An integrated quantitative PCR approach for monitoring gene and protein expression in human pluripotent and differentiated cells

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ABSTRACT

The correlation of gene and protein expression changes in biological systems has been hampered by the need for separate sample handling and analysis platforms for nucleic acids and proteins. In contrast to the simple, rapid and flexible workflow of quantitative PCR (qPCR) methods, which enable characterization of several classes of nucleic acid biomarkers (i.e. DNA, mRNA and microRNAs), protein analysis methods such as Western blotting are cumbersome, laborious and much less quantitative. However, TaqMan® Protein Expression Assays, which use the proximity ligation assay (PLA) technology, now expand the range of qPCR applications to include the detection of proteins through the amplification of a surrogate DNA template (Figure 1).

Here we describe an integrated qPCR approach for measuring relative changes in gene and protein expression from the same starting sample and on a single analytical platform that pairs TaqMan® Gene Expression Assays with TaqMan® Protein Expression Assays. To validate this approach, we used NTERA2 cells as a model system for studying changes in gene and protein biomarker (e.g. OCT3/4, NANOG, and LIN28) expression in response to retinoic acid induction. In line with expectations, changes in protein and mRNA expression were generally similar, but not identical, in both direction and timing. The data presented establish a general paradigm for studying the relationship between the stem cell transcriptome and proteome.

MATERIALS AND METHODS

Retinoic acid induction. NTERA2 cells (4X10⁶) were cultured in T75 flasks in the presence or absence (untreated control) of 10uM trans-retinoic acid (RA) for 28 days as illustrated in Figure 2. Cells were harvested by scraping at multiple time points and analyzed for protein and gene expression. After 28 days, the cells were harvested and replated on PDL/laminin coated plates (BD Biosciences) in the presence of 1uM cytosine D-arabinofuranoside and 10uM uridine (both from Sigma-Aldrich) for 21 days at which time the RA-treated cells were harvested for protein and gene expression analysis. Only the treated cells survived the replating procedure and showed morphological changes indicative of neuronal differentiation.

Sample preparation. NTERA2 cell lysates were prepared with Applied Biosystems' Protein Expression Sample Prep Kit and assayed directly for protein (see Figures 3 and 4). In parallel, crude cell lysates were processed for RNA with the PARIS™ kit (Applied Biosystems) and subsequently treated with TURBO DNA-*free*™ DNAse (Applied Biosystems). The PARIS™ mirVana kit (Applied Biosystems) was used for mircoRNA purification.

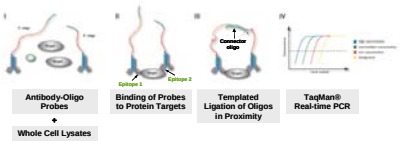
TaqMan® Protein Expression Assays and Reagent Kits. All TaqMan® Protein Expression Assays and associated reagents kits were obtained from Applied Biosystems. TaqMan® Protein Expression Assays were carried out using cell lysate dilutions of 500, 125, 32 and 0 cells per reaction as described in Figure 4. NCAM1 and ALCAM assays were prepared from biotinylated antibodies obtained from R&D Systems.

TaqMan® Gene Expression Assays and Reagent Kits. mRNA assays and One-Step RT-qPCR reagents were obtained from Applied Biosystems. Gene Expression Assays were performed according to manufacturer's instructions. No RT controls verified the removal of genomic DNA after DNAse treatment. microRNA assays and Multiplex RT and qPCR reagents were obtained from Applied Biosystems.

Real-time PCR System. qPCR assays were performed on a StepOnePlus™ Real –Time PCR System (Applied Biosystems).

Data analysis. C_T values were determined from amplification plot using a threshold of 0.2. Protein expression fold changes between untreated and treated samples were determined by first calculating ΔC_Ts (C_T for cell input minus C_T for no cell input) for each lysate dilution point and for each protein target . ΔC_Ts were then plotted vs. cell input per assay reaction. The slopes from the resulting plots were used to determine fold changes. All fold changes (up and down) are relative to day 0 (untreated cells). Gene expression fold changes between untreated and treated samples were determined by first calculating ΔC_Ts (C_T for each target minus C_T for ACTB [mRNA], RNU48 [miRNA]) for each sample. Gene expression changes were calculated relative to day 0 using the $\Delta\Delta C_T$ method.

Figure 1. TaqMan® Protein Expression Assay Schematic Overview



- Two discrete antibodies labeled with different oligonucleotides are brought into proximity on binding to 2 different epitopes on their target
- Proximity of the two oligonucleotides allows the connector oligo to bridge the two oligonucleotides
- Ligation then creates a single DNA molecule, which is detected by TaqMan® real-time PCR

Figure 2. Retinoic Acid (RA) Induced Differentiation

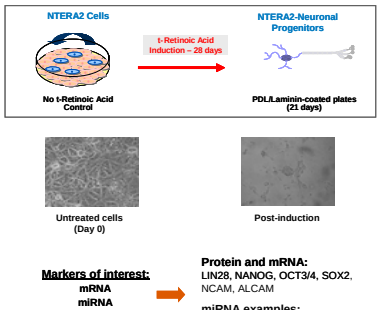


Figure 3. Sample Preparation Workflows by Sample Type

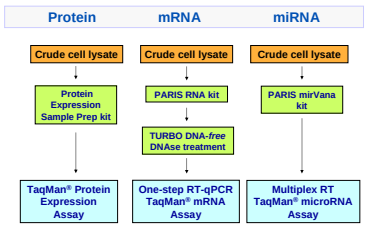
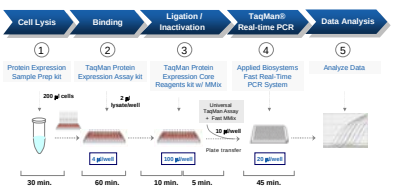


