Expression of oncogenic microRNAs: analysis by next-generation qPCR system

Abstract
Real-time PCR–based gene expression analysis requires instrument platforms that can deliver accurate and reproducible results. In this study, researchers from Sapienza University of Rome compared the performance of oncogenic microRNAs (miRNAs) with Applied Biosystems™ TaqMan™ Gene Expression Assays. The miRNAs inhibit protein synthesis by repressing translation of target mRNAs. The target mRNAs were run on both the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System and the Applied Biosystems™ StepOnePlus™ Real-Time PCR System. A shift of target mRNAs to lighter fractions of polysomes in the density gradients was noted. Using density gradients from HL-60 cell lines treated with vitamin D3 (VitD), RNA was isolated and used for gene expression assays. Results showed similar sensitivity and reproducibility between the QuantStudio 5 Real-Time PCR System and the StepOnePlus instrument.

Customer summary
Dr. Alessandro Fatica is an associate professor at the Department of Biology and Biotechnology at the Sapienza University of Rome. His area of focus since 2003 has been the study of microRNAs in cell differentiation of acute myeloid leukemia.

Project background
Acute myeloid leukemia is a heterogeneous hematopoietic malignancy, characterized by complete or partial blockage at different stages of the differentiation of myeloid progenitor cells, which has been mainly attributed to chromosomal abnormalities.

Dr. Fatica’s research interests are focused on defining the roles of noncoding RNAs in normal and abnormal differentiation of myeloid cells (granulocytes and monocytes) and long noncoding RNAs (lncRNAs) that play important roles in gene regulation. The goals of the lab are to investigate the biology of lncRNAs and to analyze and understand their functions and expression in normal and pathological conditions. In particular, he and his colleagues want to study how lncRNAs can influence myelopoiesis and to identify their target mRNAs in order to understand the molecular networks involved in the alternate control between cell growth and differentiation.
Method
In this study, the researchers tested polysome fractions from both HL-60 cell lines treated with VitD, which induces monocytic differentiation, and control untreated cells (CTR). RNAs from input cells and from polysome fractions were isolated. During the testing, six TaqMan Gene Expression Assays (miR-125b, U6, IRF4, GAPDH, HPRT, and pri-miR-223) were run on total RNA extracted from CTR and VitD-treated HL-60 cells. In addition, the same targets were analyzed with RNA from polysome density gradients (12 fractions) from both CTR and VitD cells. All experiments were analyzed in parallel between the QuantStudio 5 Real-Time PCR System and StepOnePlus System, under the same conditions (Fast TaqMan Assay) and the results were compared.

Results
Relative gene expression of total RNA
TaqMan Gene Expression Assays were tested on total RNA extracted from HL-60 CTR and VitD-treated HL-60 cells with Applied Biosystems™ TaqMan™ Fast Master Mix according to the manufacturer’s protocol. In each reaction, 15 ng of DNA was utilized. For mRNA levels of IRF4 and pri-miR-223, data were normalized to HPRT mRNA, and for mRNA level of miR-125b, data were normalized to U6 mRNA. Relative gene expression was calculated by the 2^ΔΔCt method. As shown in western blot analysis (Figure 1), IRF4 protein levels increased during VitD treatment without a concomitant increase in HPRT levels (Figure 1A), indicating a real increase in protein levels. The IRF4 (insert 4) mRNA level did not increase over the same time period (Figure 1B), indicating that the increase in IRF4 protein levels was due to posttranscriptional regulation.

Figure 1. Gene expression detected from total RNA extract of HL-60 cells. (A) The expression of IRF4 protein was analyzed by western blot analysis of protein extracted from VitD-treated HL-60 cells, using HPRT as a reference protein. (B) Expression analysis of pri-miR-223, IRF4 and miR-125b on StepOnePlus system (left) and QuantStudio 5 Real-Time PCR system (right), showing fold expression changes at 0 hours and 72 hours for each target.

Applied Biosystems QuantStudio 3 and 5 Real-Time PCR Systems overview

<table>
<thead>
<tr>
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<th>QuantStudio 3 Real-Time PCR System</th>
<th>QuantStudio 5 Real-Time PCR System</th>
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<tbody>
<tr>
<td>Block format</td>
<td>96-well (0.1 or 0.2 mL)</td>
<td>96-well (0.1 or 0.2 mL) or 384-well</td>
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<tr>
<td>Temperature</td>
<td>Three independent temperature zones (VeriFlex™ blocks)</td>
<td>Six independent temperature zones (VeriFlex blocks)</td>
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<tr>
<td>Excitation source/filters</td>
<td>Bright white LED/4 coupled filters</td>
<td>96-well: bright white LED/6 decoupled filters, up to 21 filter combinations 384-well: bright white LED/5 coupled filters</td>
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<tr>
<td>Software</td>
<td>Simple software allows for quick and easy experiment setup with preoptimized protocol templates for new users</td>
<td>Built-in software features to support compliance with 21 CFR Part 11 FDA guidelines at no additional cost</td>
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TaqMan Gene Expression Assays
TaqMan Gene Expression Assays consist of a pair of unlabeled PCR primers and a TaqMan probe with an Applied Biosystems™ FAM™ or VIC™ dye label on the 5’ end, and a minor groove binder (MGB) and a nonfluorescent quencher (NFQ) on the 3’ end. Over 1.3 million predesigned TaqMan Gene Expression Assays covering 23 species are available in single tube, 96-well plate, 384-well microfluidic card, and Applied Biosystems™ OpenArray™ formats. Custom TaqMan Gene Expression Assays can also be designed to study any gene or splice variant in any organism.

Assay design and ordering information is available at thermofisher.com/taqman
RNA extracted from equal volumes of single fractions of polysome density gradients was reverse transcribed and analyzed with TaqMan Gene Expression Assays and Applied Biosystems™ TaqMan™ Universal Master Mix. Gene expression was analyzed by calculating the percentage of the $2^{-\Delta\Delta C_T}$ value for each target in a single fraction over all fractions. Figure 2 shows the relative expression of miR-125b, run on both StepOnePlus and QuantStudio 5 systems. Both instruments show comparable results and have similar sensitivity and reproducibility.

Figure 2. Comparison of miR-125b expression levels determined by different real-time PCR systems. Expression of miR-125b is compared between control HL-60 cells (CTR) and cells treated with VitD at 250 ng/mL by (A) StepOnePlus and (B) QuantStudio 5 Real-Time PCR Systems.

Conclusion
Quantitation of gene expression on extracted RNA showed comparable performance on the QuantStudio 5 Real-Time PCR System and on the StepOnePlus instrument. The two real-time PCR systems have similar sensitivity and reproducibility, demonstrating that the QuantStudio 5 Real-Time PCR System can easily be implemented in labs where StepOnePlus Real-Time PCR Systems have been used previously. The QuantStudio 3 and 5 Real-Time PCR Systems provide our latest advancements in touch-screen usability, allowing researchers to stay connected to their data easily. These new instruments provide additional benefits like enhanced multiplexing capabilities, on-board memory, programmable pause, and ability to lock workflows and PIN-protected end-user accounts on Thermo Fisher Cloud. They are designed for both new and experienced users who need simple and affordable real-time PCR systems without compromising performance or quality.
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