High-Throughput Nanovolume qPCR

Next-Generation Platform Combines Microarray Throughput with Sensitivity

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Gene-expression profiling is an integral part of biomarker discovery as well as discovery and validation of new therapeutic targets to support drug development. Initially, profiling the intracellular range of transcript abundance relied on hybridization-based technology, such as microarrays or spotted cDNA arrays, or sequencing-based technology such as SAGE. Due to inherent technical limitations, neither method provides an accurate quantification of the broad range of transcript levels present in a given sample.

This is particularly true in the case of low-abundance transcripts due to the fact that signal-to-noise ratios decrease exponentially with the decrease of transcript number. In contrast, the signal-to-noise ratio is constant in real-time PCR facilitating the accurate measurement of low-abundance transcripts. Typically, large changes in gene expression can be underestimated or entirely missed by hybridization-based technologies.

Quantitative PCR (qPCR)-based gene-expression profiling is the current gold standard for the precise monitoring of a selected set of genes to validate results of a hybridization-based microarray experiment. However, the overall limitations of qPCR and hybridization-based technologies have created a need for a next-generation platform capable of combining the advantages of these two technologies, while addressing their shortcomings, in a single system.

WaferGen Biosystems (www.wafergen.com) has developed a platform for rapid quantitative gene-expression analysis. This technology, referred to as the SmartChip™ Real-Time PCR System, is equipped with a high-density, rapid-cycling configuration capable of providing high-throughput levels, while offering discovery and validation capabilities in a single step.

The platform performs thousands of nanoscale quantitative PCR assays in a single run, essentially combining the screening capability of microarrays with the sensitivity of qPCR. This enables life scientists to identify a broad spectrum of expressed genes in a single day, while still discriminating small changes in expression. The SmartChips can be configured to analyze large numbers of genes on a single sample, or interrogate many targeted sets of genetic markers on many samples at once.

Whole-genome assay time may be significantly reduced with the SmartChip system as compared to several days or months with microarrays or real-time PCR, respectively. Additionally, it is estimated that this platform will generate expression results 48 times faster and at 50% less cost than conventional methods.

The SmartChip system also incorporates a number of ease-of-use features including content-ready, high-density chips containing 5,000–30,000 nano-wells with gene panels optimized for cancer, toxicology, and whole genome. The platform requires a small sample size as compared to other technologies and offers real-time detection and sophisticated read-out options while assuring detection sensitivity and temperature uniformity across chips.

The SmartChip platform consists of content-ready SmartChips, a sample dispenser, a real-time PCR instrument, and analysis

Advantages of SmartChip Real-Time PCR System for Expression Analysis

	Microarray	SmartChip	Mechanism
Sensitivity (starting copy number)	100,000	3–10	Hybridization vs. amplification
	2 to 3 logs	6 logs	Real-time data acquisition during cycling vs. end point hybridization data collection
Specificity	<90% homology	<98% homology	PCR primer design/uniplex assay
Precision	<20% CV	St Dev<0.2 or <3% CV	Fewer steps from sample to results
Throughput	Whole transcriptome ~25K genes (~5–7.5K detected)	Whole transcriptome ~25K genes	High-density nano-wells
Assay Time	Days	Day	Cycling

