Roche Applied Science
Technical Note No. LC 15/2002

LightCycler

Selection of Housekeeping Genes

Purpose of this Note

Relative quantification is a powerful technique that is commonly used to study RNA gene expression. In relative quantification the expression of a target gene is measured with respect to a stably expressed reference gene (so-called housekeeping gene); the two gene levels are expressed as a ratio.

The search for an ideal housekeeping gene can be cumbersome and time-consuming. However, housekeeping genes must meet certain criteria before they can be effective reference genes.

This Technical Note gives specific guidelines for selecting housekeeping genes to be used as reference genes. It also recommends a basic procedure for setting up relative quantification applications.

Note: For detailed information on performing relative quantification with the LightCycler System, see Roche Applied Science Technical Note No. LC 13/2001 (*Relative Quantification*).

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1. General Introduction

Definitions

For maximum clarity, we will use the following definitions in this Note:

Term	Definition
Reference Nucleic Acid	An unregulated nucleic acid that is found at constant copy number in all samples. It is used to normalize sample-to-sample differences (also called endogenous control).
• **Housekeeping Gene**	For mRNA quantification. A gene that is expressed constitutively and at the same level in all samples to be analyzed.
• **Single Copy Gene**	For gene dosage quantification. Only one copy of the gene is found in all samples.
Pseudogene	Processed genes, often found in multiple copies, without exon - intron structure; non-functional sequences resulting from mutation events.
Calibrator	A sample that is used to normalize final results. To normalize the sample values, the expression ratios (target:reference) of all samples are divided by the expression ratio of the calibrator. Therefore, the calibrator must have a constant ratio of target gene to reference gene.
External Standard	It has a known concentration (*e.g.*, copies or ng/µl) and is used to generate a standard curve for the quantification of unknowns. Serial dilutions of the standard are amplified in the same LightCycler run as the target, but in separate capillaries.
Relative Standards	Series of dilutions, containing target and reference nucleic acids (relative amounts), that is used to determine the fit coefficients (efficiencies) of a relative standard curve.
Control	It is used for verification of a result and not for the quantification of unknowns; its concentration may not even be known.
• **Internal Control**	The control is amplified in the same capillary as the target or the standard; it may be endogenous or exogenous.
• **Exogenous Control**	The control is added to the PCR mixture.

Note: The term "internal standard" or "control gene" is frequently used in the literature as a synonym for endogenous reference genes, such as housekeeping genes or single copy genes (see definitions above).

2. Principles of Relative Quantification

Introduction

The study of biological regulation usually involves gene expression assays and frequently requires quantification of RNA. In the past, conventional gel- or blot-based techniques were used for these assays. However, these techniques often could not provide the speed, sensitivity, dynamic range, and reproducibility required by current experimental systems. In contrast, real-time PCR methods, especially the LightCycler Instrument, can easily meet these requirements.

Reverse transcription PCR (RT-PCR) is a common and powerful tool for highly sensitive RNA expression level profiling. For quantitative analysis of gene expression, RT-PCR often uses housekeeping genes as endogenous controls against which the expression level of a target gene can be normalized:

$$\frac{\text{Amount of target}}{\text{Amount of housekeeping RNA or single copy gene}}$$

Note: For the determination of gene dosages, we recommend using endogenous single copy genes as reference genes.

Normalization to a Reference Gene

In normalization, the amount of a target gene in each sample is divided by the amount of a reference gene in the same sample, thereby correcting for qualitative and quantitative differences caused by:

- Variations in initial sample amount or nucleic acid recovery
- Possible RNA degradation in sample material
- Differences in sample and/or nucleic acid quality
- Variations in sample loading or pipetting errors
- Variations in cDNA synthesis efficiency

Therefore, the amplification of housekeeping gene sequences serves two main purposes: to determine the availability of relatively intact RNA (cDNA) in the sample and to normalize the quantification of the target transcript.

