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Advances in the Molecular Detection of ABC Transporters Involved in Multidrug Resistance in Cancer

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Abstract

ATP-Binding Cassette (ABC) transporters are important mediators of multidrug resistance (MDR) in patients with cancer. Although their role in MDR has been extensively studied *in vitro*, their value in predicting response to chemotherapy has yet to be fully determined. Establishing a molecular diagnostic assay dedicated to the quantitation of ABC transporter genes is therefore critical to investigate their involvement in clinical MDR. In this article, we provide an overview of the methodologies that have been applied to analyze the mRNA expression levels of ABC transporters, by describing the technology, its pros and cons, and the experimental protocols that have been followed. We also discuss recent studies performed in our laboratory that assess the ability of the currently available high-throughput gene expression profiling platforms to discriminate between highly homologous genes. This work led to the conclusion that high-throughput TaqMan-based qRT-PCR platforms provide standardized clinical assays for the molecular detection of ABC transporters and other families of highly homologous MDR-linked genes encoding, for example, the uptake transporters (solute carriers-SLCs) and the phase I and II metabolism enzymes.

Keywords

ABC transporters; clinical diagnosis; personalized medicine; TaqMan-based qRT-PCR

1. INTRODUCTION

ATP-Binding Cassette (ABC) transporters mediate the active efflux transport of a variety of structurally and mechanistically unrelated compounds across biological membranes of the cell and numerous organelles [1,2,3]. These transmembrane proteins are involved in a wide variety of cellular processes in both physiologic and pathologic states [4,5]. They regulate lipid [6] and ion [7,8] homeostasis and play a role in the immune system, as they transport proteasomal degradation products into the endoplasmic reticulum for loading onto major histocompatibility complex (MHC) class I molecules, which subsequently present these peptides to the immune systems [9]. They act as guardians of especially sensitive organs by regulating the permeability of the blood brain barrier [10], blood testis barrier and placenta [11], and, in the kidney [12,13], liver [14] and gastrointestinal tract [15], they excrete toxins, thereby protecting the organism.

Nineteen known genetic diseases are associated with mutations in these genes [16]. The most common include cystic fibrosis, progressive familial intrahepatic cholestasis,

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adrenoleukodystrophy and Stargardt's macular dystrophy [16]. All of these deficiencies are recessive diseases, although an antimorphic mutation identified in the yeast ortholog of ABCB1 suggests that other such mutations may exist [17]. Besides mutations that can result in severe disease, a large number of single nucleotide polymorphisms have been identified in the ABC transporter genes [18]. Non-synonymous single nucleotide polymorphisms (SNPs) and also synonymous SNPs [19,20] may ultimately affect the activity of the transporters, which may explain in part the inter-individual differences in pharmacokinetic behavior of anticancer drugs, contributing to variable response to chemotherapy in cancer [18]. The identification of single nucleotide polymorphisms requires specific assays that are outside the scope of this manuscript. We point the reader to these excellent reviews: Kim and Misra [21], Ding and Jin [22], Ragoussis [23], and Voisey and Morris [24].

In cancer, ABC transporters have an important role in the pleiotropic mechanisms mediating multidrug resistance (MDR) by exporting chemotherapeutic agents from the cell [25]. While the roles of 13 ABC transporters in MDR have been fully characterized, recent studies suggest the involvement of more than 20 members out of the 48 encoded in the human genome [4,5]. Therefore, a diagnostic assay dedicated to the detection of ABC transporters is critical not only to predict MDR in patients with cancer but also to develop a fuller understanding of their biology. This review addresses the methodologies that have been applied to analyzing the mRNA expression levels of ABC transporters. Furthermore, we will discuss the recent work performed in our laboratory which has led to the identification of high-throughput TaqMan-based qRT-PCR platforms as promising standardized clinical assays for the molecular diagnosis of ABC transporters in cancer.

