

Novel Protein Assays Using TaqMan® Chemistry for the Detection and Relative Quantification of Protein Markers in Embryonic Stem Cells

Abstract

Stem cell characterization typically relies on determining the presence and amount of specific pluripotent protein markers. Current methods for detecting protein markers in stem cells include FACS, immunohistochemistry, and western blots. Each method, however, has its own limitations. These include a requirement for large sample input, lack of sensitivity, and results that are not quantitative.

The TaqMan® Protein Assay is a novel method using proximity ligation assay (PLA™) technology to detect proteins through the amplification of a surrogate DNA template. Applied Biosystems has optimized this technique for use with crude cell lysates and combined it with TaqMan® 5' nuclease chemistry to create a highly sensitive and specific process for measuring protein expression in small samples.

We have monitored the expression of the pluripotent markers OCT3/4, NANOG, SOX2, and LIN28 in stem cells. Results confirm cell stage-specific changes in protein expression of these key stem cell markers. In addition, we have developed a novel software tool to calculate relative protein quantities. The software allows for comparison of protein

expression levels between different samples, even for proteins that exhibit non-parallelism upon dilution. Our findings illustrate that the TaqMan® Protein Assay is ideally suited for the relative quantification of markers in pluripotent and differentiated stem cells.

Introduction

Human embryonic stem (ES) cells can be propagated indefinitely in culture, but will differentiate into major cell lineages under specific culture conditions. Pluripotent cells can also be derived from somatic cells that have been reprogrammed. These induced pluripotent stem (iPS) cells have many of the same characteristics as ES cells, including the ability to differentiate into various cell lineages. Understanding maintenance of pluripotency and commitment to cell differentiation on a molecular level is crucial to understanding developmental processes of pluripotent cells. The OCT4, NANOG, SOX2, and LIN28 proteins play critical roles in establishing or maintaining pluripotency in human ES cells and can be used as markers for pluripotency.

We have developed the Applied Biosystems® TaqMan® Protein Assays for the detection and relative quantification of OCT3/4, NANOG, SOX2, and LIN28 in human ES cells. TaqMan®

Protein Assays are an adapted form of PLA™, a proximity ligation assay technology that combines antibody-protein binding with real-time PCR-based detection of the reporter nucleic acid. The basic steps (Figure 1) are:

- 1. Binding of a protein target by paired assay probes.**
The assay probes are target-specific antibodies that are conjugated to oligonucleotides through a biotin-streptavidin linkage. Each oligonucleotide in the probe pair presents a 5' or 3' end; these ends are brought into proximity when the antibody components of the assay probe pair bind to two different epitopes on the target protein.
- 2. Ligation of the oligonucleotides by DNA ligase.**
The substrate for ligase is a bridge structure formed by hybridization of a third oligonucleotide to the oligonucleotide ends of the assay probe pair. This structure forms preferentially when the assay probes are in proximity to each other. Subsequent protease treatment inactivates the ligase.
- 3. Amplification and detection of the ligation product by TaqMan® real-time PCR.**
The ligation product serves as a template in the TaqMan® real-time PCR assay.