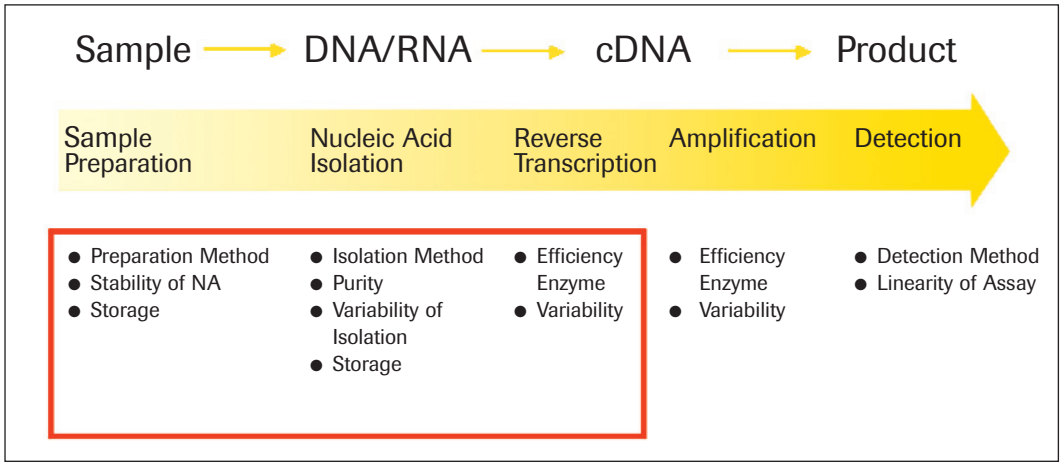

Figure 1: Factors that influence PCR amplification during sample and nucleic acid preparation.

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Different Methods

Relative quantification is one of the major applications of quantitative PCR (or RT-PCR). Quantification is relative because the result is expressed, not as an absolute amount, but as the ratio of the target (gene of interest) to a reference gene measured in the same sample. Two methods are available for determining this ratio:

- **Relative Quantification with External Standards (manual method):**
  The target concentration is expressed in relation to the concentration of a reference gene. A standard curve, based on serial dilutions of an external standard, is used to determine the concentration of the target and the reference gene. These gene concentrations are automatically calculated by the LightCycler Software. However, calculation of the target/reference ratio must be done either manually or with a spreadsheet program such as Microsoft Excel.
- **Calibrator Normalized Relative Quantification (software-based):**
  Results are expressed as the target/reference ratio of each sample divided by the target/reference ratio of a calibrator. The amounts of target and reference are a function of PCR efficiency and sample crossing point, so determination of these values does not require a standard curve in each LightCycler run. In calibrator-normalized relative quantification, accuracy is only influenced by the different amplification efficiencies of target and reference. For exact results, these efficiency differences can be corrected; for approximate results, the value for both amplification efficiencies can be assumed to be 2. This type of calculation can easily be performed with the LightCycler Relative Quantification Software, an optional software module that allows direct download of LightCycler data files.