2. METHODOLOGIES FOR mRNA DETECTION

2.1. MICROARRAYS

2.1.1. OLIGONUCLEOTIDE ARRAYS—Oligonucleotide microarrays use oligonucleotides as probes and require a 3-day process including a ribosomal RNA reduction procedure and cDNA synthesis followed by amplification and labelling by a fluorophore or biotin-conjugated deoxynucleotide. A ribosomal RNA reduction is particularly important when working with a low amount of starting material, as the procedure increases the efficiency of the cDNA synthesis that results in increasing the sensitivity of the assay. Several capture probes are required for each gene to accurately determine its expression profile. The users can choose between two approaches including a one-color or two-color assays. One-color approach involves the hybridization of a single sample to each microarray after it has been labelled with a single fluorophore (usually cyanine-3 (Cy3) or cyanine-5 (Cy5)), whereas in a two-color process, two samples (e.g. experimental and control) are labeled with different fluorophores (Cy3 and Cy5) and hybridized together on a single microarray. These microarrays allow the simultaneous analysis of from several hundred genes to the entire transcriptome. Since their development in the late nineties, they have contributed to the characterization of the molecular heterogeneity of cancers, which were before then classified based only on clinical, morphological, immunophenotypic, and cytogenetic criteria [26,27].

Multidrug resistance has been addressed by two high-density oligonucleotide microarrays using a two-color approach [28,29]. Annereau and colleagues developed a platform, the ABC-Tox chip, to assess the expression profile of 2,200 genes important in toxicological responses, complemented with probes specifically matching 36 ABC transporters as well as 70-mer oligonucleotides representing 18,000 unique human genes [28]. Several cDNA capture probes of 200 to 500 base pairs were added to the array to profile ABC transporters. This analysis required 25 µg of total RNA as starting material. The designers of this microarray investigated the mechanisms underlying camptothecin resistance in prostate

cancer cell lines. ABCC2 was found to be overexpressed in 9-nitro-camptothecin (9NC)-resistant cells compared to 9NC-sensitive cells. Although the authors speculated that the over-expression of ABCC2 might play a role during the initial steps of the drug-selection process, no further studies followed-up this finding [28]. This hypothesis remains to be demonstrated.

A different oligonucleotide microarray was developed by Huang *et al.* [29]. Their 70-mer array was comprised of 632 probes targeting (among other targets) 215 solute carrier (SLC) genes encoding uptake transporters and 40 of the 48 ABC transporters. Their array, which required only 12.5 µg of total RNA for cDNA synthesis, was used to profile the NCI-60 cell line panel. They used 12.5 µg of total RNA for cDNA synthesis and profiled the NCI-60 cell line panel. The resulting expression patterns were then correlated with potency data for a set of 119 anticancer agents with putatively known mechanisms of action [30]. ABCB5 was found to be selectively expressed in 6 of 8 melanotic melanoma cell lines and strongly correlated with 7-Cl-camptothecin ($p < 0.001$). RNA interference experiments subsequently confirmed a potential role of this transporter in camptothecin, 10-OH and 5FU resistance, whereas no change in potency was observed for mitoxantrone [29].

These studies paved the way for analyses of multidrug resistance using in-house dedicated high-throughput platforms for MDR-linked gene expression profiling. However, the amount of total RNA required for this type of analysis is substantial, precluding investigations of clinical samples. To date, protocols have been optimized that offer the capability of using only 100 ng to 1 µg of total RNA as starting material. However, a cDNA amplification step is required. Although linear amplification procedures have been developed, this process may undoubtedly introduce substantial bias due to the inconsistent amplification resulting in the significant underestimation of the initial differential gene expression profile. Cross-hybridization due to the short length of the oligonucleotides is another drawback of this technique. In summary, the length of the procedure, some technical limitations and the subsequent analysis render the use of these oligonucleotide microarrays impractical for use in most clinical settings.

2.1.2. cDNA ARRAYS—cDNA probes of 200 to 400 base pairs combine the excellent hybridization efficiency of a cDNA fragment with the ability of an oligonucleotide to distinguish between homologous transcripts. Gillet and colleagues developed an assay, the DualChip Human ABC, for the expression profiling of 38 ABC transporter genes [31]. This low density microarray carries probes designed to avoid any possible cross-hybridization [32]. The use of this array involves a straightforward 2-day process. Due to the limited number of probes, the data-processing steps are simplified and the results are readily available [32]. However, 10 µg of total RNA are required for cDNA synthesis, which again complicates large scale study based on clinical samples.