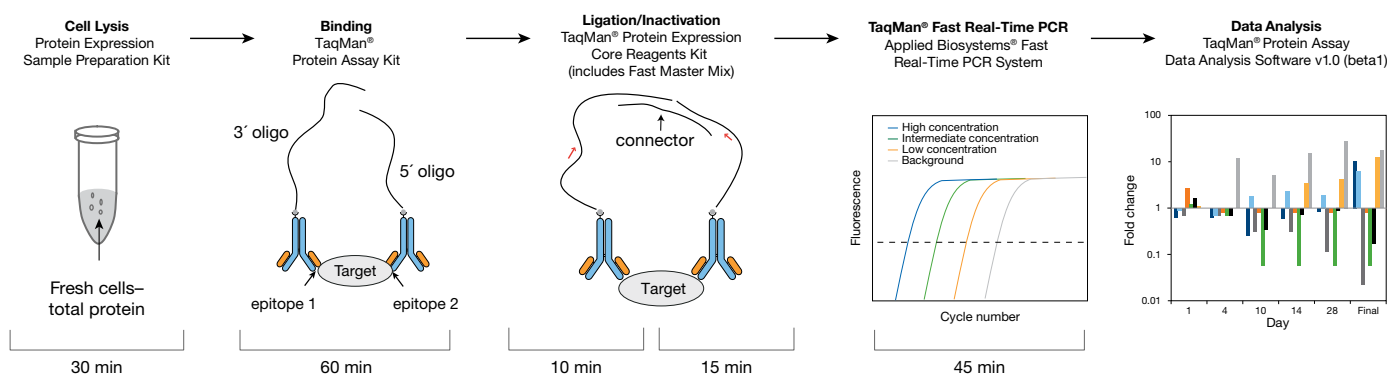


Figure 1. Overview of the TaqMan® Protein Assay Workflow.

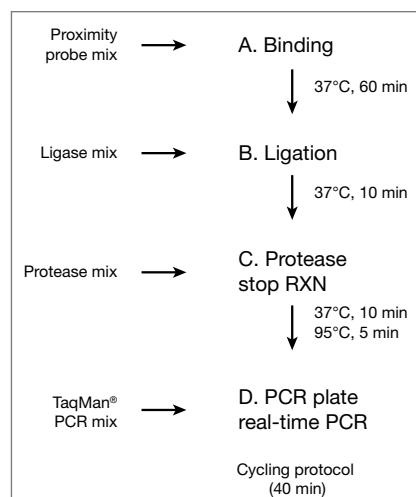


Figure 2. Overview of the TaqMan® Protein Assay Protocol.

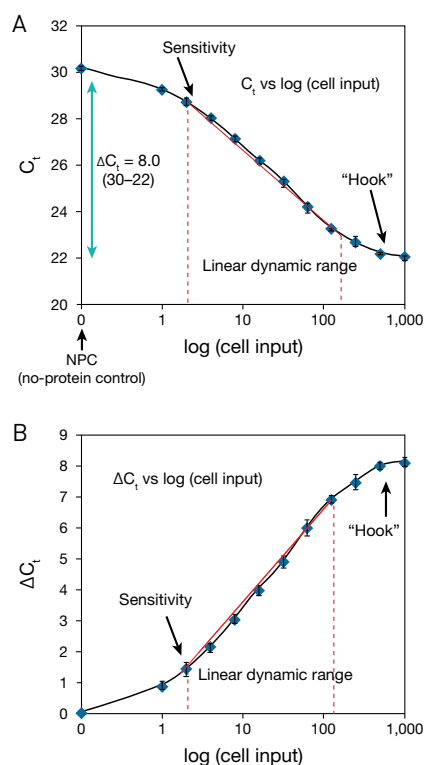


Figure 3. CSTB Expression in Raji Cell Lysates. The TaqMan® Protein Assay for CSTB was performed with 2-fold serially diluted Raji cell lysate, from 1,000 to 1 cell equivalent of input. Each data point is an average of 4 replicates. The data in A are plotted as C_t vs. log (cell input), and the data in B are the same data plotted as ΔC_t vs. log (cell input). $\Delta C_t = C_t$ [NPC (no-protein control)] – C_t (x cell input).

In this overview, the performance of the TaqMan® Protein Assays for OCT3/4, NANOG, SOX2, and LIN28 with respect to specificity, linear dynamic range, and reproducibility is reported, and the sensitivity of the assays is compared to that of western blots. In addition, representative data are given to illustrate the unique data analysis requirements and methodology.

Materials and Methods

TaqMan® Protein Assay

The workflow is detailed in Figure 2:

- 2 μ L of sample (diluted cell lysate) and 2 μ L of oligo-labeled antibody probes are mixed and incubated for 60 min at 37°C.
- 96 μ L of ligation mix is added to the binding reaction and incubated at 37°C for 10 min.
- 2 μ L of diluted protease mix is added at 37°C for 10 min, followed by heat inactivation at 95°C for 5 min.
- 9 μ L of the ligation product is combined with 1 μ L of 20X TaqMan® Protein Assay primer/probe mix and 10 μ L of 2X TaqMan® Protein Assay Fast Master Mix. Ligation products are amplified by real-time PCR using the Applied Biosystems® StepOnePlus™ Real-Time PCR System and default fast cycling parameters.