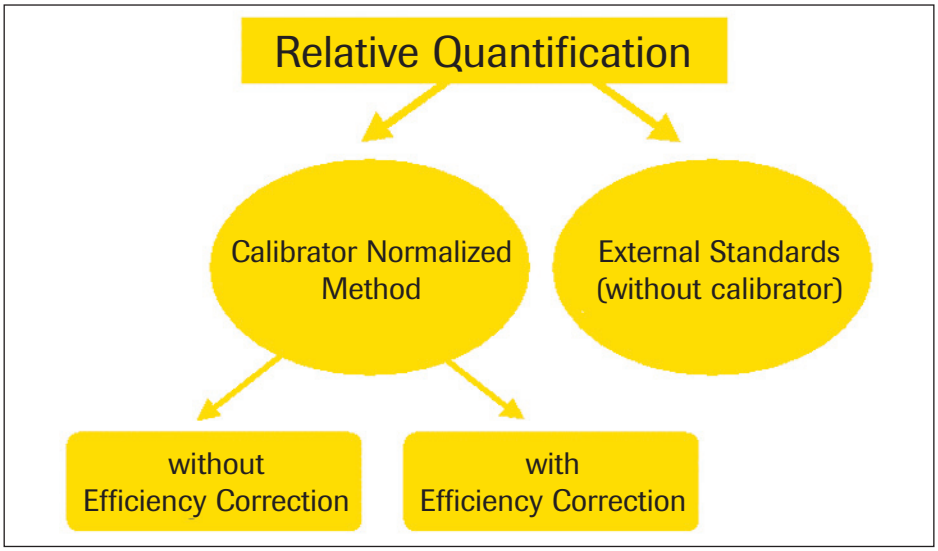

Figure 2 illustrates the two different methods for relative quantification. The LightCycler Quantification Software allows you to perform Calibrator Normalized Quantification with or without efficiency correction.

Note: For detailed information on relative quantification please refer to Technical Note No. LC13/2001.

3. Housekeeping Genes as Reference Nucleic Acids

Overview

Housekeeping genes encode proteins that are essential for maintenance of cell function. For instance, housekeeping genes which code for components of the cytoskeleton (*e.g.*, β-actin, (α-tubulin), components of the major histocompatibility complex (such as β2-microglobulin), enzymes of the glycolytic pathway (GAPDH = glyceraldehyde-3-phosphate dehydrogenase) or ribosomal subunits appear to be expressed ubiquitously. Detection of housekeeping mRNA has routinely been used to control several variables that may affect RT-PCR (see "Normalization to a Reference Gene", page 3). These endogenous controls are present in each experimental sample and therefore are perfect candidates for normalization of the final result.

Since housekeeping genes are essential for cell viability, it was generally assumed that they were expressed at similar levels in different types of cells. In fact, this was a requirement if they were to be used as reference nucleic acids. However, several reports indicate that the expression of housekeeping genes is actively regulated; levels may vary across tissues and different types of cells, during cell proliferation and stages of development, or due to experimental treatment of cells.

For example, the level of mRNA from GAPDH, a very frequently used housekeeping gene, was reported to respond to various stimuli like insulin, dexamethasone and epidermal growth factor (EGF). In addition, studies showed that certain housekeeping genes (18S rRNA and β2-microglobulin) are suitable reference genes for quantitative gene expression studies in serum-stimulated fibroblasts, while others (β-actin and GAPDH) are not.

Suitability of rRNA

Ribosomal RNAs, *e.g.* 18S rRNA or 28S rRNA, have drawbacks as reference nucleic acids because (1) they are absent in purified mRNA samples, (2) they lack polyA tails and so are not reverse transcribed by the oligo(dT) method and (3) they are much more abundant than target mRNA transcripts, which are usually rare.

Conclusion

When choosing a housekeeping gene as a reference for relative quantification, one must identify a gene whose expression level remains relatively constant for a certain experimental set-up. In fact, it is usually necessary to test a panel of housekeeping genes experimentally to find one that is not regulated in the investigated system.

Since choosing an appropriate reference gene is critical for accurate quantitative RNA analysis, the behavior of candidate genes in different cell types and cell metabolic stages should be carefully examined.

Note: Due to the increased sensitivity, reproducibility and dynamic range of real-time PCR methods, the requirements for a proper reference gene are very stringent.

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3. Housekeeping Genes as Reference Nucleic Acids, continued

Selection Criteria

Always validate reference genes when designing quantitative gene-expression studies. When selecting a suitable reference gene for relative quantification of a target gene, consider the following:

- **Expression level not regulated in the system analyzed**
  The reference housekeeping gene should not be regulated in the tissue or sample material under investigation; a stable and constant reference is required for accurate normalization. Candidate genes should be subjected to tests similar to those described in Section 4.6 of this Note.

- **RNA-specific detection**
  The primers and/or Hybridization Probes should be carefully selected to prevent detection of any sequences from contaminating traces of DNA.
  Note: Even when the primers are designed to bind only to exon/intron boundaries, amplification from processed, intron-free pseudogenes can occur and distort expression levels.

- **Expression level similar to that of target**
  The copy number of the housekeeping gene and the target gene should be similar so the measurements can be done on the same linear scale.