The authors validated the assay using cancer cell lines [31] before applying it to study clinical samples of acute myeloid leukemia (AML) [33], T-cell acute lymphoblastic leukaemia [34] and breast cancer [35]. These studies revealed that up to 30 ABC transporters may correlate with specific patterns of drug resistance [4]. However, perhaps the most significant finding was that when childhood AML patients overexpress ABCA3, they respond poorly to treatment [33]. The median expression of ABCA3 was indeed three times higher in 21 patients who had failed to achieve remission after the first course of chemotherapy than in a well-matched group of 21 patients who had achieved remission at this stage ($P = 0.023$) [33]. ABCA3 does not confer classical drug efflux; the authors speculated that it might instead be involved in the intracellular sequestration of chemotherapeutic drugs, which was later demonstrated by Chapuy and colleagues [36,37].

2.2. RT-PCR

2.2.1. GEL-BASED RT-PCR—Liu and colleagues designed a semi-quantitative assay to detect the expression of 47 ABC transporter genes [38]. Briefly, this approach consists of an oligo-dT-based reverse transcription of total RNA. The resulting cDNAs are semi-quantified using GAPDH levels as references. Normalized levels of cDNAs are used for PCR amplification. The PCR products are then separated by electrophoresis and the intensity of each band is quantified using GelPicAnalyzer software (GeneCopoeia, Inc.). This assay requires 4 µg of total RNA and was used to study the ABC transporter gene expression profiles in the adriamycin-selected breast cancer cell line MCF7 (MCF7/ADR3000) [38]. This approach has two main drawbacks: it is not reliable for detection of moderate changes in expression levels, and it is not applicable for quantitative detection of abundant mRNAs.

2.2.2. REAL-TIME RT-PCR—Real-time RT-PCR is the state-of-the-art technology for mRNA detection and quantitation. The technique is sensitive enough to enable mRNA quantitation from a single cell [39]. Moreover, its broad 10^7 -fold dynamic range allows precise quantitation of both rare and abundant mRNAs. There are two main classes of chemistries: DNA-binding agents and the Förster Resonance Energy Transfer (FRET) mechanism [40]. Sybr Green is a fluorescent dye that binds double-stranded DNA, and upon excitation emits light. As a PCR product accumulates, fluorescence increases. This method requires extensive optimization, as SYBR Green binds to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, resulting in an overestimation of the target concentration. There are two types of FRET-based probes: hydrolysis and hybridization probes. The technique, based on a hydrolysis probe (TaqMan), depends not only on FRET but also on the 5' exonuclease activity of the DNA polymerase used in the PCR. Each TaqMan probe is 15 to 25 oligonucleotides long, and is labelled with a fluorescent reporter dye at the 5' end and a quencher moiety at the 3' end. These probes are complementary to the target sequence. In the unhybridized state, the proximity of the dyes does not completely quench the fluorescence and a background fluorescence is observed. During PCR, the probe anneals specifically between the forward and reverse primer to the targeted region of the gene. The polymerase then carries out the extension of the primer and replicates the template. The 5' exonuclease activity of the polymerase cleaves the probe, releasing the reporter dye, whose intensity, as a result increases. This process repeats in every cycle and fluorescence increases proportional to the amount of probe cleavage. As opposed to Sybr Green, TaqMan probes require very little optimization. Hybridization probes solely depend on FRET. The method is based on two probes, one labelled with a fluorescent donor dye and the other with an acceptor dye. Once in close vicinity (3 to 5 base pairs), the donor dye emits energy that excites the acceptor dye, which in its turn will emit fluorescence at a different wavelength. After each cycle, additional hybridization probes can anneal, increasing the fluorescent signal [40]. To our knowledge, this technique has not been tested to profile ABC transporters.

Langmann and colleagues reported a TaqMan-based quantitative RT-PCR (qRT-PCR) in a 384-well plate format, which requires 50 ng of total RNA as starting material [41]. The authors investigated the ABC transporter gene expression profiles in 20 human tissues obtained from a commercial source. Interestingly, Nishimura and Naito released a similar study performed on 23 human tissues and only using 25 ng of total RNA. This group profiled not only all the human ABC transporters but also more than a hundred uptake transporters (SLC) with the clear objective of establishing a tissue distribution of efflux and uptake transporter mRNAs for drug development laboratories [42]. Szakacs et al. measured the expression level of the same genes in the NCI-60 cell line panel by a SYBR Green-based qRT-PCR method, and with 150 ng of total RNA [43]. Correlations were then drawn between these gene expression profiles and the growth inhibitory profiles of 1,429 candidate

