Application of quantitative real-time RT-PCR in reproductive toxicicology for detecting chemical effects on human endometrial function

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Introduction

Changes in gene expression induced by xenobiotics can be evaluated as sensitive toxicological endpoints. We apply quantitative real-time RT-PCR in the development of an *in vitro* test for the identification of reproductive toxicants interfering with embryo implantation. Our work is part of the integrated project *ReProTect* (EU) whose aim is the development of *in vitro* tests required under the new European chemicals legislation (REACH). In the research area of "Implantation" we are exploring predictive toxicological endpoints in human endometrial explants in order to detect chemical effects on endometrial receptivity. This test is a closer approach to *in vivo* conditions than primary or permanent cell cultures. Facing the low tissue amounts which are available for each incubation (approx. 20 mg) quantitative real-time RT-PCR was selected as the major analytical method.

Potential toxicological endpoints (target genes)
Leukaemia inhibitory factor (LIF)
Progesterone receptor (PR)
Estrogen receptor α (ERα)
Calcitonin
Cyclooxygenase-2 (COX-2)
Corticotrophin releasing hormone receptor 1 (CRHR1)
VEGF-receptor 2 (KDR)

Potential reference genes
Delta (5)-aminolevulinate-synthase (ALAS1)
Hypoxanthine phosphoribosyltransferase (HPRT)
Glucose-6-phosphate dehydrogenase (G6PDH)
Porphobilinogen deaminase (PBGD)

Endometrial explants culture

Endometrial tissues were obtained by aspiration curettage from premenopausal women. The endometrial biopsies were chopped into pieces of 1-2 mm/side and cultured in sex steroid supplemented medium at 37 °C in 5 % CO_2 for 6-24 hrs. Test chemicals will be administered during this incubation period and compared to negative controls with vehicle alone.

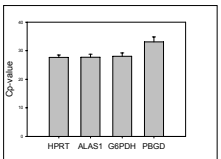
Investigation of toxicological endpoints by real-time PCR

In order to identify predictive toxicological endpoints among a broad spectrum of possible target genes, assays from the versatile Universal Probe Library (UPL; Roche) are used. Our aim is to evaluate our qPCR runs by calibrator normalized relative quantification. Up to now a calibrator originating from endometrial specimens was prepared, reference genes were selected from a set of housekeeping genes and calibrator standard curves for 3 target genes and 2 reference genes were determined.
For RNA isolation RNeasy-minicolumns (Qiagen) and for reverse transcription Superscript II (Invitrogen) were used. RNA was characterised by RNA 6000 nano LabChips (Agilent). RTQ-RT-PCR was run on a LightCycler 480 with LightCycler 480 Probes MasterMix (Roche).
Our calibrator (2 ml, divided into 10 µl aliquots) is composed of pooled cDNA samples prepared from endometrial tissues from 18 different patients. The coefficients of variation of Cp values of target and reference genes yet investigated were <5% (triplicates). Standard curves were run with 6 replicates per dilution. As possible reference genes ALAS1, HPRT, G6PDH and PBGD were studied with ALAS1 and HPRT displaying the least variation in endometrial specimens from 6 different patients.

Calibrator normalized relative quantification

$$\text{Normalized ratio} = \frac{\frac{\text{Conc. target (sample)}}{\text{Conc. reference (sample)}}}{\frac{\text{Conc. target (calibrator)}}{\text{Conc. reference (calibrator)}}}$$

Critical aspects
Selection of reference gene(s)
Preparation of calibrator:
Reproducibility of (external) standard curves
Equal efficiencies in samples and calibrator

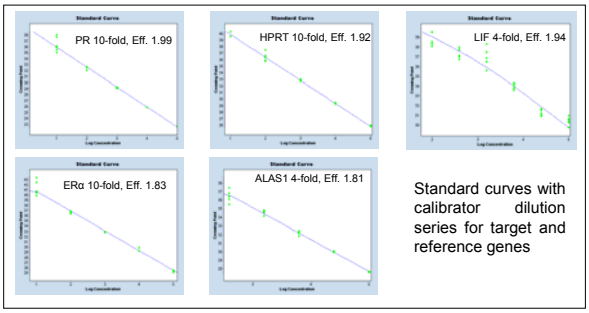


Mean Cp-values of housekeeping genes in human endometrium

Reproducibility of calibrator Cp's

LIF	30,05 ± 0.65 (n=6)
PR	22.73 ± 0.15 (n=3)
ERα	25.88 ± 0.73 (n=4)
ALAS1	28.01 ± 0.63 (n=7)
HPRT	27.20 ± 1.30 (n=4)


◄ Column graph of relative quantification analysis results (PR/ALAS1).
Blue bars: Concentration ratio
Green bars: Normalized ratio



Standard curves with calibrator dilution series for target and reference genes

Summary

(1) The Universal Probe Library is an useful and cost-efficient tool to make a broad spectrum of qPCR assays rapidly available.
(2) Prior to the application of calibrator-normalized relative quantification some time-consuming preparatory work is necessary (calibrator preparation, selection of reference genes, determination of standard curves). A critical aspect is the determination of standard curves for low and medium expressed genes (e.g. high scattering of replicates for LIF).
(3) Evaluation by calibrator normalized relative quantification works in principle for our application. Some software problems have to be cleared (e.g. handling of negative samples displaying a fluorescence signal, low sample Cp compared to the highest standard concentration).
(4) Investigations on the reproducibility of standard curves, chemical effects of test substances (e.g. RU486) and establishment of more UPL-assays (e.g. CRH receptors, cyclooxygenase-2, calcitonin) are under work.

References
1. Stavreus-Evers A et al. (2003), Development and characterization of an endometrial tissue culture system. Reprod Med Online 7: 243-9
2. Catalano RD et al. (2003), The effect of RU486 on the gene expression profile in an endometrial explant model, Mol Hum Reprod 9:465-473

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