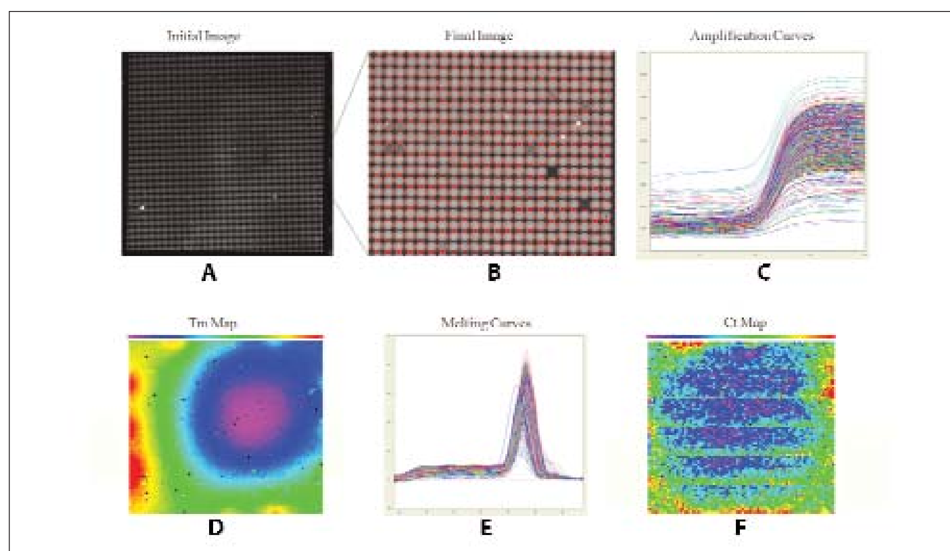

Figure 1. Human reference gene GAPDH assay was dispensed onto the entire chip covering 5,184 wells using the custom nanovolume dispenser, sealed with an adhesive film and cycled for 40 cycles followed by melting curve analysis. (A) Initial image was taken just before the cycling at room temperature. (B) The cycle 40 image is an enlarged view of a portion of the 5K SmartChip taken at the end of the annealing step at 60°C. (C) Amplification curves of 5,184 wells demonstrated the capability of the software to handle thousands of wells at a time. (D) Tm map depicts the melting curve analysis and the temperature range of the GAPDH amplicon. (E) The melting curves exhibit a single peak around the expected Tm of 84.7°C. (F) Ct Map of 5,184 wells with a StDev of 0.4 cycles.

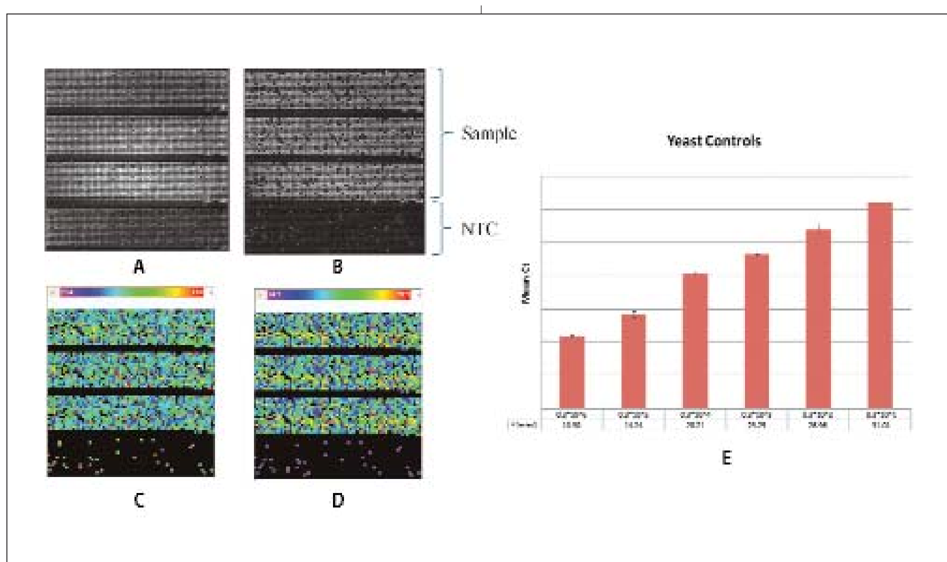

Figure 2. Primer-loaded 1000-gene SmartChip was filled with cDNA sample and master mix or master mix without cDNA as negative control, sealed and cycled for 40 cycles followed by melting curve analysis. (A) Initial image prior to cycling. (B) Image taken at cycle 40. (C) Ct map. (D) Tm map. (E) Six different exogenous yeast spike-in controls at various in-put concentrations (3—300K copies) were plotted against mean Ct values obtained for the three replicates on the SmartChip and the StDev for the error bars. The SmartChip platform has a sensitivity of three copies, dynamic range of five logs, and CV of <3%.