anticancer drugs tested against the NCI-60 cell line panel [44]. This database allowed the identification of lead compounds in the early stages of drug development that are and that are not ABC transporter substrates. It also revealed molecules with collateral sensitivity, whose activity is potentiated, rather than antagonized, by ABC transporters [43]. Later, Langmann and associates reported the development of a new assay allowing the quantification of all human ABC transporters by a platform named “Human ATP-Binding Cassette Transporter TaqMan Low-Density Array”. Instead of 50 ng of total RNA, this assay requires only 2 ng per gene profiled [45]. The authors applied this assay to map the expression profile of these genes in normal human epidermal melanocytes and several cell lines originating from primary and metastatic melanomas [46].

One must bear in mind that SYBR Green cannot distinguish between specific and non-specific products accumulated during PCR. Therefore, follow up assays are needed to validate results. TaqMan-based qRT-PCR is superior to SYBR Green, as the technique is more specific and sensitive. Throughput is an important parameter in any application in a clinical setting or in large scale translational research. In this regard, TaqMan-based qRT-PCR performed in a 96 or 384-well format is obviously more tedious and requires more sample material (total RNA) and reagents than the TaqMan Low Density Array (TLDA).

3. TOWARDS A STANDARD DIAGNOSTIC ASSAY FOR TRANSLATIONAL RESEARCH

The literature on the role of ABC transporters in the clinic is conflicting [5,47] and one of the main reasons for this is the limitations of the assays that have been used. The establishment of a standard assay allowing the precise discrimination of highly homologous genes using a small amount of sample would help to produce a clearer, more unified picture of MDR [48]. Moreover, it would undoubtedly lead to progress not only in understanding the mechanisms governing MDR but also in the translation of this knowledge to clinical practice, especially in personalized medicine [49].

Recently, our laboratory conducted a study to identify the best technology for transcript analysis and more specifically, a technology capable of precisely discriminating highly homologous genes such as ABC transporters [50]. The NCI-60 cell line panel was our model of choice, as a database is available comprising the transcriptome expression profile obtained from oligonucleotide microarrays, the ABC transporter gene expression profile established from Sybr Green qRT-PCR and the cytotoxic potencies of 1,429 anticancer drugs against these 60 cancer cell lines (CellMiner database (<http://discover.nci.nih.gov/cellminer/>)). We then appraised the accuracy of these two assays along with the TLDA in the discrimination of the ABC transporter genes. The data showed that TLDA provides more sensitivity, yielding a larger dynamic range of measurement, and is the technology best suited for accurately quantitating individual genes in the ABC transporter superfamily in a high-throughput format [50] (Fig. (1)). In order to demonstrate these observations, we generated a database similar to that reported by Szakacs and colleagues [43], with the aim of eliminating compounds that are efflux-transporter substrates in the early stages of drug development. Correlating the ABC transporter gene expression profiles obtained by the TLDA with the cytotoxic potencies of 1,429 anticancer drugs led to the identification of additional anticancer agents that display ABC transporter-mediated drug resistance, which were poorly ranked in our previous database using SYBR Green qRT-PCR [50]. For instance, we showed that saframycin A, a quinone antitumor antibiotic, is a potent substrate of ABCC1, whereas ABCG2-overexpressing cells are resistant to sparoxomycin A1, a pyrimidinylpropanamide antibiotic with antitumor properties. Besides anticancer agents, the analysis revealed other previously unexplored compounds that are substrates of these ABC transporters, such as NSC265473, which was identified as a substrate of ABCG2

[50]. Furthermore, the ability to make predictions regarding substrates of three different ABC transporters (ABCB1, C1, G2) using mRNA expression data argues strongly that mRNA levels accurately reflect the presence of at least these three functional transporters in the NCI-60 cells. Nevertheless, transcript expression alone can obviously not suffice for inferring the functionality of the transporter as post-transcriptional regulation could affect interpretation of the gene expression profiles.

