Identifying Protein Biomarkers for Testicular Cancer Research Using Proximity Ligation Assays

TaqMan® Protein Expression Assays

Researchers in the Department of Pathology at Erasmus University Medical Center in Rotterdam, The Netherlands, are using TaqMan® Protein Expression Assays, an adaptation of proximity ligation assay (PLA™) technology, to quantify protein biomarkers for pluripotency using small quantities of human stem cell and testicular germ cell samples. Such biomarkers can potentially be used to identify and characterize malignant cells. The TaqMan® Protein Expression Assays have provided this group with advantages over currently used technology in that they are quick, use minimal sample, are highly reproducible, and can be used with biofluids.

Pluripotency Biomarkers

Human embryonic stem cells and germ cells of the developing testis express a number of protein markers associated with pluripotency (e.g., OCT3/4, NANOG, SOX2, LIN28), reflecting their common derivation from the embryonic inner cell mass. Studies have shown that malignant testicular germ cell tumors (TGCTs) also express these markers, a highly unique expression profile among malignant tumors.

Identifying Early Stages of Testicular Cancer

Dr Leendert Looijenga and colleagues at Erasmus University Medical Center (Rotterdam, The Netherlands) have demonstrated that OCT3/4, a pluripotent embryonic stem cell marker, can be used as a marker for early stages of testicular cancer [1]. Additional studies in this laboratory have highlighted other pluripotent embryonic stem cell markers, e.g., NANOG and SOX2, that may also be key factors in TGCT identification [2,3].

Early detection of TGCT is imperative for its treatment; early-stage TGCT can be treated effectively by local low-dose irradiation with limited side effects. Undetected TGCT will progress and potentially becomes insensitive to irradiation, resulting in the need for surgery and chemotherapy for treatment.

The current standard for early detection of TGCT relies on histological examination of testicular tissue. However, Dr Looijenga's laboratory has demonstrated that the use of immunohistochemical (IHC) staining for OCT3/4 is significantly more informative than sample morphology alone. Further development of this technique has, for the first time, led to the direct detection of OCT3/4 in TGCT cells from semen samples [4]. Although Dr Looijenga's IHC

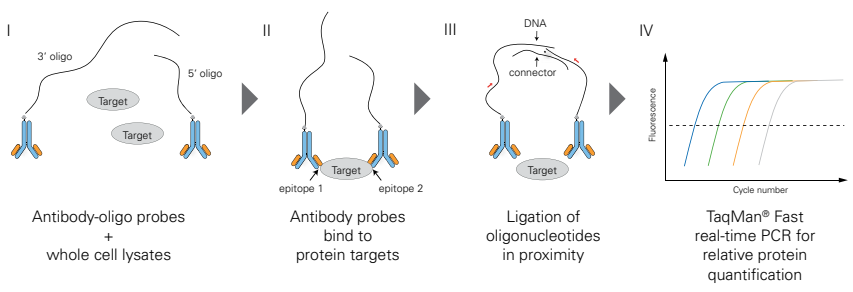


Figure 1. TaqMan® Protein Expression Assay Technology. The TaqMan® Protein Expression Assay is a homogeneous assay based on two antibodies, each conjugated to an oligonucleotide through a streptavidin–biotin linkage. When the two conjugated antibodies bind to their target protein and are in close proximity, the oligonucleotides can be ligated, serving as the template for real-time PCR amplification and quantification.

method in semen has the potential to decrease the invasiveness of the assay, it has yet to address cost, and qualitative and quantitation issues.

Proximity Ligation as an Inexpensive, Highly Reproducible, Quantitative Protein Measure

To this end, Dr Looijenga and colleagues Ad Gillis and Hans Stoop have tested the ability of the new Applied Biosystems® protein expression technology, the TaqMan® Protein Expression Assay, for detection of a number of pluripotency markers, e.g., OCT3/4, NANOG, SOX2, and LIN28, in human embryonic stem cell lines as well as in representative cell line models for the different stages of TGCT. These assays enable relative protein quantitation using affinity-purified antibodies combined with a real-time PCR detection system (Figure 1), and take only 3.5 hours to perform (see *The Specificity and Sensitivity of TaqMan® Assays Now, for Stem Cell Protein Research*, page 3, for a description of the workflow).

The results for TGCTs and derived cell lines with known expression of OCT3/4 and SOX2 showed that these pluripotency markers are indeed an informative tool for identification of malignant stem cells (seminoma and embryonal carcinoma cells, and their common precursor, carcinoma *in situ* cells). The findings corroborated observations made using IHC (Figure 2), while providing quantitation, speed, and the ability to be performed with much smaller sample inputs. The TaqMan® Protein Expression Assays also showed an outstanding level of reproducibility.