  For instance, rare target transcripts may not be amplified when the RNA is partially degraded, whereas amplification of highly abundant housekeeping genes (*e.g.* β-actin, 18S rRNA) still occurs, leading to false negative results.
  Note: Avoid using a lower amount of template for the amplification of the reference gene than for the amplification of the target; changing the starting template concentration will produce non-identical experimental conditions for target and reference. (For example, dilution of an inhibitor will occur in the PCR of the reference, but not in the PCR of the target).

4. Procedure for Selecting a Suitable Housekeeping Gene

Overview of Selection Procedure

To identify a suitable candidate for a housekeeping gene we recommend following the selection procedure below:

1. Define the sample material to be used in quantifying the target of interest.
2. Identify a couple of likely candidates for an unregulated housekeeping gene (literature search).
3. Search for RNA-specific primer sequences that guarantee DNA-free amplification and/or carefully design Hybridization Probes for RNA-specific detection.
4. Prepare RNA/cDNA.
5. Optimize PCR conditions to produce optimal fluorescence signals and (ideally) to allow both amplifications (target and reference) to be run with the same LightCycler program settings.
6. Determine whether the candidate housekeeping genes are regulated in the sample material selected, then choose an appropriate (unregulated) housekeeping gene.
7. Perform relative quantification analysis.

Advantages of Housekeeping Gene Selection Set

If you are planning to use Hybridization Probes for the assay, you can simplify the above procedure by using the Roche LightCycler Human Housekeeping Gene Selection Set (Cat. No. 3 310 159). This set detects five housekeeping genes, which span a wide range of expression levels (low - medium). Specifically developed primer/probe mixes (included in the set) guarantee RNA-specific detection. The set also contains convenient ready-to-use detection mixes and a positive control for each gene. These housekeeping genes may be amplified under a wide range of PCR conditions (*i.e.*, T annealing = 55° - 60°C), so several can be used together in one LightCycler run as references for a single target gene.

The benefits of the Housekeeping Gene Selection Set are even available for a SYBR Green I assay system. In that case, combine both assay formats:

- The target gene is amplified and quantified in one run with the LightCycler Instrument programmed for SYBR Green I. The reaction mix could be *e.g.* the LightCycler FastStart Master SYBR Green I mix.
- The housekeeping gene is amplified in a second run with the LightCycler Instrument programmed for Hybridization Probes. For quantification, use the hybridization probe/primer mixes and the prediluted standard solutions included in the five different Housekeeping Gene Sets.

After the two runs, the two independently determined quantification values are used to calculate the relative ratio of target to housekeeping gene. Make the experimental conditions for the two LightCycler runs as similar as possible to minimize run-to-run variations.

Note: For further details please refer to Technical Note No. LC 16/2002.

4.1 Sample Material

Examples of Sample Material

Select the sample material of interest, *e.g.*:

- cell cultures
- scientific biopsy material
- other biological samples (blood, lymph node sections, paraffin embedded tissue)
- stimulated vs. unstimulated cell lines
- normal tissue vs. malignant tissue; metastatic vs. nonmetastatic cells

Note: If necessary, use macro- or microdissection techniques for isolating tumor tissue, thereby minimizing the dilution effects of normal tissue surrounding the tumor.

4.2 Literature Search

Suggestion

Check publications in the appropriate field for possible reference gene candidates. Focus especially on housekeeping genes that are unregulated in the sample material of interest.

Examples

The table below contains a list of various housekeeping genes, their genomic structure and their known regulation in some types of tissue.

Gene	Genomic structure/pseudogenes	Regulation *e.g.*
ß-actin	multigene family, >20 genes; 1 active locus 20 pseudogenes	↑: homones of tyroid gland ↑: stomach tumor
γ-actin	multigene family, pseudogenes	
GAPDH	multigene family; 10-30 genes; >200 in mouse mostly pseudogenes	↑: lung, pancreatic, colon cancer ↑: insulin, EGF
5.8S, 18S, 28S RNA	pseudogenes	
ß2-microglobulin	no pseudogenes	↑: Non-Hodgkin lymhoma abnormal expression in tumors
G6PDH	no pseudogenes	↑: kidney, stomach tumor ↑: hormones, oxidant stress, ↑: growth factors
PBGD	no pseudogenes	
aldolase	pseudogenes	

Selected Literature

Goidin D, et al (2001) *Ribosomal 18S RNA Prevails over GAPDH and β-Actin Genes as Internal Standard for Quantitative Comparison of mRNA Levels in Invasive and Noninvasive Human Melanoma Cell Subpopulations.* Analytical Biochemistry **295**: 17-21.

