

Analysis of MicroRNA and Protein Expression on a Single Platform Using the ViiA[™] 7 Real-Time PCR System

Measuring the effect of changes in microRNA (miRNA) activity on downstream protein expression in cells has been encumbered by the need for separate sample processing and analysis approaches, largely due to the very different properties of nucleic acids and proteins. Additionally, traditional protein analysis methods such as western blotting continue to be laborious and semiquantitative at best. New TaqMan[®] Protein Assay technology addresses these issues with a novel approach employing proximity ligation and enables a simple, rapid, and flexible real-time PCR workflow to be directly applied to protein quantitation. By pairing TaqMan[®] MicroRNA Assays with TaqMan[®] Protein Assays, an integrated approach for measuring relative changes in miRNA and protein expression from the same starting tissue sample (processed separately for nucleic acid and protein extraction) can now be performed on a single analytical platform, the ViiA[™] 7 Real-Time PCR System. Building on six generations of real-time PCR systems, the ViiA[™] 7 instrument provides new features designed for high-productivity real-time PCR. We used this integrated approach to study the relationship



between miRNA expression and the proteome using relevant biomarkers in a pluripotent human embryonal carcinoma cell line, NTERA2, upon differentiation to neuronal cells.

Assess the Relationship Between miRNA Expression and the Proteome

miRNA transcripts are becoming frequently used as biomarkers for assessing, monitoring, and/or determining the state of a cell because of the ubiquitous role they play in posttranscriptional gene regulation and the relative ease with which they can be analyzed. However, the link between miRNA expression and the relative abundance of their putative mRNA targets and encoded proteins in many cases has remained untested. Now, by pairing TaqMan® MicroRNA Assays with TaqMan® Protein Assays, relative changes in miRNA and protein expression can be measured from a single sample on a single analytical platform, the ViiA[®] 7 Real-Time PCR System.

To validate this approach, we used a pluripotent human embryonal carcinoma cell line (NTERA2) as a model system to test miRNA and protein expression correlation and determine fold changes. Protein assays were performed for two stem cell pluripotency markers (OCT3/4 and LIN28) and two differentiation markers (NCAM1 and ALCAM), and run using reagents provided in the new TaqMan® Protein Assays Open Kit on the ViiA[™] 7 Real-Time PCR System. Changes in expression of global miRNA and key pluripotency species, such as the miR-302 cluster, and differentiation markers, including miR-125b and miR-145, were measured using the TaqMan® Array MicroRNA Card A (version 2.0), again on the ViiA[™] 7 Real-Time PCR System.

Materials and Methods

Retinoic Acid Induction

NTERA2 cells (4 x 10⁴) were cultured in T-75 flasks in the presence or absence (untreated control) of 10 μ M trans-retinoic acid (RA) for 10 days. Cells were harvested at multiple time points (days 0, 1, 4, and 10).

Sample Preparation

NTERA2 cell lysates were prepared using the Applied Biosystems® Protein Expression Sample Prep Kit and assayed directly for protein. In parallel, cells were processed for miRNA using the Ambion® *mir*Vana[®] PARIS[®] Kit.

TaqMan® Assays and Reagent Kits

The TaqMan® Array MicroRNA Card was run as recommended by the manufacturer. Total RNA was reverse transcribed and preamplified using Megaplex[™] Primer Pools. cDNA was mixed 1:1 with TaqMan® Universal Master Mix II and pipetted into each of the 8 loading ports on the TaqMan® array card. A total of 377 miRNAs and 3 endogenous references were quantified.

TaqMan® Protein Assays were carried out using NTERA2 cell lysate dilutions representing 500, 125, 32, and 0 cells per reaction. TaqMan® Protein Assays are an adapted form of a proximity ligation assay (PLA[™]) technology [1,2] that combines antibody-protein binding with real-time PCR-based detection of the reporter nucleic acid sequence (Figure 1).

