Quantitative end-point RT-PCR gene expression measurement using the Agilent 2100 Bioanalyzer and standardized RT-PCR

Application

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Abstract

This application note describes how the Agilent 2100 bioanalyzer can be used in combination with Standardized RT (StaRT)-PCR reagents from Gene Express National Enterprises (G.E.N.E.), Inc. (Huntsville, AL, USA) to obtain highly reproducible, standardized gene expression measurement with RT-PCR. The internal standards used in StaRT-PCR control for variable loading of PCR products into electrophoresis channel, and allow quantitative gene expression measurement at the endpoint of PCR, without real-time analysis. Gene expression measurement of 15 genes was more reproducible with the Agilent 2100 bioanalyzer than with the ABI Prism 310 Genetic Analyzer or agarose gel electrophoresis methods. The mean coefficient of variance (CV) for these three methods was 0.26, 0.39 and 0.50, respectively.

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Introduction

We combined the Agilent 2100 bioanalyzer with a quantitative method for standardizing gene expression, Standardized RT (StaRT)-PCR (Willey et al, *Am. J. Resp. Cell Mol. Biol.*, 19, 6, 1998), to produce a sensitive, reproducible method for quantitative end-point PCR measurement of gene expression. The Agilent 2100 system markedly simplifies electrophoresis. There is no need to prepare agarose gels and, following electrophoresis, immediate results are provided by PCR product quantification software. The internal standards used in StaRT-PCR control for variable loading of PCR products into electrophoresis channels, and allow quantitative gene expression measurement at the end point of PCR, without real-time analysis. StaRT-PCR used with agarose gel electrophoresis has been validated in independent studies (Loitsch et al, *Clinical Chemistry*, 45, 619, 1999; Allen et al, *Am. J. Resp. Cell Mol. Biol.*, 21, 693, 1999; Mollerup et al, Cancer Research, 59, 3317, 1999).

StaRT-PCR reagents for over 400 genes are now available commercially through Gene Express National Enterprises, Inc.* (www.genexnat.com). Reagents for an additional 2000 genes are in production. In a blinded inter-laboratory study of common samples, this method provided excellent reproducibility (manuscript in preparation). Thus, all laboratories using a common mixture of internal standards for StaRT-PCR will be able to enter gene expression data into a common gene expression databank. Such a databank has been established at G.E.N.E., Inc.

StaRT-PCR reactions include cDNA from a cell or tissue sample of interest and a standardized mixture of competitive template internal controls for each gene to be measured. Quantitation is based on a ratio of each endogenous gene (referred to as native template [NT]) PCR product to the corresponding shorter internal standard competitive template (CT) product. Until recently, the primary method used to size, separate and detect NT and CT StaRT-PCR products has been agarose gel electrophoresis. This method is available to most laboratories without the purchase of additional equipment. However, recently available alternative methods for electrophoresis, including the Agilent 2100 bioanalyzer have the potential to markedly increase throughput and reproducibility. Additionally, if StaRT-PCR is to have broad application, it is important to determine whether electrophoresis and analysis of StaRT-PCR products with different instruments will provide comparable data. This question was addressed by comparing StaRT-PCR expression data for 15 genes obtained following electrophoretic separation of PCR products by either the usual agarose gel, the Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA), or the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

* E.L.C., K.A.W., D.A.W. and J.C.W. each have a significant financial interest in Gene Express, Inc. which produces and markets reagents for StaRT-PCR.

