How to do successful gene expression analysis using real-time PCR

Jan Hellemans and Jo Vandesompele
Biogazelle, Zwijnaarde, Belgium

experiment design

Power analysis

qPCR experiments require a sufficient number of biological samples to be included so meaningful and statistically significant results can be obtained. All too often researchers use too few samples, preventing them from drawing strong conclusions. Power analysis may overcome this type of failures.

Sample vs gene maximization

Most qPCR experiments involve more measurements than fit in a single plate. When spreading reactions across plates one can choose to either distribute genes and keep samples clustered in a plate (sample maximization), or to cluster genes and distribute samples (gene maximization). In a relative quantification study, researchers are typically interested in comparing expression levels for a given gene among samples. Since sample maximization minimizes technical variation between samples it is the preferred set-up.

Whenever samples have to be spread across plates, inter-run calibrators have to included to detect and correct for unwanted run-to-run variation.

Plate layout

Thinking about a proper plate layout, with samples and assays ordered in rows and columns enabling the use of multi-channel pipets, streamlines plate filling and annotation and reduces the risk of pipeting mistakes. It is acceptable to have empty wells in a plate.

sample preparation

RNA extraction

RNA extraction is the start of all gene expression studies. Quality and yield are important. Depending on the assay type, RNA samples may require DNase treatment to prevent gDNA coamplification.

RNA quality control

RNA quality has a profound impact on the results, in terms of the significance of differential expression, variability of reference genes and sample classification performance using a multi-gene signature.

RNA quality is assessed in terms of integrity and purity. Integrity can be evaluated by means of inspection of the 18S & 28S ribosomal RNA peaks or by performing a 5'-3'mRNA ratio test. The SPUD assay can be used for measuring sample purity.

Ideally, all samples are pure and fully intact. If this is not possible, it is important to compare samples of similar quality.

Pre-amplification

The amount of available RNA may be insufficient to achieve a sufficient sensitivity or to screen all genes of interest. Sample pre-amplification (whole transcriptome or target specific) may offer a way out.

assay design and validation

Design

Intron-spanning assays have the advantage that they may avoid gDNA co-amplification provided the gene of interest contains introns that are sufficiently large. Exonic designs on the other hand may use a larger design space (all exons rather than a limited set of exon pairs) increasing the design success rate. Probes are advocated for their increased detection specificity and allow for qPCR multiplexing. A well designed SYBR assay may however provide the same level of specificty at a lower cost.

In view of the wide range of transcript variants it is of importance to design assays in the gene region that is included in the majority of transcripts.

In-silico validation

The specificity of designs can be evaluated in-silico with dedicated tools. BiSearch is our preferred tool because it allows for controlling the number of mismatches that would still allow qPCR amplification.

To avoid allelic bias or drop out, one has to make sure no SNPs are located at the 3' end of the primer binding region.

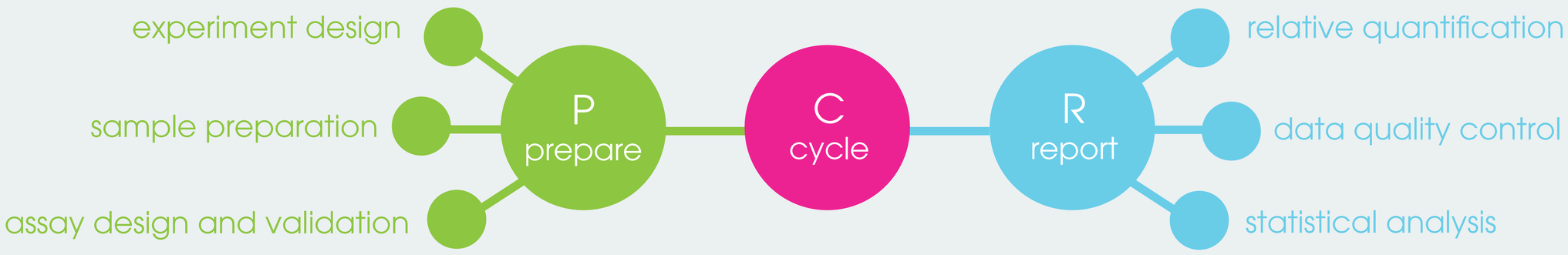
Secondary structures in the primer binding region are to be avoided because they may negatively impact PCR performance.