ViiA[™] 7 Real-Time PCR System and Data Analysis

Real-time PCR assays were performed on the ViiA[™] 7 Real-Time PCR System [3]. C_t values were determined from amplification plots, using a threshold of 0.2. Fold changes in miRNA expression

at 1, 4, and 10 days, relative to day 0, were determined by first calculating ΔC_{i} (C, value for the target minus C, value for the RNU48 endogenous reference) for each sample. Changes in miRNA expression were calculated relative to day 0 (untreated cells), using the $\Delta\Delta C_{1}$ method [4]. Fold changes in protein expression between untreated and treated samples were determined by first calculating ΔC_{i} (C, value for cell input minus C, value for no cell input) for each lysate dilution and for each protein target. ΔC , values were then plotted vs. cell input per assay reaction. The slopes from the resulting plots were used to determine fold changes using Applied Biosystems® ProteinAssist™ Software. All fold changes are relative to day 0 (untreated cells).

Easily and Accurately Monitor miRNA and Protein Levels During Stem Cell Differentiation

TaqMan® Protein Assays were used to determine the relative expression of the corresponding four target proteins in untreated and RA-treated NTERA2 cells (Figure 2). As expected, the levels of OCT4 and LIN28



Figure 1. TaqMan® Protein Expression Assays Provide a Simple, Rapid, and Sensitive Method for Relative Protein Quantification in Cell Lysates. Proteins can typically be detected in reactions containing 500 or fewer cell equivalents. Compared to typical protein detection methods such as western blotting, the TaqMan® Protein Assay requires less sample input, is more sensitive, and has a simpler and faster workflow. The TaqMan® Protein Assay is a homogeneous process that involves the following 3 steps: (1) binding of paired assay probes to a protein target in whole cell lysates, (2) templated ligation of the oligonucleotides in proximity, and (3) real-time PCR detection. The ligation product serves as a template in the TaqMan® real-time PCR assay. No wash steps are required, and results are obtained within 3.5 hours.

declined in RA-treated cells with increasing differentiation. In contrast, the differentiation markers NCAM1 and ALCAM were detected at higher levels in RA-treated NTERA2 cells compared to untreated cells.

TaqMan® MicroRNA Assays were used to generate miRNA expression signatures in untreated and RA-treated NTERA2 cells (Figure 3). The expected miRNA signatures associated with the differentiation process were observed. For example, the temporal changes in expression of specific mature miRNAs such as miR-145 during RA induction correlated with suppression in OCT3/4 protein expression, supporting the recently described link between OCT3/4 and miRNA-mediated cellular differentiation [5]. miR-125b showed up-regulation consistent with its role in the differentiation of human neuronal cells [6]. The miR-302 cluster demonstrated down-regulation associated with loss of pluripotency [7].

TaqMan[®] Assays and the ViiA[™] 7 Real-Time PCR System Help Identify Trends in miRNA and Protein Expression Profiles

Overall, the miRNA and protein expression profiles correlate with their biological roles in terms of timing and direction of changes over the course of RA induction.

TaqMan® Protein Assays enable relative protein quantification and correlation to miRNA expression results using one analytical platform, and provide a general paradigm for studying the relationship between the stem cell transcriptome and proteome [8]. To learn more, visit

www.appliedbiosystems.com/proteinassays.

TaqMan® Array MicroRNA Cards and Megaplex[™] Primer Pools enable the profiling of hundreds of miRNAs from minimal sample input, with the broad dynamic range and specificity of TaqMan® real-time PCR. To learn more, visit miRNA.appliedbiosystems.com.

The ViiA[™] 7 Real-Time PCR System is an ideal platform for TaqMan[®] Assays, offering a simple, flexible system that can be easily adapted to your research [3]. Combined



Figure 3. Monitor Global and Specific miRNA Levels on the ViiA™ 7 Real-Time PCR System. Relative quantification for a panel of miRNAs [treated cells at day 10 minus untreated cells day 0) is shown. Negative fold-change values denote a decrease in expression in cells at day 10 of treatment relative to untreated cells at day 0, while positive fold-change values denote an increase in expression. RNU48 was used as the endogenous control for normalization. with the unsurpassed performance of TaqMan® Assays and Arrays, the platform enables reliable results with every experiment. For more information, visit www.appliedbiosystems.com/viia7.

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Figure 2. Monitor Protein Levels on the ViiA[™] 7 Real-Time PCR System. Relative quantification was monitored for 4 proteins (pluripotency markers OCT3/4 and LIN28, and differentiation markers ALCAM and NCAM1) during RA induction of NTERA2 cells, using TaqMan[®] Protein Assays. Expression changes are relative to day 0 (untreated).

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