburo N, Sabino E, Carrasco-Avino G, et al. Evaluation of a high throughput method for the detection of mutations associated with thrombosis and hereditary hemochromatosis in Brazilian blood donors. PLoS One. 2015;10:e0125460.

[40] Iskakova A, Romanova A, Aitkulova A, Sikhayeva N, Zholdybayeva E, Ramanculov E. Polymorphisms in genes involved in the absorption, distribution, metabolism, and excretion of drugs in the Kazakhs of Kazakhstan. BMC Genet. 2016;17:23.

[41] Mo S, Liu Y, Wang S, Bo X, Li Z, Chen Y, et al. Exploring the efficacy of paternity and kinship testing based on single nucleotide polymorphisms. Forensic Sci Int Genet. 2016;22:161–168.

[42] Allen A, Taylor M, McKeown B, Curry A, Lavery J, Mitchell A, et al. Compilation of a panel of informative single nucleotide polymorphisms for bovine identification in the Northern Irish cattle population. BMC Genet. 2010;11:5.

[43] Fernandez M, Goszczynski D, Liron J, Villegas Castagnasso E, Fernández M, Lirón J, et al. Comparison of the effectiveness of microsatellites and SNP panels for genetic identification, traceability and assessment of parentage in an inbred Angus herd. Genet Mol Biol. 2013;36:185–191.

[44] Pomeroy R, Duncan G, Sunar Reeder B, Ortenberg E, Ketchum M, Wasiluk H, et al. A low-cost, high-throughput, automated single nucleotide polymorphism assay for forensic human DNA applications. Anal Biochem. 2009;395:61–67.

[45] Wang J, Lin M, Crenshaw A, Hutchinson A, Hicks B, Yeager M, et al. High-throughput single nucleotide polymorphism genotyping using nanofluidic Dynamic Arrays. BMC Genomics. 2009;10:561.

[46] Kerr S, Campbell A, Murphy L, Hayward C, Jackson C, Wain L, et al. Pedigree and genotyping quality analyses of over 10,000 DNA samples from the generation Scotland: Scottish family health study. BMC Med Genet. 2013;14:38.

[47] Anonymous. Regulation (EC) No 178/2002 of the European Parliament and of the Council, of 28 January2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJEC. 2016;31:1–24.

[48] Capoferri R, Bongioni G, Galli A, Aleandri R. Genetic control of conventional labeling through the bovine meat production chain by single nucleotide polymorphisms using real-time PCR. J Food Prot. 2006;69:1971–1977.

[49] Fernandez M, Rogberg Munoz A, Liron J, Goszczynski D, Fernández M, Rogberg Muñoz A, et al. Effectiveness of single-nucleotide polymorphisms to investigate cattle rustling. J Forensic Sci. 2014;59:1607–1613.

[50] Pozzi A, Previtali C, Cenadelli S, Gandini L, Galli A, Bongioni G. Genetic traceability of cattle using an OpenArray genotyping platform. Anim Genet. 2016;47:133–134.

[51] Catanese G, Montes I, Iriondo M, Estonba A, Iudicone D, Procaccini G. High resolution SNPs selection in Engraulis encrasicolus through Taqman OpenArray. Fish Res. 2016;177:31–38.

[52] Stevanato P, Broccanello C, Biscarini F, Del Corvo M, Sablok G, Panella L, et al. High-throughput RAD-SNP genotyping for characterization of sugar beet genotypes. Plant Mol Biol Rep. 2013;32:691–696.

[53] Biscarini F, Stevanato P, Broccanello C, Stella A, Saccomani M. Genome-enabled predictions for binomial traits in sugar beet populations. BMC Genet. 2014;15:87.

[54] Stevanato P, Trebbi D, Panella L, Richardson K, Broccanello C, Pakish L, et al. Identification and validation of a SNP marker linked to the gene HsBvm-1 for nematode resistance in sugar beet. Plant Mol Biol Rep. 2015;33:474–479.

[55] Stevanato P, De Biaggi M, Broccanello C, Biancardi E, Saccomani M. Molecular genotyping of "Rizor" and "Holly" rhizomania resistances in sugar beet. Euphytica. 2015;206:427–431.

[56] Chen H, Edwards R, Tucci S, Bu P, Milsom J, Lee S, et al. Chemokine 25-induced signaling suppresses colon cancer invasion and metastasis. J Clin Invest. 2012;122:3184–3196.

[57] Javelaud D, Dewolf D, Grigorenko E, Mauviel A. Simultaneous TaqMan® Q-PCR quantitation of hundreds of cancer-related genes in melanoma cell lines using the OpenArray® technology. J Invest Dermatol. 2012;132:S125.

[58] Li P, Grigorenko E, Funari V, Enright E, Zhang H, Kim H. Evaluation of a high-throughput, microfluidics platform for performing TaqMan™ qPCR using formalin-fixed paraffin-embedded tumors. Bioanalysis. 2013;5:1623–1633.