StaRT-PCR

StaRT-PCR was performed using previously published protocols with G.E.N.E. system 1a gene expression kit (Gene Express National Enterprises, Inc., Huntsville, AL). Briefly, a master mixture containing buffer, $MgCl_2$, dNTPs, cDNA from normal human bronchial epithelial cells (lot 17378, American Type Culture Collection, Rockville, MD), CT mixture from G.E.N.E. system 1a kit and Taq polymerase (Promega, Madison, WI) was prepared. Aliquots of this mixture were dispensed into tubes containing gene-specific primers and cycled either in a Rapidcycler (Idaho Technology, Inc., Idaho Falls, ID) or PTC-100 block thermal cycler with heated lid (MJ Research, Inc., Incline Village, NV) for 35 cycles. In each protocol the denaturation temperature was 94 °C, the annealing temperature was 58 °C, and the elongation temperature was 72 °C. All StaRT-PCR reagents had been optimized for the same PCR conditions. NT and CT PCR products from the Rapidcycler were separated and evaluated on an agarose gel or in the Agilent 2100 bioanalyzer as described below. Primer pairs used for amplification in the PTC-100 block thermal cycler consisted of one fluorescently labeled primer and one unlabeled primer for each gene. These NT and CT PCR products were separated and analyzed in an ABI Prism 310 Genetic Analyzer.

Electrophoresis and quantitation

Agilent 2100 bioanalyzer

For analysis with the Agilent 2100 bioanalyzer, the DNA 7500 LabChip® kit was used. Following amplification in the Rapidcycler, 1 µl of each 10 µl PCR reaction was loaded into a well of a chip prepared according to protocol supplied with the DNA 7500 LabChip kit. Briefly, 9 µl gel-dye matrix was loaded into the chip in one well and the chips were pressurized for 30 seconds. Two additional wells were filled with gel-dye matrix and the remaining wells were loaded with 5 µl each of molecular weight marker. One microliter of DNA ladder was loaded into a ladder well and 1 µl of PCR product was loaded into each sample well. The chip was vortexed and placed into the Agilent 2100 bioanalyzer. DNA 7500 assay was run, which applies a current sequentially to each sample to separate products. DNA is detected by fluorescence of the intercalating dye in the gel-dye matrix. NT/CT ratios were calculated from the area under the curve for each PCR product and a size correction was made since an intercalating dye was used to detect DNA. The area under the curve values for each NT and CT was entered into the G.E.N.E., Inc. spread sheet. Alternatively, the concentration values, which are based on the areas, can be used to calculate the NT/CT ratio.

Agarose gel electrophoresis

Following amplification in the Rapidcycler, PCR products were loaded directly onto 4 % agarose gels (3:1 NuSieve: SeaKem) containing 0.5 µg/ml ethidium bromide. Gels were electrophoresed for approximately one hour at 225 V and visualized with a Foto/Eclipse image analysis system (Fotodyne, Hartland, WI). Digital images were saved on a Power Mac 7100/66 computer and Collage software (Fotodyne) was employed for densitometric analysis.

Quantification of gene expression was determined according to the G.E.N.E., Inc. manual. These mathematical steps are programmed as formulas into a spread sheet available directly or downloadable from G.E.N.E., Inc. so that only the raw data values for each NT and CT for each gene must be entered. The gene expression values then are calculated automatically.

Capillary electrophoresis

PCR products amplified with fluorescent primers in the PTC-100 block thermal cycler were detected by capillary electrophoresis in an ABI Prism 310 Genetic Analyzer under denaturing conditions. One microliter of each PCR reaction or 1 ml of a mixture of PCR reactions was combined with 9 ml formamide and 0.5–1 ml of ROX 1000 size marker. Samples were then heated to 94 °C for 5 minutes

and flash-cooled in an ice slurry. Samples were loaded onto the machine and electrophoresed at 15 kV, 60 °C for 35-45 minutes using POP4 polymer and filter set D. The default injection parameters of 15 kV, 5 seconds were used. The fragment analysis software GeneScan (Applied Biosystems, Inc., Foster City, CA,) was used to determine peak sizes in base pairs and peak heights which were used to calculate NT/CT ratios. No size correction was performed since each DNA molecule was tagged with one fluorescent marker from one labeled primer. The area under the curve values for each NT and CT was used for calculations.

Statistical analysis

All statistical analyses were conducted using SAS version 6.11 (SAS Institute, Cary, NC). A GLM test followed by Duncan analysis was used to test for significant differences in gene expression values obtained by the three methods of detection within the same cDNA. Differences were considered statistically significant if the P value was less than 0.05.

