

A novel quantitative PCR-based protein detection method (TaqMan® protein expression assay) for OCT3/4, NANOG, SOX2 and LIN28 in human germ cell tumors (GCTs).

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Introduction

Pluripotency regulation of embryonic stem (ES) cells is dependent on a number of factors, including OCT3/4, NANOG, SOX2 and LIN28. These are also identified as highly informative markers for the detection of the various stages of human GCTs, especially seminoma, embryonal carcinoma (EC, the malignant counterpart of human ES cells), and their common precursor carcinoma *in situ* (CIS) (1,2). Expression analyses have been mainly investigated using Northern blot analysis, (quantitative) RT-PCR, as well as Western blotting and Immunohistochemistry. A direct and quantitative protein analysis on small amounts of cells has not been applied so far. Here we present TaqMan® Protein Expression Assay data, which is based on a novel detection method, referred to as PLA™, a Proximity Ligation Assay technology (3).

Materials and Methods

PLA determines the presence of a protein quantitatively using affinity-purified antibodies combined with a real time PCR-detection system (Figure 1), suitable to be applied on cell lysates between 10-500 cell equivalents. The workflow (Figure 2) consists of: 1) cell lysis (sample prep); 2) incubation of antibody-oligo-probes; 3) ligation; 4) real time PCR detection. In total, it takes about 4 hours to complete.

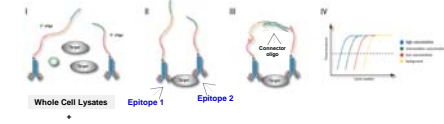


Figure 1. Schematic representation of the proximity ligation technology.

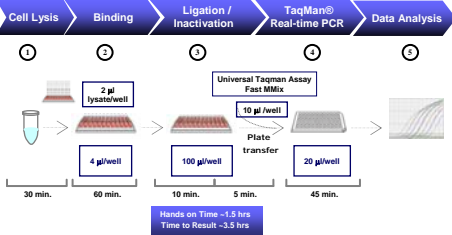


Figure 2. Workflow for TaqMan® Protein Expression Assays.

Results

No OCT3/4, NANOG, SOX2, and LIN28 protein is detected in a negative control (FS1 cells), although protein is present (CSTB) (Figure 4, upper left panel), while the other cells show presence of the proteins in various concentrations. High reproducibility is demonstrated (variation of less than 0.2 Cts between technical replicates, being less than 1% CV.

Cell-line	% positive stained cells (IHC) on cytoplasm			
	LIN28	NANOG	SOX2	OCT3/4
FS-1	0%	0%	0%	0%
NCCIT	98%	22%	98%	74%
2102Ep	89%	86%	87%	65%
TCam2	81%	94%	5%	82%
NT2	88%	72%	91%	78%

Table 1: IHC analysis of a series of well characterized GCT-derived cell lines, with known protein content, were investigated.

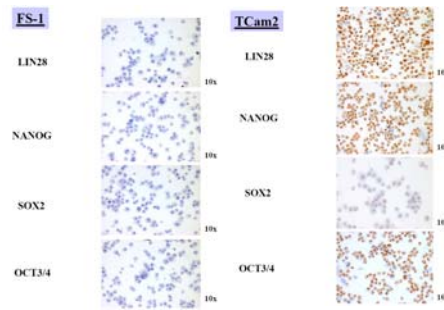


Figure 3. Immunohistochemical findings for FS1 and TCAM2.

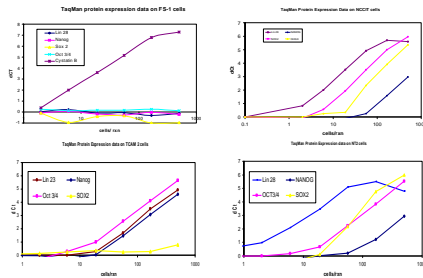


Figure 4. TaqMan® Protein Expression Analysis data for hOCT3/4, hSOX2, hNANOG, hLIN28 & h CSTB markers in FS-1, NCCIT, TCAM-2 and NT2 cell lines.

The sensitivity of the method, i.e., identification of undifferentiated cells, depends on the target used: the most sensitive being LIN28 (30 cell equivalents), followed by OCT3/4 and SOX2 (both at 60) and NANOG (100). A summary of the relative expression levels is shown

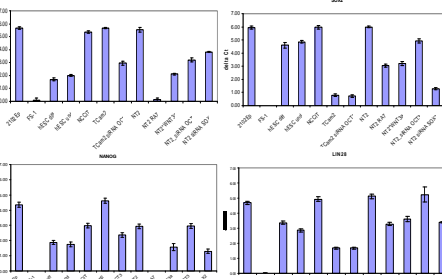


Figure 5. Relative protein levels of OCT3/4, SOX2, LIN28 and NANOG in a series of well characterized GCT-derived cell lines, cultured under different conditions, i.e., undifferentiated / differentiated / siRNA treatment. OCT3/4, Sox2 and Nanog at 500 cell equivalents and Lin28 at 56 cell equivalents. The lower cell input used for Lin28 detection is due to the hook effect observed at high protein concentrations.