[59] Song D, Cui M, Zhao G, Fan Z, Nolan K, Yang Y, et al. Pathway-based analysis of breast cancer. Am J Transl Res. 2014;6:302–311.

[60] Ciarloni L, Hosseinian S, Monnier Benoit S, Imaizumi N, Dorta G, Ruegg C, et al. Discovery of a 29-gene panel in peripheral blood mononuclear cells for the detection of colorectal cancer and adenomas using high throughput real-time PCR. PLoS One. 2015;10:e0123904.

[61] Kim H, Halabi S, Li P, Mayhew G, Simko J, Nixon A, et al. A molecular model for predicting overall survival in patients with metastatic clear cell renal carcinoma: results from CALGB 90206 (Alliance). EBioMedicine. 2015;2:1814–1820.

[62] Patel S, Wu Y, Bao Y, Mancebo R, Au Young J, Grigorenko E. TaqMan® OpenArray® high-throughput transcriptional analysis of human embryonic and induced pluripotent stem cells. Methods Mol Biol. 2013;997:191–201.

[63] Koh W, Pan W, Gawad C, Fan HC, Kerchner G, Wyss Coray T, et al. Noninvasive in vivo monitoring of tissue-specific global gene expression in humans. Proc Natl Acad Sci U S A. 2014;111:7361–7366.

[64] Forreryd A, Johansson H, Albrekt A, Lindstedt M. Evaluation of high throughput gene expression platforms using a genomic biomarker signature for prediction of skin sensitization. BMC Genomics. 2014;15:379.

[65] de Boer M, Berg S, Timmermans, Martijn J T N, den Dunnen J, van Straalen N, Ellers J, et al. High throughput nano-liter RT-qPCR to classify soil contamination using a soil arthropod. BMC Mol Biol. 2011;12:11.

[66] Harty B, Krishnan A, Sanchez N, Schioeth H, Monk K, Schiöth H. Defining the gene repertoire and spatiotemporal expression profiles of adhesion G protein-coupled receptors in zebrafish. BMC Genomics. 2015;16:62.

[67] Robic A, Larzul C, Grindflek E, Chevillon P, Hofer A, Fève K, et al. Molecular characterization of the porcine TEAD3 (TEF-5) gene: examination of a promoter mutation as the causal mutation of a quantitative trait loci affecting the androstenone level in boar fat. J Anim Breed Genet. 2012;129:325–335.

[68] Bonacic K, Campoverde C, Sastre M, Hachero Cruzado I, Ponce M, Manchado M, et al. Mechanisms of lipid metabolism and transport underlying superior performance of Senegalese sole (Solea senegalensis, Kaup 1858) larvae fed diets containing n-3 polyunsaturated fatty acids. Aquaculture. 2016;450:383–396.

[69] Rosa R, Mesquita R, Kalil S, Pohl P, Capelli Peixoto J, Braz G, et al. Exploring the immune signalling pathway-related genes of the cattle tick Rhipicephalus microplus: from molecular characterization to transcriptional profile upon microbial challenge. Dev Comp Immunol. 2016;59:1–14.

[70] Tierney R, Shannon Lowe C, Fitzsimmons L, Bell A, Rowe M. Unexpected patterns of Epstein-Barr virus transcription revealed by a high throughput PCR array for absolute quantification of viral mRNA. Virology. 2015;474:117–130.

[71] Ambros V. microRNAs: tiny regulators with great potential. Cell. 2001;107:823–826.

[72] Tanriverdi K, Kucukural A, Mikhalev E, Tanriverdi S, Lee R, Ambros V, et al. Comparison of RNA isolation and associated methods for extracellular RNA detection by high-throughput quantitative polymerase chain reaction. Anal Biochem. 2016;501:66–74.

[73] Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci. 2008;105:10513–10518.

[74] Jang J, Simon V, Feddersen R, Rakhshan F, Schultz D, Zschunke M, et al. Quantitative miRNA expression analysis using Fluidigm microfluidics dynamic arrays. BMC Genomics. 2011;12:144.

[75] Keller A, Leidinger P, Bauer A, ElSharawy A, Haas J, Backes C, et al. Toward the blood-borne miRNome of human diseases. Nat Methods. 2011;8:841–843.

[76] Mooney C, Raoof R, El Naggar H, Sanz Rodriguez A, Jimenez Mateos E, Henshall D, et al. High throughput qPCR expression profiling of circulating microRNAs reveals minimal sex- and sample timing-related variation in plasma of healthy volunteers. PLoS One. 2015;10:e0145316.

[77] Hudson J, Duncavage E, Tamburrino A, Salerno P, Xi L, Raffeld M, et al. Overexpression of miR-10a and miR-375 and downregulation of YAP1 in medullary thyroid carcinoma. Exp Mol Pathol. 2013;95:62–67.