Empirical validation

Every assay has to be validated in the lab, even after having done in-silico quality assessment. This also applies for the majority of commercial assays that have not yet been lab validated.

Specificity can be evaluated by amplicon size analysis and, for SYBR assays, the interpretation of melt curves. Co-amplification of homologous sequences may be missed with these methods.

Amplification efficiencies can be assessed by measuring a serial dilution series. Good assasys have efficiencies in the 90%-110% range.



Numerous critical issues in the workflow need to be addressed before biologically meaningful and trustworthy conclusions can be drawn. Here, we review the entire workflow from the planning and preparation phase, over the actual real-time PCR cycling experiments to data-analysis and reporting steps. This process can be captured with the appropriate acronym PCR: plan/prepare, cycle and report. The key message is that quality assurance and quality control are essential throughout the entire RT-qPCR workflow.

relative quantification

Efficiency correction

The delta-delta-Cq quantification method assumes 100% amplification efficiency. For suboptimal assays the use of the Pfaffl or qBase model, supporting variable efficiencies is recommended.

Multiple reference gene normalization

It has been clearly demonstrated that the use of single non-validated reference genes results in suboptimal or even plain wrong results. The qBase quantification model combines the information of multiple reference genes for proper normalization.

Inter-run calibration

To detect and correct for technical run-to-run variation so called inter-run calibrators may be included in the experiment. These identical samples should yield the same result in different runs; observed technical variations can subsequently be used to correct all measurements.

Error propagation

Plotting the error bar for relative quantitites involves more than simply calculating the standard deviation on the Cq values of PCR replicates. Errors on the gene of interest and the reference gene(s) have to be properly combined and propagated throughout calculations to end up with the correct error on the final results.

data quality control

Amplification and melt curves

For each assay the amplification curves of all samples should run in parallel, having a similar sigmoidal shape.

For SYBR assays, the derivate melt curve can be used to detect non-specific amplification or primer dimer formation.

Replicates

Depending on the required precision, some variation in replicate Cq values can be tolerated. Objectively identified bad PCR replicates (outlier, known pipetting issue, abnormal curve) should be excluded from analysis.

Negative controls

Ideally, negative controls should not result in any amplification signal. It may be acceptable to accept some signal for negative controls as long as their Cq value is sufficiently larger than those of the other samples. Requiring a 5 cycle difference corresponds to accepting ~3% non-specific signal.

Reference gene expression stability

Even after having selected the best set of reference genes in a geNorm pilot study, their stability should be verified in subsequent experiments. Both the geNorm M-value and the CV on normalized relative quantities of reference genes can be used for this.

statistical analysis

Biological replicates

It is important not to draw statistical conclusions on PCR replicates. A representative, independent and sufficiently large set of biological replicates is needed (see power analysis).

Log transformed relative quantities

Many of the commonly applied statistical tests, including the t-test and ANOVA, rely on the normality of the data. Relative gene expression levels are known to not follow a normal distribution. Log transformation will make these data more symmetrical, thereby approaching normality.

Test selection

Selecting a statistical test involves more than just personal preferences. The wrong test may fail to prove the real impact of your study. Wizards such as those integrated in qbase+ may help selecting the proper test.

Multiple testing correction

The cut-off of 0.05 to call a result significant limits the chance for false positive conclusions to 5 %. When testing multiple hypotheses (genes), this 5 % applies to every test, resulting in a much larger overall false positive risk. This problem can be corrected for by means of multiple testing correction, typically Bonferonni for small numbers of genes and false discovery rate control for larger screening studies.

Further reading: S. Derveaux, J. Hellemans and J. Vandesompele. How to do successful gene expression analysis using real-time PCR. Methods, 2010.

Biogazelle NV
Technologiepark 3
B-9052 Zwijnaarde, Belgium
www.biogazelle.com
info@biogazelle.com