Schmittgen T, Zakrajsek B (2000) *Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR.* J. Biochem. Biophys. Methods **46**: 69-81.

Selvey S, et al (2001) *β-Actin - an unsuitable internal control for RT-PCR.*
Molecular and Cellular Probes **15**: 307-311.

Zhong H and Simons J W (1999) *Direct Comparison of GAPDH, ß-Actin, Cyclophilin, and 28S rRNA as Internal Standards for Quantifying RNA Levels under Hypoxia.*
Biochemical and Biophysical Research Communications **259**: 523-526.

4.3 Primer/Hybridization Probe Design

RNA-specific Detection

As depicted in Figure 3, primer (a) and probes (b) can be designed to discriminate between RNA and contaminating DNA. They span exon-intron boundaries, so they do not detect signals from co-amplified DNA.

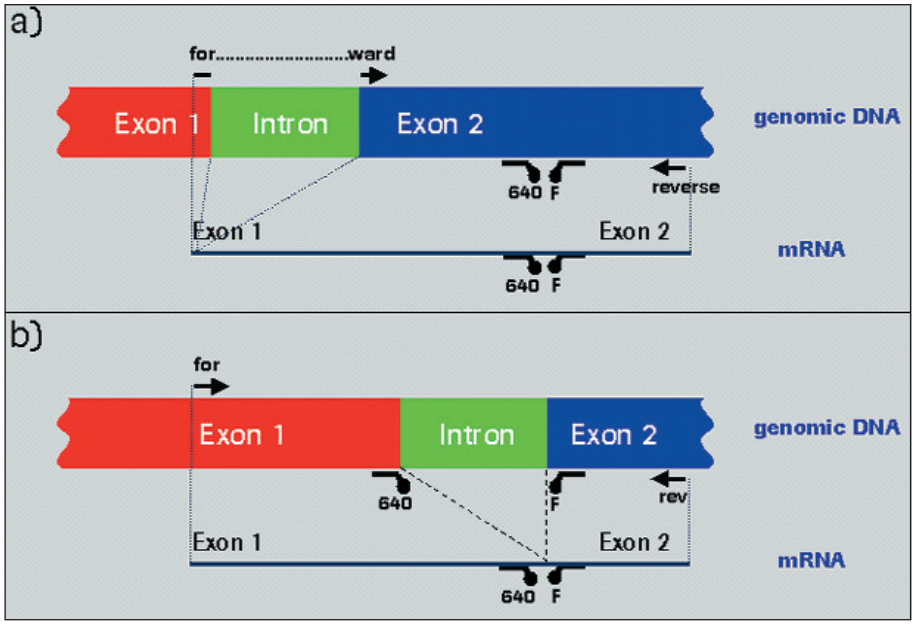

Figure 3: RNA-specific primers and probes

Pseudogenes

The literature describes numerous instances of near-copies of protein-coding genes. These pseudogenes may cause problems because they usually do not have introns. Thus, even primer sets that span exon-intron boundaries can amplify specific products of identical size in genomic DNA (from pseudogenes) and total RNA (as in figure 4). Hence, be careful when choosing a gene to serve as a reference, since co-amplification and detection of a corresponding pseudogene may influence the final result.

Note: Pseudogenes were probably originally duplicates of active genes that were re-integrated into the genome, but sequence drift has altered them. They show significant homology to an active gene but carry mutations (as little as one nucleotide) that prevent expression. Because these regions produce no known proteins, they are called pseudogenes.