In addition, EC cell lines with siRNA-mediated inhibition of OCT3/4, SOX2, and LIN28 are investigated. Immunohistochemistry of the NT2 cells after siRNA OCT3/4 treatment is shown (Figure 6) as well as the TaqMan® protein expression results (Figure 7).

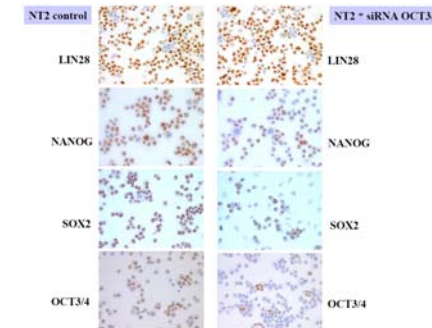


Figure 6. Immunohistochemistry on NT2 cells and NT2 cells after treatment with an OCT3/4 siRNA, the reductions in percentages of positive cells are: LIN28: 2%, NANOG: 6%, SOX2: 45%, OCT3/4: 67%.

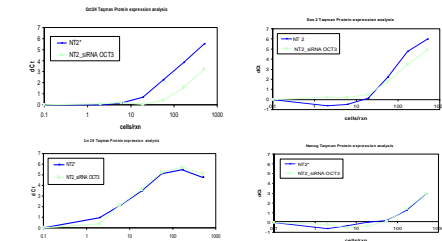


Figure 7. TaqMan® protein expression results of the NT2 subclones before and after siRNA OCT3/4 treatment.

NT2 cells are also investigated after exposure to retinoic acid (RA) for 7 days, a well known system for differentiation induction (Figure 8 for immunohistochemistry) as well as the TaqMan® protein expression results (Figure 9). The reductions in percentages of positive cells by IHC are: LIN28: 46%, NANOG: 72%, SOX2: 68%, OCT3/4: 78%.

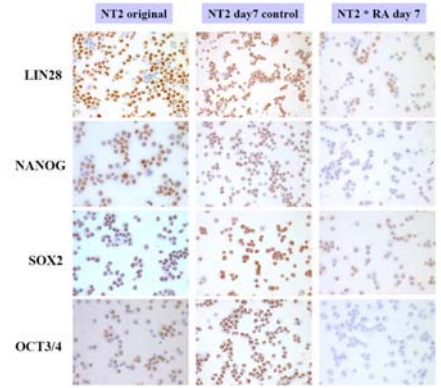


Figure 8. Immunohistochemistry before and after RA of NT2.

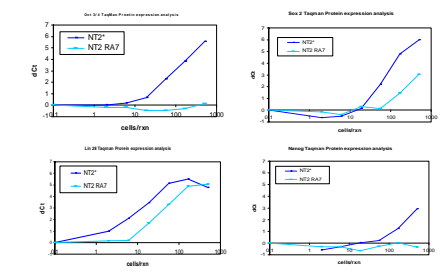


Figure 9. TaqMan® protein expression results of the NT2 subclones before and after 7 days of retinoic acid (RA) treatment.

In addition to the cell lines, a similar approach is performed on a series of primary frozen (and formalin-fixed, paraffin-embedded) GCTs (Figure 10).

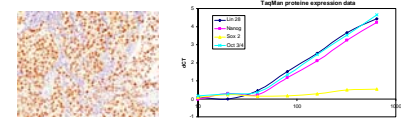


Figure 10. Representative results of OCT3/4 immunohistochemistry and TaqMan® protein expression on all four targets on a primary frozen seminoma.

Conclusion

TaqMan® Protein Expression Assays, which uses PLA™, is a highly reproducible and quantitative method for the detection of protein in small samples from both cell lines and tumors. It allows a fast investigation of the presence of proteins, even in a combined set up. This technique will be highly informative in the investigation of biological samples for the presence of specific proteins, as well as for studying experimental model, both *in vitro* and *in vivo*, in which protein level changes must be monitored. It is suitable to be applied to investigate relationships between mRNA, micro RNA and protein levels.

References

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- de Jong J et al. Differential expression of SOX17 and SOX2 in germ cells and stem cells has biological and clinical implications. *J Pathol* 2008;215:21-30.
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