Samples

The samples were prepared from NTERA2 and Raji cells using the Protein Expression Sample Preparation Kit. NTERA2 is a pluripotent human embryonal carcinoma cell line [ATCC® Number CRL- 1973™] and can be induced to differentiate along neuroectodermal lineages upon exposure to trans-retinoic acid (tRA). NTERA2 expresses the stem cell markers OCT3/4, NANOG, SOX2, and LIN28. The Raji cell line is a lymphoblast-like cell line derived from a Burkitt's lymphoma [ATCC® Number CCL- 86™]. The Raji cell line does not express the stem cell markers and therefore serves as a negative control for the OCT3/4, NANOG, SOX2, and LIN28 assays. Both Raji and NTERA2 cells do express CSTB and ICAM1 and so serve as positive controls for the TaqMan® Protein Assay.

Westerns

Cells were resuspended in SDS-PAGE buffer and loaded onto NuPAGE® Novex® Bis-Tris Gels (Invitrogen). Gels were transferred to

0.2 μ m nitrocellulose membranes and probed with 0.1 μ g/mL primary antibodies. The primary antibodies are the same antibodies used in the TaqMan® Protein Assays. After incubation with a secondary antibody, proteins were visualized with chemiluminescence. Digital images were obtained after 5 min exposures using Alpha Innotech software.

Results

Representative TaqMan® Protein Assay data are shown in Figure 3. Because the TaqMan® Protein Assay is performed without any wash steps, there is always some background ligation occurring, even in the absence of target protein (NPC: no-protein control). In addition, the absolute C_t values may change slightly from day to day. Therefore the data are best represented by plotting ΔC_t vs. log (cell input) [Figure 3B], where ΔC_t is calculated as C_t [NPC (no-protein control)] – C_t (x cell input). The resulting sigmoidal curve is more representative of an immunoassay rather than a gene expression TaqMan® Assay, and includes a "hook" that can occur in immunoassays such as ELISAs when too much antigen is present.

To demonstrate specificity and sensitivity, TaqMan® Protein Assays for OCT3/4, LIN28, NANOG, and SOX2 were performed with 2-fold serially diluted NTERA2 and Raji cell lysates. The data demonstrate that the stem cell marker proteins are readily detected in 100–500 cells of NTERA2 and are undetectable in the Raji negative control cells [Figure 4]. Depending on the assay, the sensitivity ranges from 10 to 35 cells/well (Table 1).

The signal to noise, linear range, and limit of detection of the OCT3/4, LIN28, NANOG, SOX2, ICAM1, and CSTB TaqMan® Protein Assays are shown in Table 1. The data were obtained from replicate assays ($n = 5$) performed on different days and by multiple operators. The data exhibit run-to-run and operator-to-operator consistency and demonstrate that the assays are robust and reproducible.

Western blotting is routinely used for protein detection, although it is cumbersome to perform and does not yield quantitative data. The OCT3/4, LIN28, NANOG, and SOX2 proteins from NTERA2 lysates were visualized by western blotting using the same antibody-

