Quantification of mRNA Expression using DNA-based Standard Curve RT-qPCR Methods

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1 INTRODUCTION

Gene expression analyses are bioanalytically relevant for quantifying expression strength of vector-based gene therapeutics (TK/PK), as well as for establishing efficacy (PD) of oligonucleotide-based therapeutics (Figure 1). Reverse transcription quantitative PCR (RT-qPCR) is often the method of choice for performing gene expression assessments, with alternative methodologies including branched DNA assays (QuantiGene), NanoString or droplet digital PCR (ddPCR).

RT-qPCR methods vary in technical considerations and analytical design:

- one-step or two-step
- oligo dT, random, or gene specific primers
- SYBR or probe-based
- singleplex or multiplex reactions
- relative to a DNA/RNA calibration standard
- relative to reference genes (ΔΔCt, Livak and Schmittgen, 2001)

We have developed a simple and efficient workflow for validating RT-qPCR assays for mRNA quantification using a single-step, DNA standard curve-based RT-qPCR method.

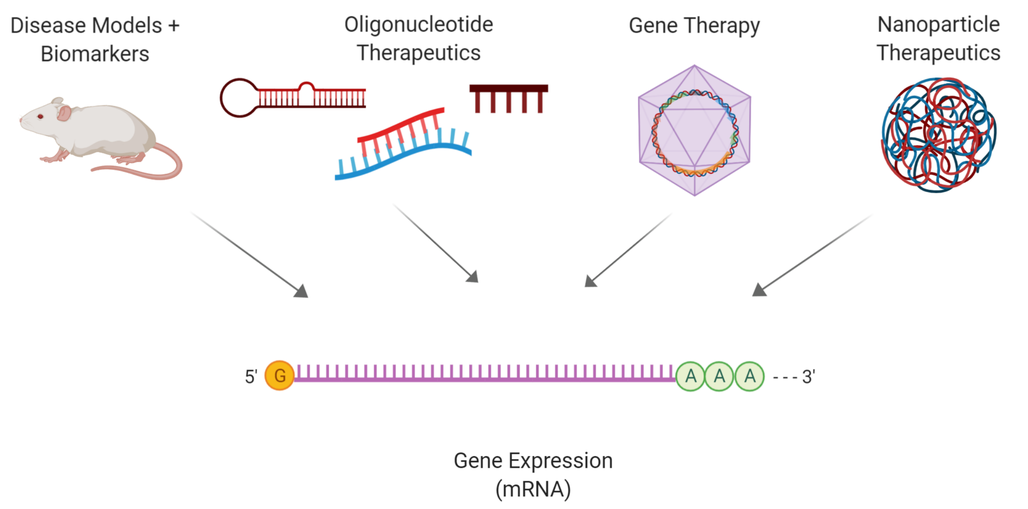


Figure 1: Gene expression modulation in disease and in response to therapeutic intervention.

2 MATERIALS AND METHODS

RNA Extraction performed using a phenol-chloroform based method (TRIzol Reagent, Invitrogen)

Figure 2a: Workflow for the preparation of purified RNA from tissues.

DNase-treatment using the DNA-free DNA Removal Kit (Ambion)
RNA quantification using fluorescence-based Quant-iT RiboGreen RNA Assay Kit (Invitrogen)

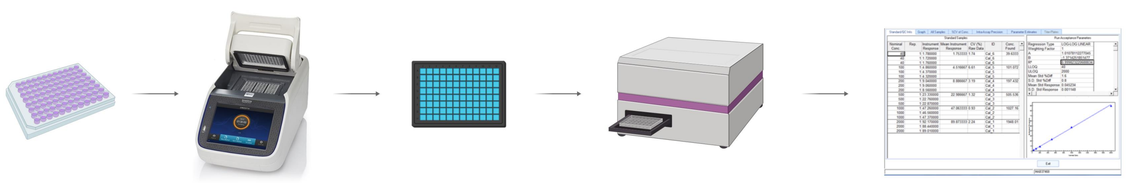


Figure 2b: Workflow for RNA processing prior to RT-qPCR analysis.

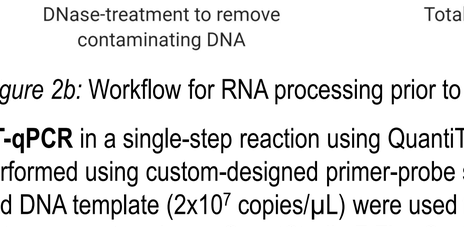
RT-qPCR in a single-step reaction using QuantiTect Multiplex RT-PCR Kit (Qiagen). Detection of target can be performed using custom-designed primer-probe sets or off-the-shelf assays. For this case-study, a Bio-Rad assay and DNA template ($2x10^7$ copies/µL) were used for Gene X quantification in mouse liver and placenta. Data was captured on a QuantStudio 7 Flex System and analysed in Watson LIMS.