Further analysis was performed to determine the best data source to establish MDR gene signatures [51]. Here again, we used the NCI-60 cell line panel as a model and the expression profiles of 380 MDR-linked genes [52] obtained by both TLDA and an oligonucleotide microarray (CellMiner database (<http://discover.nci.nih.gov/cellminer/>)). These data were compared across a variety of p-value thresholds. This analysis showed that the gene expression profiles obtained from TLDA allow a more accurate classification of the NCI-60 cell lines into the 9 cancer types they represent, than the profiles obtained by microarrays. Indeed, 71% of the cell lines were correctly classified at $p=0.05$ and 69% at $p=0.001$, whereas the expression profiles of the same genes obtained from microarray analysis classified the 60 cancer cell lines with 66% accuracy at 0.05 and 61% at 0.001. We observed that confining the analysis to only ABC transporter genes on the TLDA yields less accurate classification (53%, $p=0.05$ and 29%, $p=0.001$) and microarray analysis of the same genes provides the worst results (36%, $p=0.05$, with no classification obtained at $p=0.001$) (data not shown) [51].

In another study, we investigated the MDR-linked gene expression profiles in 40 clinical samples of hepatocellular carcinoma (HCC) [53]. These samples, studied earlier, were divided into two groups based on the genome-wide expression profile obtained from an oligonucleotide microarray [54]. The patients in the A-group were found to have lower survival compared to the patients in the B-group [54]. When comparing the results from both TLDA and microarray platforms for genes differentially expressed between the A and B groups, we found that there was moderate correlation of the fold differences between the TLDA and microarray platforms (Pearson correlation 46.4%, $p<2.2\times 10^{-16}$). TLDA shows a much larger extent of measured fold ratios than microarray (-13.2 to 24.7 compared to -1.3 to 1.2 for microarray). Additionally, the number of differentially expressed genes with false discovery rate (FDR) <0.05 is much smaller for the microarray (2 genes) compared to TLDA (45 genes). Neither of the two genes detected as differentially expressed by microarray were detected by TLDA. This striking result indicates the inability of gene expression profiles obtained by microarray to distinguish among members of a family of highly homologous genes (data not shown) [53].

A diagnostic tool for translational research or personalized medicine needs to be sensitive and specific. It should also be robust (i.e., it should be capable of providing data that are reproducible). In addition to these criteria, the throughput of the assay is critical. TLDA allows the simultaneous profiling of 384 genes, but can analyze only one sample at a time. However, a new TaqMan-based qRT-PCR nanofluidic platform or dynamic array has been developed. This platform, the BioMark 48.48 Dynamic Array, requires much smaller quantities of reagent than other approaches, requires fewer pipetting steps, is less labor intensive, and most important, uses nanoscale reaction mixtures. Moreover, it allows the simultaneous profiling of 48 genes in 48 samples. We used it to assess ABC transporter gene expression profiles in the NCI-60 panel [50]. Although preamplification is a requirement for gene expression analysis with this platform, both the BioMark 48.48 and TLDA platforms produced gene profiles that correlated well for ABC transporter genes, as 39 of the 48 ABC transporters showed Pearson correlations of >0.80 [50] (Fig. (2)).

4. CONCLUSION

ABC transporter-mediated drug resistance has been extensively studied through *in vitro* models, which led to the characterization of 13 MDR-linked transporters. Nevertheless, their role in clinical multidrug resistance has been subject to controversy. We have identified two major confounding factors in clinical studies: (1) poorly selected patient cohorts and, (2) limitations of the methodology used to detect gene expression.

The best scenario for studying multidrug resistance in the clinic would require paired samples that are untreated (taken at diagnosis) and treated (taken at relapse). Unfortunately, this design is unachievable for solid tumors, mainly due to ethical concerns related to the invasive nature of biopsies in cancer patients undergoing chemotherapy. In leukemia, despite the relatively non-invasive procedure related to blood sampling, very few paired samples are available in clinical repositories. Therefore, the trend is to analyze samples selected “by default,” rather than by design.

Two principal methodologies have been discussed in this manuscript: microarrays and qRT-PCR. Although whole-genome arrays have been used over the last decade to study cancers, multidrug resistance was addressed through dedicated in-house assays, often used once or only in a handful of studies. We did learn a great deal from these arrays, as they highlighted a new prognostic marker (ABCA3), a transporter specifically expressed by melanotic melanomas (ABCB5) and the potential involvement in MDR of more than half of the entire ABC transporter superfamily. However, technical limitations are a major impediment to our understanding of the role of these efflux transporters in clinical MDR. Some of the technical hurdles involve cross-hybridization issues, the poor sensitivity and specificity of microarrays, and the fact that Sybr Green-based qRT-PCR requires extensive optimization to reduce the propensity of the dye to bind indiscriminately to any double-strand DNA.