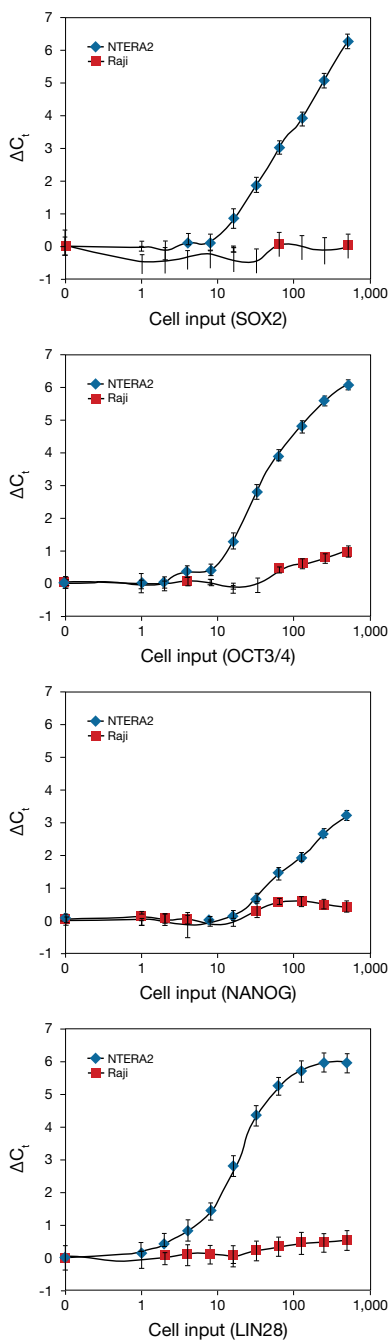


Figure 4. TaqMan® Protein Assays for Pluripotency Markers. The TaqMan® Protein Assays for OCT3/4, SOX2, NANOG, and LIN28 were performed with 2-fold serially diluted NTERA2 (blue) or Raji (red) cell lysates, from 500 to 0.7 cell equivalent of input. Each data point is an average of four replicates. The pluripotency markers are readily detected in NTERA2 cell lysates and are undetected in Raji cell lysates.

Table 1. Statistical Analysis of Expression Data From NTERA2 and Raji Cells.

Assay	Signal/noise (ΔC_t) [†]	Linear range (log)	Limit of detection (cell input)
	Avg* (StDev)	Avg* (StDev)	Avg* (StDev)
LIN28 ¹	5.78 [0.33]	1.20 [0.00]	7.81 [0.00]
NANOG ¹	2.88 [0.65]	1.20 [0.00]	34.37 [17.12]
OCT3/4 ¹	5.81 [0.40]	1.20 [0.00]	21.87 [8.57]
SOX2 ¹	5.78 [0.88]	1.26 [0.14]	28.12 [7.00]
CSTB ²	7.07 [0.46]	2.10 [0.21]	3.90 [2.39]
ICAM1 ²	7.92 [0.52]	1.20 [0.21]	34.38 [17.12]

* n = 5. † $\Delta C_t = C_t$ (cell input at maximum end of linear range) – C_t (no cell input).
¹ = measured in NTERA2 lysate. ² = measured in Raji lysate.

ies used in the TaqMan® Protein Assays (Figure 5). A robust signal is seen only with 100,000 cells/lane, and the limit of detection is only 10,000–30,000 cells/lane. The table in Figure 5 highlights the major advantages of the TaqMan® Protein Assay over western blotting, including sample input requirements, workflow, limit of detection, and quantification output.

The data generated by the TaqMan® Protein Assays pose a unique challenge for calculating relative quantification. For gene expression TaqMan® Assays, relative quantification is calculated using $E^{-\Delta\Delta C_t}$, where E represents the efficiency of the PCR reaction. To determine the relative amount of a target in a test sample with respect to a reference sample, it is not necessary that the efficiency of the PCR reaction be equal to 100% [E = 2]. The only requirement is that the efficiency of the PCR reaction be the same for both the reference and test sample. The efficiency is calculated using $E = 10^{[-1/\text{slope}]}$ and slope is obtained from the C_t vs. log (template) standard curve.

With the TaqMan® Protein Assays, the slope of ΔC_t vs. log (cell input) curve is dependent on the PCR reaction as well as the antibody/antigen binding reaction. Because no wash steps are used with the TaqMan® Protein Assays, the antibody/antigen binding reac-

tion is influenced by the cellular matrix. As a result, for any given assay, the slopes of ΔC_t vs. log (cell input) curves for different samples may be very different. Consequently, the standard $E^{-\Delta\Delta C_t}$ method for calculating relative quantification cannot be used for TaqMan® Protein Assays. A new method was developed to calculate relative quantities of proteins in samples with different ΔC_t vs. log (cell input) slopes. The method uses a quantification threshold (QT), or a ΔC_t threshold that is used to calculate the fold difference between the test and reference samples.

The following experiment with NTERA2 cells is presented to illustrate the functionality of and necessity for the QT method. NTERA2 cells were treated on day 0 with tRA to induce differentiation, and cells lysates were prepared on day 0 and on days 14 and 28 after treatment. The TaqMan® Protein Assay for LIN28 was performed on the three samples, each diluted 3-fold from 500 to 0.7 cell equivalents/well. The C_t results were imported into the TaqMan® Protein Assay Data Analysis Software, and the resulting ΔC_t vs. log (cell input) curves are shown in Figure 6. The software automatically calculates the linear dynamic range, and the slopes for each of the curves are different: 4.0, 2.7, and 1.8 for days 0, 14, and 28, respectively. Because of the