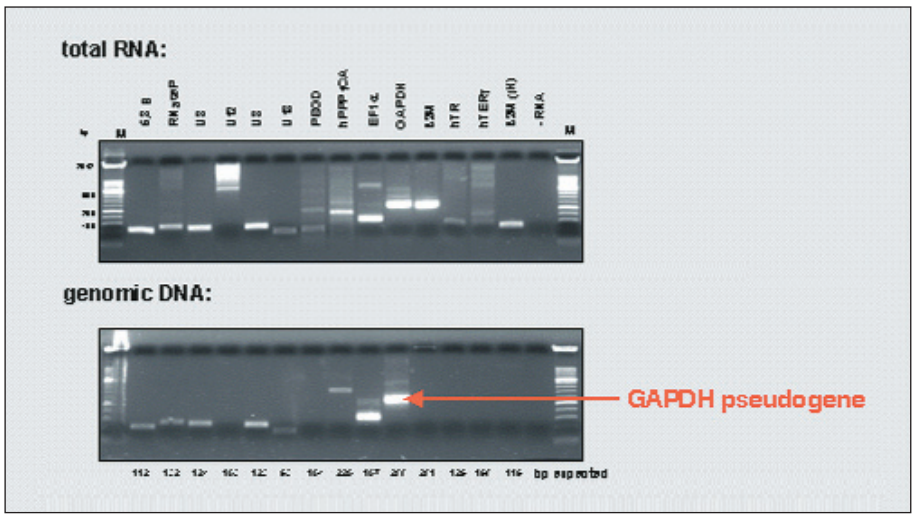

Figure 4: Appearance of a GAPDH pseudogene in gel electrophoresis.

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LightCycler Housekeeping Gene Selection Set

Roche offers a LightCycler Housekeeping Gene Selection Set to help identify a suitable reference for relative quantification of a particular target gene. The set includes primers and Hybridization Probes to detect five different housekeeping genes:

- Genes expressed at a low level
  h-HPRT: Hypoxanthine phosphoribosyltransferase (1 –10 molecules per cell)
  h-PBGD: Phorphobilinogen deaminase (10 –100 molecules per cell)
- Genes expressed at a moderate level
  h-ALAS: 5-Aminolevulinate synthase (approx. 500 molecules per cell)
  h-G6PDH: Glucose-6-phoshate dehydrogenase (100 –1,000 molecules per cell)
  h-β2M: β_2-Microglobulin (1,000 – 10,000 molecules per cell)

The primer and probes detection mixes in the set were specifically designed to detect only the mRNA of the housekeeping gene. Tests show that they do not detect pseudogenes or sequences in (contaminating) genomic DNA. The primer/probe sets will anneal at any temperature between 55°C and 60°C, allowing detection conditions to be adjusted for optimum analysis of the target gene.

4.4 Nucleic Acid Isolation

RNA Purification

Purify total RNA or messenger RNA from the sample material. We recommend using the following Roche products:

- MagNA Pure LC System or
- High Pure RNA isolation kits.

For most applications (*e.g.*, RT-PCR from a total RNA template), use 20 – 400 ng of total RNA per reaction. Special applications may require a different amount of total RNA; this should be determined empirically.

cDNA Synthesis

If you opt for a two-step RT-PCR:

- Perform cDNA synthesis in a block cycler with a heated lid.
- Hexamer priming is recommended, however oligo(dT) priming may also be used.
- In parallel with the RNA to be analyzed, run a negative control (no template control = water instead of RNA).
- Use the following Roche products to ensure high quality cDNA:
  - 1st Strand cDNA Synthesis Kit for RT-PCR (AMV)
  - Expand Reverse Transcriptase
  - C. therm. Polymerase for Reverse Transcription
    [for GC-rich templates; works with specific or oligo (dT) priming]

The advantage of the 2-step protocol is that the cDNAs for the target gene and the housekeeping gene come from the same pool of cDNA. Because the RT step is inherently variable, using identical conditions for cDNA synthesis of both target and reference may give better results.

Note: Alternatively, RT-PCR can be performed in one step with cDNA synthesis and PCR occurring in the same LightCycler Capillary. For one step RT-PCR, use *e.g.* LightCycler RNA Master Hybridization Probes.