Results

Comparison of three methods of electrophoresis

Gene expression values for 15 genes were obtained by three methods of electrophoresis from the same cDNA, as described. Figure 1 on page 5 depicts representative results obtained by each of the three electrophoresis methods. The agarose gel shows the familiar band pattern (figure 1A). Although some of the bands are distorted a good resolution and quantification can be obtained. The intensity of the bands, which is used for the calculation of the gene expression, is determined by appropriate software from the gel image. The Agilent 2100 bioanalyzer obtains signals in digital format. The electrophoretic traces of 12 samples are shown in figure 1 B. Alternatively, these data can also be displayed as a gel like image. The ABI 310 Genetic Analyzer also acquires digital data. Figure 1 C shows several electropherograms from pooled PCR reactions. In each case, NT bands appear above CT bands. Quantification of gene expression was determined according to G.E.N.E., Inc. protocol. The formula calculating gene expression in the form of molecules/10^6 molecules was incorporated in an MS Excel file. Each gene was measured with each method. Genes measured by all three methods in figure 1 are β-actin, CDC2, E2F1, E2F4, p18 and TNF receptor. No statistically significant differences between values obtained by the three methods were detected for 14/15 genes. Gene expression values for one gene, E2F5, obtained by each electrophoresis method were significantly different from each other. The most likely reason that E2F5 values differed between methods is that the CV for each method was so low. If the coefficient of variance (CV) for each method (agarose gel, ABI Prism 310, Agilent 2100 bioanalyzer) had been at the average level for each method , there would not have been a significant difference in the means measured for E2F5 (figure 2 on page 6).