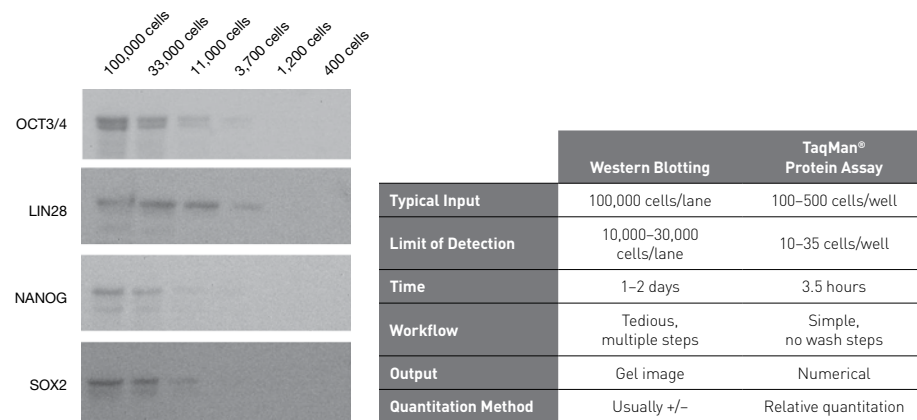


Figure 5. Western Blotting Vs. TaqMan® Protein Assay.

slope differences, the $E^{-\Delta\Delta C_t}$ method cannot be used, and the relative quantification is instead based on QT. The QT is set at a minimum, but not necessarily the lowest ΔC_t value above the noise of the assay. The default QT value of 2 is routinely used for most data sets. The relative quantification is calculated from the x-intercept (cell input) of the ΔC_t vs. log (cell input) curves and the QT:

$$\text{Relative quantification (RQ)} = \frac{\text{x-intercept at QT (ref)}}{\text{x-intercept at QT (test)}}$$

Using the TaqMan® Protein Assay Data Analysis Software, the relative quantification of LIN28 in NTERA2 cells after 14 and 28 days of tRA treatment as compared to day 0 is calculated as 0.29 and 0.1, respectively. The downward trend of LIN28 protein expression in tRA-treated NTERA2 cells is expected and matches that of the LIN28 mRNA expression (data not shown).

Summary

TaqMan® Protein Assays provide a simple, rapid, and sensitive method for protein relative quantification in cell lysates. The TaqMan® Protein Assays for the pluripotent stem cell markers OCT3/4, NANOG, SOX2, and LIN28 are robust and demonstrate specificity and sensitivity. Compared to western blotting, the TaqMan® Protein Assay requires less sample, is more sensitive, and has a simpler and faster workflow. Moreover, the TaqMan® Protein Assay allows users to determine relative protein quantities and enables correlation to mRNA and or miRNA expression results using the same analytical platform.

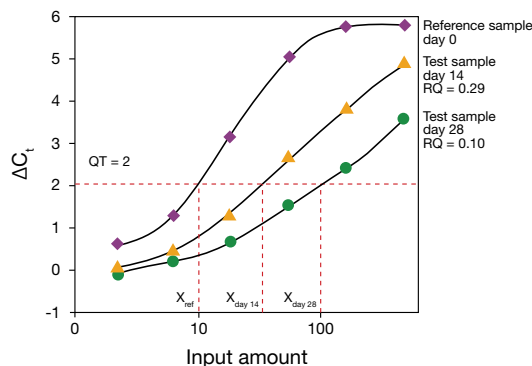


Figure 6. Relative Quantification Using TaqMan® Protein Assay Data Analysis Software. TaqMan® Protein Assays for LIN28 were performed with cell lysates from NTERA2 cells collected after 0, 14, and 28 days of tRA treatment. The quantification threshold (QT) is used to calculate the fold difference between the reference and test samples at the x-intercept (cell input) of the ΔC_t vs. log (cell input) curves. The relative expression of LIN28 at days 14 and 28 post-treatment as compared to day 0 was 0.39 and 0.1, respectively.

Acknowledgments

We would like to thank Elana Swartzman, Shiaw-Min Chen, Mark Shannon, Chad Mooney, Julie Kuykendall, and David Ruff of Applied Biosystems for their contributions to this application note.

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Printed in the USA. 02/2010 Publication 127AP13-01 O-090460