Novel Protein Expression Assays using qPCR for the Detection and Relative Quantification of Protein Markers in Stem Cells

Applied Biosystems

Elana Swartzman, Mark Shannon, Shiaw-Min Chen, Chad Mooney, Julie Nguyen, Caren Dhanatya, Tina Settineri and David Ruff
Applied Biosystems, Foster City, CA 94404

ABSTRACT

Stem cell characterization typically relies on determining the presence and amount of specific pluripotent protein markers. Current methods for detection of protein markers in stem cells include FACS, immunohistochemistry and western blots. Each method, however, has its own limitations such as requirement for large sample input, lack of sensitivity, or is not inherently quantitative.

TaqMan® Protein Expression Assay is a novel method using PLA™, a Proximity Ligation Assay technology, to detect proteins through the amplification of a surrogate DNA template.

PLA is a three-step process that involves:
1. binding of paired antibody-oligonucleotide probes to a protein target in whole cell lysates
2. templated ligation of the oligonucleotides in proximity
3. qPCR detection

TaqMan® Protein Expression Assay Schematic Overview

Antibody-Oligo Probes	Binding of Probes to Protein Targets	Templated Ligation of Oligos in Proximity	TaqMan® Real-time PCR
Whole Cell Lysates			
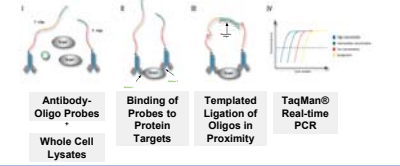
We have optimized this technique in crude cell lysates using a simple, one-step sample lysis protocol to release all classes of proteins, and combined it with TaqMan chemistry to create a highly sensitive and specific process for measuring protein expression in small samples

We have developed TaqMan Protein Expression Assays for OCT3/4, NANOG, SOX2 and LIN28 and supporting reagents to monitor the relative protein levels in human embryonal stem cells. The assays are robust, sensitive, and require 100x less cells than Westerns. As with traditional gene expression TaqMan assays, the data obtained from the TaqMan Protein Expression Assays are readily amenable to relative quantification. Consequently, the TaqMan Protein Expression Assays are ideally suited for the relative quantification of markers in pluripotent and differentiated stem cells.

Figure 1
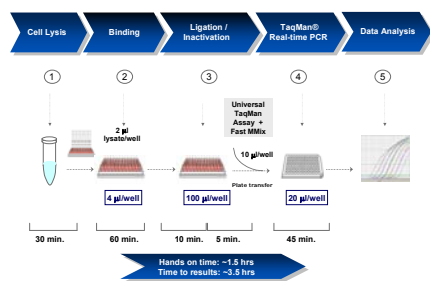**TaqMan® Protein Expression Assay Detailed Workflow**

| Cell Lysis | Binding | Ligation / Inactivation | TaqMan® Real-time PCR | Data Analysis |

Hands on time: ~1.5 hrs
Time to results: ~3.5 hrs

Table 1   TaqMan Protein Expression Reagents

TaqMan Protein Expression Assay Kits (6 individual kits)		Protein Expression Lysate Control Kits (2 individual kits)	
		NTERA2	Raji
		Human embryonal carcinoma stem cell line	Human Burkitt's lymphoma cell line
Stem cell pluripotency markers	human SOX2	✓	X
	human OCT3/4	✓	X
	human LIN28	✓	X
	human NANOG	✓	X
Control markers	human ICAM1	✓	✓
	human CSTB	✓	✓
TaqMan Protein Expression Core Reagents kit	Required with each Protein Expression Assay Kit. Contains all the reagents needed to run the assays, including ligase, universal TaqMan Assay, and TaqMan Protein Expression Fast Master Mix		
Protein Expression Sample Prep Kit	One step lysis of cultured cells		

MATERIALS and METHODS

TaqMan Protein Expression Assay
- The workflow is detailed in Figure 1
- The kits listed in Table 1 provide all the necessary reagents to perform the TaqMan Protein Expression Assay, from cell lysis to real-time PCR.
- In addition to the four stem cell markers: SOX2, OCT3/4, LIN28 and NANOG, two markers that serve as controls are available: ICAM1 and CSTB.
- NTERA2 and Raji cell lysates serve as positive and/or negative controls. The expression pattern of the 6 markers in the two control lysates are shown in Table 1.