PCR Standardization

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based RNA analysis using a system such as the Experion (**Bio-Rad Laboratories**) or the Bioanalyzer 2100 (**Agilent Technologies**). This technique is fast, highly standardized, uses a small amount of total RNA, and is automated, including the data analysis.

Each system uses a quality index scale to represent the level of degradation in a sample. The Agilent RNA Integrity Number (RIN) is a number from 1 to 10 derived by an algorithm that takes into account eight portions of the electrophoretic trace. The Bio-Rad RNA Quality Indicator (RQI), also a number from 1 to 10, is generated by an algorithm that takes into account three portions of the electrophoretic trace and then compares the electropherogram of RNA samples to a series of standard degraded RNA samples.

The two quality indices are correlated and can generally be used interchangeably as reliable indicators of RNA quality[7]. The PCR-based 3´:5´ mRNA integrity assay[8] constitutes an additional RNA quality-assessment tool, although its practical usefulness remains to be determined.

Ideally, a threshold that delineates the quality of RNA required to produce reliable results should be established for each study. For example, in a study of more than 700 biopsies from neuroblastoma patients, the average values for samples with acceptable quality were a difference in Cq of 2.36 for the 3´:5´ assay and an Experion-derived RQI of 7.4, while both of these indices show a wide distribution of values across the samples (*Figure 4*)[9].

A general guideline has also been suggested that recommends an RIN of at least 5 to obtain reliable RT-qPCR results[10]. Finally, inhibition of reverse-transcription or PCR should be checked by dilution of the sample or use of a universal inhibition assay such as SPUD[11].

Conclusion

qPCR and RT-qPCR are powerful core technologies that impel the advances made in our understanding of basic biological and disease processes as well as underpin the field of molecular diagnostics. However, the combination of ease of use and lack of rigorous standards of practice has resulted in widespread misinterpretation of data and consequent publication of erroneous conclusions.

The RNA quality issue highlighted in this article is just one example of the many crucial parameters that must be addressed by guidelines that shift the focus of concern

software. To date, 5,184-well and primer loaded SmartChips have been fabricated and used to measure ~1,000 genes in total RNA samples derived from matched normal and lung tumor tissue (*Table*).

Results

Researchers have been able to demonstrate complete reproducibility across 5,184 wells with the SmartChip system. In one test, a reaction mix containing all the components for a GAPDH real-time PCR assay was assembled and dispensed into 5,184 wells in a SmartChip using the system's custom nanodispenser. Data collected following 40 cycles demonstrated the platform's ability to simultaneously achieve consistent results across thousands of wells (*Figure 1*).

Additional tests have demonstrated the SmartChip platform's sensitivity and dynamic range. In one experiment (*Figure 2*), researchers showed that the SmartChip system possesses a sensitivity of three copies and a dynamic range of five logs.

Additional testing has confirmed the system's ability to perform and meet analytical performance specifications, while demonstrating the miniaturization of reaction volumes from 10 µL to 100 nL. Real-time results were generated in under three hours for ~1,000 genes in triplicate with 300–500 ng of starting total RNA. Furthermore, researchers successfully tested total RNA samples derived from matched normal and tumor tissues samples from the lung.

SmartChip Research Programs

The SmartChip system is currently being used in gene-expression research at the University of Pittsburgh Medical Center and the University of Texas Southwestern Medical Center. These research programs are designed to identify and validate therapeutically relevant gene-expression biomarkers in chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, lung cancer, and wound healing.

Additionally, a SmartChip genotyping research collaboration has been established with scientists in the Institute for Genome Sciences and Policy at Duke University Medical Center. The focus of this program is the validation of SNPs that are related to breast cancer as well as an examination of the impact that validated SNPs have on patients' disease prognosis and response to treatment.

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