3 METHOD VALIDATION

Reference Standard and RNA Linearity

Limit of quantification/detection (LOQ/LOD)

Inter-/Intra-Assay Accuracy and Precision



RNA Levels in Individual Control Samples

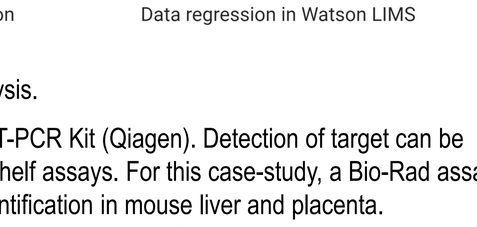


Figure 3: Plate map templates for characterising assay performance. Cal – Calibration Standard. WB – Water Blank. No RT – No Reverse Transcriptase. QC – Quality Control (Upper Limit, High, Mid, Low, Lower Limit). S – Sample.

4 RESULTS

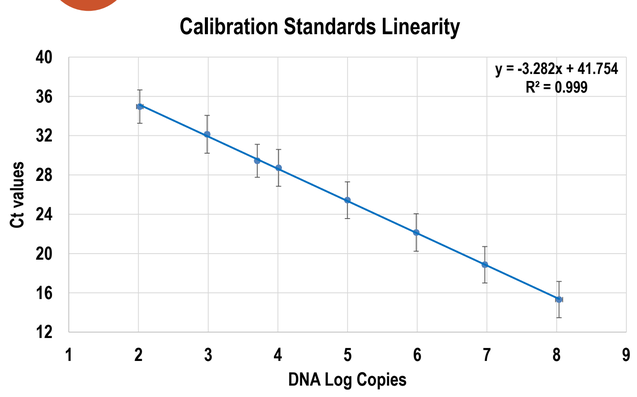


Figure 4a: Calibration standards linearity. Lot to lot variation impact on precision (Ct signal) but not on accuracy (concentration).

Assay Performance

	Accuracy (RE)	Precision (CV)
Intra-Assay	± 1.4%	≤ 0.6%
Inter-Assay	± 0.7%	≤ 0.6%

Table 1: Intra-/Inter-assay accuracy and precision results based on single reference standard lot numbers.

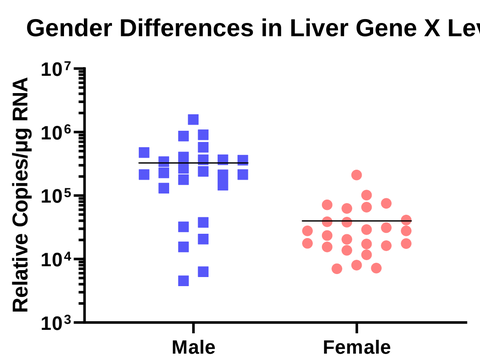


Figure 4b: Non-treated liver gene expression in 25 male and 25 female mice. Assessment of control levels and gender differences.

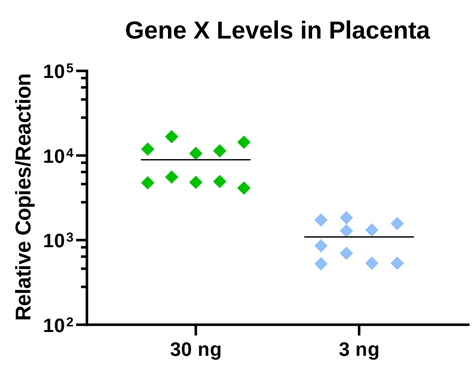


Figure 4c: Non-treated placenta gene expression in 10 female mice at 2 concentrations, showing linear fold-change. Reduction of input RNA simulates mRNA knockdown expected in treated animals.

Application criteria:

- Linearity is assessed in 2 samples for each matrix. LOQ/LOD is assessed once with DNA standard.
- Intra-/inter-assay accuracy and precision is assessed on 6 occasions by at least 2 analysts.
- Control sample analysis should be performed for each matrix and gender, at 2 RNA concentrations.
- Calibration standards should be analysed in triplicate wells; QCs, samples and blanks in duplicate wells.
- At least 6 calibrators, 66% of QCs and one QC at each level should meet the acceptance criteria.
- The coefficient of determination r^2 should be ≥ 0.990. The amplification efficiency should be 90-110%.
- Precision (%CV) of Ct values should be ≤ 3%. Accuracy (%RE) of log copies should be within ±10%.
- Water blanks and No RT controls containing RNA should be < LOD.

5 CONCLUSION

A single-step RT-qPCR is advantageous over a two-step protocol based on: reduced potential for contamination, lower variability due to single-reaction setup, lower effects from RNA integrity due to gene-specific primer-probe sets (Fleige and Pfaffl, 2006), as well as time-efficient and simple setup, reducing the likelihood of process errors. The minimum-required testing parameters described in this workflow allow gene expression assays to be validated in a straightforward and reproducible manner, using any target sequence and custom primer-probe sets.

References:

Livak and Schmittgen, Methods, 2001,25(4):402-8, DOI: 10.1006/meth.2001.1262
Fleige and Pfaffl, Molecular Aspects of Medicine, 2006, 27:126-139, DOI: 10.1016/j.mam.2005.12.003