The establishment of a specific and sensitive standard assay, capable of discriminating highly homologous genes is critical to a better understanding of MDR mechanisms. Recent studies identified TaqMan-based qRT-PCR as a superior method for ABC transporter gene expression profiling [45,50,55]. Previously unpublished data have been included in this review to highlight the inability of oligonucleotide arrays to distinguish differences in expression of ABC transporters in clinical hepatocellular carcinoma samples. Finally, the need for high-throughput assays was discussed. Two assays, TLDA and Biomark 48.48 were compared and found to generate similar gene expression profiles. TLDA allows the study of 384 genes in one sample, whereas Biomark 48.48 can profile 48 genes in 48 samples. This assay, which is based on a nanofluidic system, was recently upgraded to study 96 genes in 96 samples. We believe that TaqMan-based qRT-PCR has the potential to provide comparable results across test sites and users in a way that microarrays, at least for the field of MDR, have not been able to achieve.

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Figure 1. Correlation of ABCB1 and ABCG2 expression profiles from three distinct platforms
 Expression profiles for ABCB1 across all 60 cell lines were compared between: (A) SYBR Green and microarray; (B) TLDA and microarray; (C) TLDA and SYBR Green. Identical comparisons were performed for ABCG2 expression profiles: (D) SYBR Green and microarray; (E) TLDA and microarray; (F) TLDA and SYBR Green. The data show that TLDA provides more sensitivity, yielding a larger dynamic range of measurement. The coefficient of correlation is given for each comparison. Values for both the X and Y axes are normalized intensity data and presented in log base 2. Reprinted from Orina et al. 2009, *Mol Cancer Ther* **8**: 2057–2066.

Figure 2. Correlation of ABCB1, ABCC1 and ABCG2 gene expression profiles obtained from the TLDA and BioMark 48.48 Dynamic Array

Expression profiles for ABCB1 (A), -C1 (B) and -G2 (C) across all NCI-60 cancer cell lines were compared between TLDA and BioMark platforms. Values for both the X and Y axes are normalized intensity data and presented in log base 2. Reprinted from Orina et al. 2009, Mol Cancer Ther 8: 2057–2066.

Table 1
Gene expression profiling platforms used to assess ABC transporter gene expression

Gene expression profiling platform	Total RNA requirement	Advantages	Disadvantages	Commercial sources*
Microarrays				
Oligonucleotide arrays	From 100 ng to 25 µg per array	Whole transcriptome study	Lengthy procedure, linear pre-amplification, cross-hybridization	Affymetrix, Agilent, Life Technologies, GE Healthcare, Illumina, Roche, NCI Operon
cDNA arrays	10 µg per array	Simple procedure and data processing	Large amount of required starting material, cDNA does not allow full discrimination of highly homologous genes	Eppendorf, Miltenyi Biotec
RT-PCR				
Gel-based RT-PCR	4 µg per assay		Semi-quantitative, not reliable for detection of moderate changes in expression levels, not applicable to quantitative detection of abundant mRNAs	Produced in the lab
Real-Time RT-PCR				
Sybr-Green based RT-PCR	From 50 to 200 ng per gene	Low amount of starting material, easy laboratory set-up	Requires extensive optimization, limited specificity and sensitivity, tedious procedure	Life Technologies (Applied Biosystems), Roche, SABiosciences
TaqMan-based RT-PCR	From 20 to 50 ng per gene	Low amount of starting material, easy laboratory set-up, few optimizations, high specificity and sensitivity	High cost, tedious procedure	Life Technologies (Applied Biosystems), Roche
High-throughput TaqMan-based RT-PCR platforms				
TaqMan Low Density Array	2 ng per gene	High throughput and limited handling due to automated system	High cost	Life Technologies (Applied Biosystems)
BioMark 96.96	50 ng per array	Low cost compared to TLDA, nanofluidic system (low amount of reagent and starting material required)	Linear pre-amplification required	Fluidigm

* List not exhaustive.