Figure 4. TaqMan® Protein Expression Assay Workflow



RESULTS

TaqMan® Protein Expression Assay Results:
Profile of untreated NTERA2 cell lysate

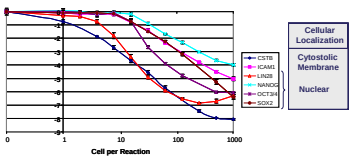


Figure 5. TaqMan® Protein Expression Assay results for 4 pluripotent stem cell markers (LIN28, NANOG, OCT3/4, SOX2) and 2 controls (CSTB, ICAM1), with 2-fold dilution series of NTERA2 cell lysate. Plot of ΔC_T (C_T with cell input minus C_T with no cell input) vs. cells per reaction. Lower limit of detection for the assays is 1-32 cells. Linear dynamic range for quantification is 1-2 logs.

As shown in Figure 6 (below), 3 of the protein markers (NANOG, OCT3/4, and LIN28) show decreased expression by Day 4 of RA treatment. NANOG and OCT3/4 are no longer detectable by Day 4 and 10, respectively. LIN28 continues to be expressed, but at lower levels and is no longer detectable in differentiated cells (Final day). SOX2 levels are maintained throughout RA induction, but are sharply decreased in differentiated cells.

CSTB and ICAM1 protein levels are relatively constant throughout the period of RA treatment, but are elevated in differentiated cells. NCAM1 is detected at low levels in untreated cells compared to differentiated cells, while ALCAM is not detectable at Day 0. Relative levels of NCAM1 gradually increase over time, while ALCAM levels are only increased noticeably at day 14 and thereafter.

For the mRNA assays (Figure 7, below), the relative levels of NANOG, OCT3/4 and LIN28 mRNA are all decreased by day 10 of the RA induction timecourse. Levels of NANOG and OCT3/4 are noticeably reduced by day 4. In contrast, levels of ICAM1, NCAM1, and ALCAM increase throughout the induction period, while the relative abundance of CSTB remains constant.

TaqMan® Protein Expression Assay Fold-change Results:
Time-course of RA induction and differentiation

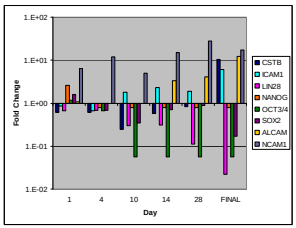


Figure 6. Fold-change summary for 8 proteins (4 pluripotency markers, 2 controls, 2 differentiation markers [NCAM1, ALCAM]) during RA induction of NTERA2 cells. Relative fold differences between samples were determined as described in Materials and Methods. Expression changes are relative to Day 0 (untreated). Negative values denote lower relative expression, while positive values denote higher relative expression.

TaqMan® Gene Expression Assay Fold-change results:
Time-course of RA induction and differentiation

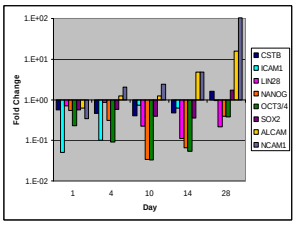


Figure 7. Relative expression changes for 8 target transcripts over the course of RA induction of NTERA2 cells. ACTB was used at the endogenous control for data normalization. Negative values denote a decrease in expression relative to Day 0 (untreated), while positive values denote an increase in expression.

Changes in miRNA levels at Day 5 post-RA induction

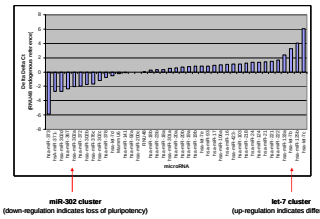


Figure 8. Relative expression changes ($\Delta\Delta C_T$s) for a panel of microRNAs (treated cells, Day 5 minus untreated, Day 0). Negative values denote a decrease in expression in cells at day 5 of treatment relative to untreated cells, while positive values denote an increase in expression.

CONCLUSIONS

1. **TaqMan® Protein Expression Assays provide a simple, rapid and sensitive method for protein relative quantification in cell lysates**
   - **Proteins can typically be detected in reactions containing 500 or less cell equivalents**
2. **TaqMan® Protein Expression Assays were used to determine the relative expression of 8 target proteins in untreated, RA-treated, and differentiated NTERA2 cells**
   - **As expected, OCT4, NANOG, and LIN28 were detected in untreated cells but their levels declined or became undetectable in RA-induced or differentiated cells**
   - **In contrast, other proteins, such as ICAM1, NCAM1, and ALCAM, were detected at much lower levels in untreated NTERA2 cells compared to RA-induced and differentiated cells**
3. **TaqMan® Gene Expression Assays were used to determine the relative expression of 8 target transcripts in untreated and RA-treated NTERA2 cells**
   - **As expected, OCT4, NANOG, SOX2, and LIN28 transcripts were detected at high levels in untreated cells and decreased in RA-induced cells**
   - **In contrast, transcripts for ICAM1, NCAM1, and ALCAM increased with RA-treatment, while levels of CSTB remained relatively constant**
4. **Overall, the protein expression profiles correlate well with gene expression profiles in terms of timing and direction of changes over the course of RA induction**
5. **Temporal changes in expression of specific mature microRNAs during RA induction also correlate well with temporal changes in LIN28 expression, supporting the link between LIN28 and microRNA-mediated cellular differentiation**

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LICENSING & TRADEMARKS

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