[78] Nadal E, Truini A, Nakata A, Lin J, Reddy R, Chang A, et al. A novel serum 4-microRNA signature for lung cancer detection. Sci Rep. 2015;5:12464.

[79] Eriksen AHM, Andersen R, Pallisgaard N, Sorensen F, Jakobsen A, Hansen TF, et al. MicroRNA expression profiling to identify and validate reference genes for the relative quantification of microRNA in rectal cancer. PLoS One. 2016;11:e0150593.

[80] Remon J, Alvarez Berdugo D, Majem M, Moran T, Reguart N, Lianes P. miRNA-197 and miRNA-184 are associated with brain metastasis in EGFR-mutant lung cancers. Clin Transl Oncol. 2016;18:153–159.

[81] Kara M, Yumrutas O, Ozcan O, Celik O, Bozgeyik E, Bozgeyik I, et al. Differential expressions of cancer-associated genes and their regulatory miRNAs in colorectal carcinoma. Gene. 2015;567:81–86.

[82] Matthaei M, Hu J, Kallay L, Eberhart C, Cursiefen C, Qian J, et al. Endothelial cell microRNA expression in human late-onset Fuchs' dystrophy. Invest Ophthalmol Vis Sci. 2014;55:216–225.

[83] Denk J, Boelmans K, Siegismund C, Lassner D, Arlt S, Jahn H, et al. MicroRNA profiling of CSF reveals potential biomarkers to detect Alzheimer`s disease. PLoS One. 2015;10:e0126423.

[84] Jensen M, Andersen R, Christensen H, Nathan T, Kjeldsen J, Madsen J. Circulating microRNAs as biomarkers of adult Crohn's disease. Eur J Gastroenterol Hepatol. 2015;27:1–1044.

[85] Benson EA, Eadon MT, Desta Z, Liu Y, Lin H, Burgess KS, et al. Rifampin regulation of drug transporters gene expression and the association of microRNAs in human hepatocytes. Front Pharmacol. 2016;7:111.

[86] Leuenberger N, Robinson N, Saugy M. Circulating miRNAs: a new generation of anti-doping biomarkers. Anal Bioanal Chem. 2013;405:9617–9623.

[87] Gonzales T, Kulow M, Park D, Kaspar C, Anklam K, Pertzborn K, et al. A high-throughput open-array qPCR gene panel to identify, virulotype, and subtype O157 and non-O157 enterohemorrhagic Escherichia coli. Mol Cell Probes. 2011;25:222–230.

[88] Dhoubhadel B, Yasunami M, Yoshida L, Thi HAN, Morimoto K, Dang DA, et al. A novel high-throughput method for molecular serotyping and serotype-specific quantification of Streptococcus pneumoniae using a nanofluidic real-time PCR system. J Med Microbiol. 2014;63:528–539.

[89] Ishii S, Kitamura G, Segawa T, Kobayashi A, Miura T, Sano D, et al. Microfluidic quantitative PCR for simultaneous quantification of multiple viruses in environmental water samples. Appl Environ Microbiol. 2014;80:7505–7511.

[90] Grigorenko E, Fisher C, Patel S, Chancey C, Rios M, Nakhasi H, et al. Multiplex screening for blood-borne viral, bacterial, and protozoan parasites using an OpenArray platform. J Mol Diagn. 2014;16:136–144.

[91] Looft T, Johnson T, Allen H, Bayles D, Alt D, Stedtfeld R, et al. In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci U S A. 2012;109:1691–1696.

[92] Tseng M, Fratamico P, Bagi L, Delannoy S, Fach P, Manning S, et al. Diverse virulence gene content of Shiga toxin-producing Escherichia coli from finishing swine. Appl Environ Microbiol. 2014;80:6395–6402.

[93] Su J, Wei B, Ou Yang W, Huang F, Zhao Y, Xu H, et al. Antibiotic resistome and its association with bacterial communities during sewage sludge composting. Environ Sci Technol. 2015;49:7356–7363.

[94] Xu L, Ouyang W, Qian Y, Su C, Su J, Chen H. High-throughput profiling of antibiotic resistance genes in drinking water treatment plants and distribution systems. Environ Pollut. 2016;213:119–126.

[95] WaferGen [Internet]. 2016. Available from: http://www.wafergen.com/products/smartchip-real-time-pcr [Accessed: 2016/07/18]

[96] Alfredo Martinez J, Milagro F, Martínez JA. Genetics of weight loss: A basis for personalized obesity management. Trends Food Sci Technol. 2015;42:97–115.

[97] Tosches M, Bucher D, Vopalensky P, Arendt D. Melatonin Signaling Controls Circadian Swimming Behavior in Marine Zooplankton. Cell. 2014;159:46–57.