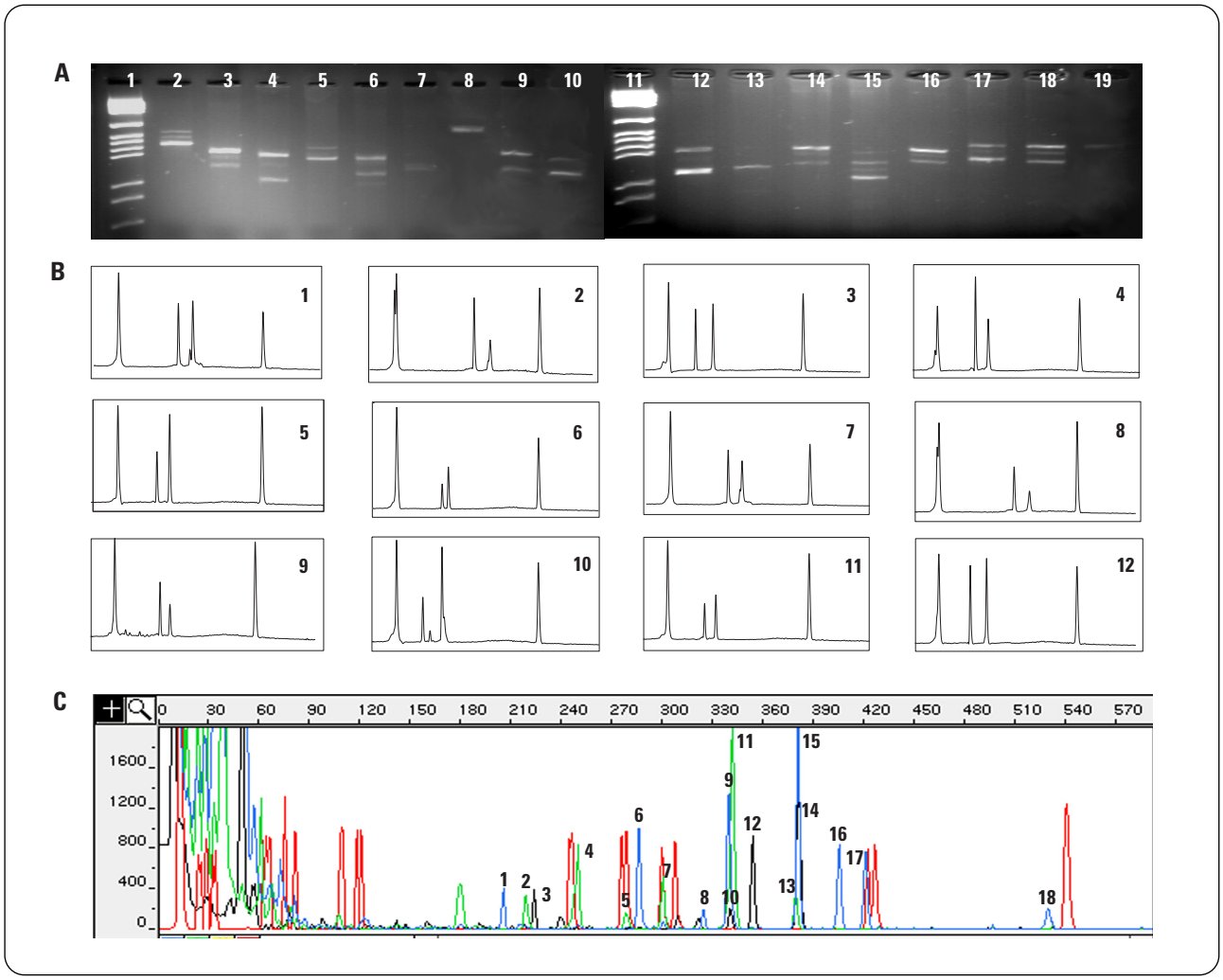

Figure 1

Representative results of three electrophoresis methods. For A - C, aliquots of BEC 17378, cDNA were StaRT-PCR amplified with primers for different genes.

A.

Agarose gel analysis of, BEC 17378 cDNA, and G.E.N.E. system 1A CT mix E. 1, pGEM size marker; 2, β-actin; 3, c-*myc*; 4, CDC2; 5, E2F1; 6, E2F4; 7, E2F5; 8, GAPDH; 9, Jun B; 10, MAD; 11, pGEM size marker; 12, p18; 13, p19; 14, p21; 15, p53; 16, PCNA; 17, RB; 18, SPR1; 19, TNF receptor. In each case, NT bands appear above CT bands.

B.

Agilent 2100 bioanalyzer, electropherograms 1 - 6, BEC 17378 and G.E.N.E. System 1A CT Mix D. electropherograms 7 - 12, BEC 17378 and G.E.N.E. System 1A CT mix E.
1, β-actin; 2, GAPDH; 3, CDC2; 4, Jun B; 5, p21; 6, TNF receptor; 7, β-actin; 8, GAPDH; 9, E2F1; 10, E2F4; 11, MAD; 12, p18. For each graph, time in seconds on X-axis, fluorescence intensity on Y-axis, marker standard peaks are seen on the far left and right and the CT and NT peaks are between the markers on the left and right respectively.

C.

ABI Prism 310 Genetic Analyzer, BEC 17378 and G.E.N.E. system 1A CT mix E.
1, p18 CT; 2, p53 CT; 3, CDC2 CT; 4, E2F4 CT; 5, p53 NT; 6, SPR1 CT; 7, RB CT; 8, p18 NT; 9, E2F1 CT; 10, TNF receptor CT; 11, E2F4 NT; 12, CDC2 NT; 13, RB NT; 14, TNF receptor NT; 15, SPR1 NT; 16, E2F1 NT; 17, β-actin CT; 18, β-actin NT.

Reproducibility

Three or more replicate measurements were made for 44/45 gene expression values presented in figure 2. Reproducibility was best with the Agilent 2100 bioanalyzer (CV = 0.26). The results for agarose gels are similar to those reported for other studies in which standardized, quantitative RT-PCR was used (Willey et al, Am. J. Resp. Cell Mol. Biol., 19, 6, 1998, Loitsch et al, Clinical Chemistry, 45, 619, 1999, Rots et al, Leukemia, 14, 2166, 2000).