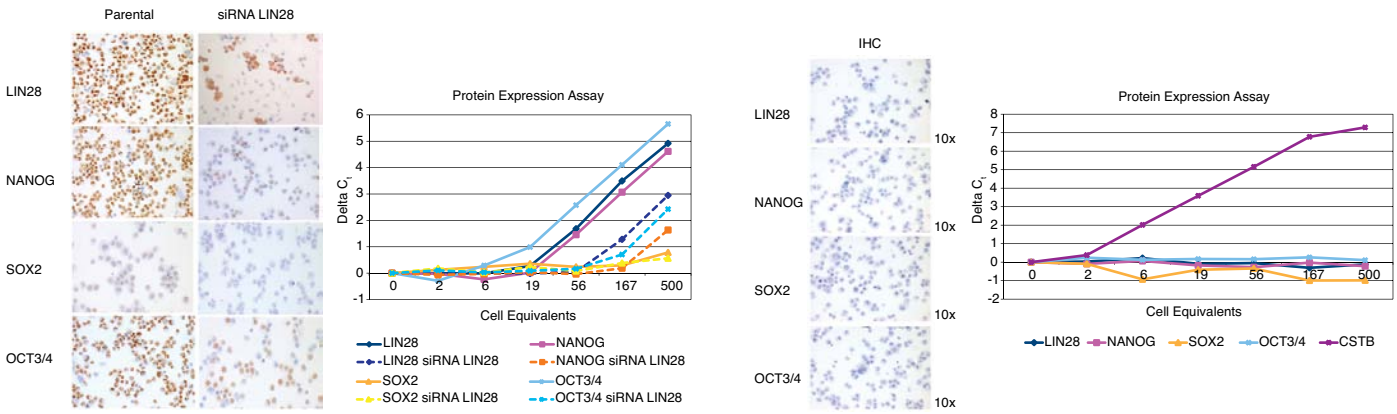
Dr Looijenga and colleagues have also used TaqMan® Protein Expression Assays as an effective method for monitoring protein knockdown in TGCT cell lines with siRNA-mediated inhibition of OCT3/4, SOX2, and LIN28, using *Silencer*® Select siRNAs. "It is faster and uses significantly less material than western blotting. In addition, analysis of the effect on other proteins can be performed on the same sample, which is of high biological potential. Moreover, correlation analysis with mRNA levels (performed by TaqMan® real-time RT-PCR) can be easily included," noted Dr Looijenga.

Looking Forward: Multiprotein Analysis From Fluids, Not Biopsies

In the future, Dr Looijenga sees using TaqMan® protein expression technology for further characterization of pluripotency marker knockdown, and for performing multiprotein analysis. He also hopes to develop the technique such that easily obtainable body fluids can be used in place of biopsies for biomarker analysis. Dr. Looijenga notes, "The development of TaqMan® Protein Expression Assays will result in a significant step forward in the investigation of the biology of many proteins, including those involved in stem cell regulation, both normal and malignant, using limited amounts of material, in a standardized setup".

References

1. Looijenga LR et al. (2003) POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. *Cancer Res* 63:2244–2250.
2. Hart AH et al. (2005) The pluripotency homeobox gene NANOG is expressed in human germ cell tumors. *Cancer* 104:2092–2098.
3. de Jong J et al. (2008) Differential expression of SOX17 and SOX2 in germ cells and stem cells has biological and clinical implications. *J Pathol* 215:21–30.
4. van Casteren NJ et al. (2008) Noninvasive detection of testicular carcinoma in situ in semen using OCT3/4. *Eur Urol* 54:153–160.



Panel A. TCam2 Seminoma.

Panel B. FS-1 (negative control).

Figure 2. TaqMan® Protein Expression Assays Corroborate IHC Staining Results. Both immunohistochemistry (IHC) and TaqMan® Protein Expression Assays were performed on TCam2 cells (seminoma cell line) for LIN28, NANOG, SOX2, and OCT3/4 proteins, 4 common biomarkers of the pluripotent state. The 4 biomarker proteins were present in various concentrations. The effect on these 4 pluripotent markers was also examined after transfecting the TCam2 cells with a Lin28 siRNA. The TaqMan® Protein Expression Assays were highly reproducible (variation <0.2 C_t between technical replicates). None of these specific proteins was detectable in a negative control (FS-1 cells), though protein was present (as shown by detection of CSTB, a cystatin, considered a housekeeping protein).