Instrumentation
The TaqMan Protein Expression Assay has been validated for use on the following instruments from Applied Biosystems using default cycling fast or standard cycling parameters:
StepOne Plus™
7500 Fast
7900 HT Fast
7900 HT Standard

Westerns
- Cells were resuspended in SDS-PAGE buffer and loaded onto NuPAGE® Novex® Bis-Tris Gels (Invitrogen).
- Gels were transferred to 0.2 µ nitrocellulose membranes and probed with 0.1 µg/mL primary antibodies. The primary antibodies are the same antibodies used in the TaqMan Protein Expression Assays
- After incubation with a secondary antibody, proteins were visualized with chemiluminescence. Digital images were obtained after 5 min exposure using Alpha Innotetech software.
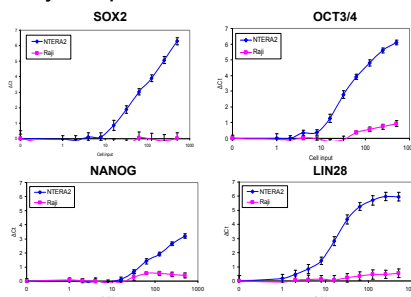
RESULTS

Figure 2. Representative TaqMan Protein Expression Assay Data
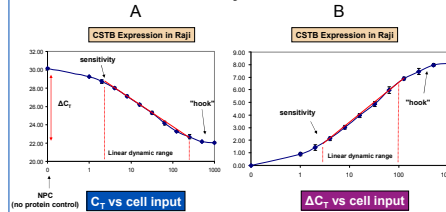
- The TaqMan Protein Expression Assay for CSTB was performed with 2 fold serially diluted Raji cell lysate (from 1000 to 1 cell input). Each data point is an average of 4 replicates.

- There is always some background ligation occurring when no protein is present (NPC). The data is therefore normalized by subtracting the background signal.
    - Figure 2a is plotted as C_T vs cell input and Figure 2b is plotted as ΔC_T vs cell input. The ΔC_T is calculated as: C_T (NPC [no protein control]) – C_T (x cell input)

- The "hook" effect occurs when too much antigen is present and is typically observed in immunoassays, such as ELISAs
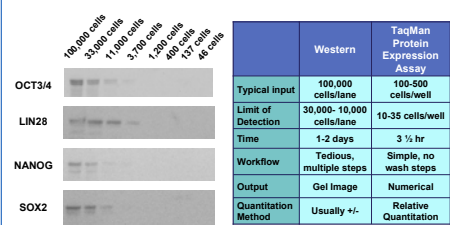
Figure 3
Stem Cell Marker TaqMan Protein Expression Assays are Specific and Sensitive

SOX2   OCT3/4   NANOG   LIN28

TaqMan Protein Expression Assays for the stem cell markers were performed with 2 fold serially diluted NTERA2 and Raji cell lysates (from 500 to 1 cell input). Each data point is an average of 4 replicates.

The stem cell marker proteins are detected in 10-500 cells of NTERA2 and are not detected in Raji cells.

Figure 4
Western vs TaqMan Protein Expression Assay

	Western	TaqMan Protein Expression Assay
Typical input	100,000 cells/lane	100-500 cells/well
Limit of Detection	30,000- 10,000 cells/lane	10-35 cells/well
Time	1-2 days	3 ½ hr
Workflow	Tedious, multiple steps	Simple, no wash steps
Output	Gel Image	Numerical
Quantitation Method	Usually +/-	Relative Quantitation

The same antibodies used for the TaqMan Protein Expression Assays were used for detection of OCT3/4, LIN28, NANOG, and SOX2 in NTERA2 cell lysates by Western blotting. No proteins were detected with Raji cell lysates (data not shown).

Table 2
Signal/Noise, Linear Range and Limit of Detection of the TaqMan Protein Expression Assays

| 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
#ΔC_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

The data was obtained from replicate assays (n = 5) performed on different days and by multiple operators and exhibits run-to-run operator-to-operator consistency.

Figure 5
TaqMan Protein Expression Assay using hESC and NSC Cell Lysates
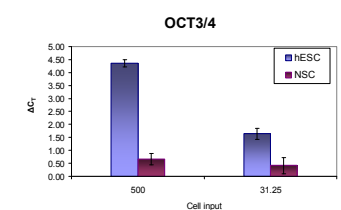
OCT3/4

The TaqMan Protein Expression OCT3/4 assay was performed with human embryonal stem cell (hESC) and neural stem cell (NSC) lysates. The NSCs were derived from hESCs using a neural differentiation protocol. As expected, OCT3/4 is readily detectable in hESCs, with even as little as 31 cells, and is absent in NSCs.

The lysates were kindly provided by Alana J. Toro-Ramos and Jennifer Moore from the Stem Cell Research Center, Rutgers University.

CONCLUSIONS

1. New TaqMan Protein Expression Assays using PLA technology provide a new alternative for protein analysis in small amounts of cell culture samples:
   - Simple one step cell lysis sample prep
   - Minimal Sample Input (10-500 cells per well)
   - Easy workflow similar to RNA expression methods

2. Assay measures stage-specific quantitative changes in human stem cell proteins for stem cell characterization & differentiation

For additional TaqMan Protein Expression Assay applications with demonstration of mRNA/protein correlation and relative quantitation measurements, see posters 1230 and 1123.

REFERENCES

1. Fredriksson S., Gullberg, M., Jarvius, J., Olsson, C., Pietras, K., Gústafsdóttir, S. M., Östman, A., and Landegren, U. Protein detection using proximity-dependent DNA ligation assays. *Nature Biotechnology* **20**, 473 - 477 (2002).

2. The International Stem Cell Initiative. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nature Biotechnology* **25**, 803 - 816 (2007).

127PR23-01