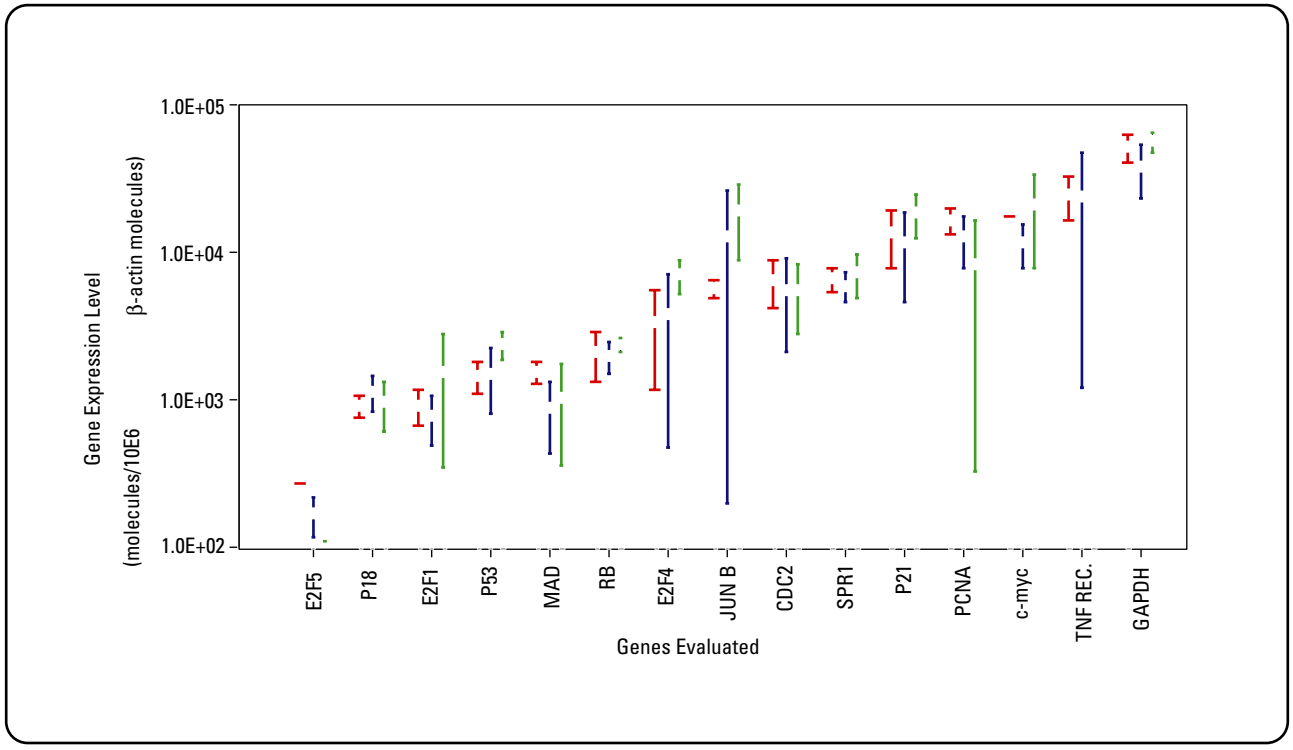

Figure 2
Reproducibility of expression measurements obtained by Agilent 2100 bioanalyzer CE (red), agarose gel electrophoresis (green) or ABI Prism 310 CE (blue). Triplicate measurements were obtained for each gene by each method except for TNF REC. by ABI Prism 310 CE. The SD for each triplicate measurement is plotted with gap in each bar representing the mean expression value. Data from Agilent 2100 bioanalyzer are presented by bars with wider ends.

Conclusions

StaRT-PCR in combination with Agilent 2100 bioanalyzer electrophoresis provides results statistically similar to those obtained with agarose gel or ABI 310 electrophoresis, with better reproducibility.

StaRT PCR combined with the Agilent 2100 bioanalyzer provides an excellent method for quantitative RT-PCR gene expression measurement for the following reasons:

- The analysis can be performed at the end-point of PCR without need for real-time measurement at each cycle of PCR.
- It is more sensitive because quantification can be performed at the first cycle that the bands are detectable without need for multiple points to generate a slope.
- Data are standardized and may be entered into a common data bank.
- High reproducibility.

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