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PCR Set-up

Reference and target genes should be amplified from the same cDNA pool. However, most quantification assays will require amplification of target and reference genes in separate capillaries because the amount of available template for the two differs dramatically. If the two genes are amplified in the same reaction tube and the amounts of starting template differ greatly, competition will usually limit target amplification to a dynamic range of only 3 – 5 magnitudes.
Note: Only if the dynamic range of target expression is naturally limited (variations of only 10- to 100-fold) should you consider a multiplex approach to amplify target and reference in the same capillary; in that case, define all reaction parameters very carefully.

Storage

Check the quality of RNA, then store samples in aliquots. To minimize degradation, store DNA/cDNA at -15° to -25°C; RNA at -70°C or below. Stabilize low concentrations (especially of RNA samples) with carrier nucleic acid (*e.g.*, MS2 RNA, 10 ng/µl). Use only nuclease-free labware, sterile aerosol-resistant pipette tips and siliconized tubes.

4.5 LightCycler PCR Optimization

Overview

Initially, PCR conditions (LightCycler Instrument program, annealing temperature, $MgCl_2$ concentration, cDNA concentration, and so forth) for each application should be established experimentally. First optimize all PCR parameters for the target reaction, following the recommendations listed below. After selecting all parameters for amplification of the target, adapt the reference gene amplification to these "target conditions", so both PCRs can be run with the same LightCycler program settings.

Tip: For determination of assay conditions, always use Roche LightCycler ready-to-use reagents.
Note: For further details on optimization, see Roche Molecular Biochemicals Technical Note No. LC 2 – 5/99 and LC 9/2000 (*Optimization Strategy*). In addition, consult the pack inserts of the LightCycler Kits for PCR or RT-PCR and the LightCycler Operator´s Manual.

$MgCl_2$ - Titration

Always vary the $MgCl_2$ concentration in the first optimization experiment:
- 1 – 5 mM $MgCl_2$ for DNA assays
- 4 – 8 mM $MgCl_2$ for RNA assays
  Exception: Reactions which use LightCycler RNA Master Mixes are dependent on $Mn(OAc)_2$ rather than $MgCl_2$.

Template

Start with at least three different dilutions of the sample material, *e.g.*:
cDNA undiluted - 1:10 diluted - 1:100 diluted - ...

During this dilution test, you can identify any inhibitory effects in the undiluted sample material by comparing the crossing point (Cp) values of the different dilutions.

To determine the linear measurement range for each gene, find what dilution series produces equal ΔCps between dilutions [*e.g.* ΔCp (undiluted – 1:10) = ΔCp (1:10 – 1:100) = 3.3]. [The optimum Cp change (ΔCp) between undiluted and 1:10 dilutions is 3.3 ⇒ Efficiency of PCR = 2.]

Always determine the linear measurement range for both the target and housekeeping PCRs. The overlapping dilution range (*i.e.,* that produces equal (Cps for both target and housekeeping PCRs) defines the linear measurement range for the assay.

Note: For further information, refer to Technical Note No. LC 16/2002.

Always include some controls, such as:
- no template control (NTC)
- RT-minus control
- positive control (if available)

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Primers and Hybridization Probes

General guidelines for primer and probe design:

- Amplicon length should not exceed 1000 bp; short amplicons are recommended for best results
- Always use highly purified (HPLC-purified) primers and probes
- Start with these concentrations:
  - Primers: 0.5 µM each
  - Hybridization Probes: 0.2 µM each
- Avoid sequences that would allow formation of duplexes between primers and/or Hybridization Probe pairs

Note: For the design of primers and Hybridization Probes use Roche LightCycler Probe Design Software or follow the guidelines in Technical Notes No. LC 1/update 2002 and LC 6/99, as well as the LightCycler Operator´s Manual.

Further Optimization

Assay performance can be improved by doing the following:

- Annealing temperature: optimize in 1° – 2°C steps
- Program variations: decrease temperature transition rate during elongation to 2° – 5°C/s
- Primer concentration: optimize within the range of 0.3 – 1.0 µM each
- Hybridization Probe concentration: optimize within the range of 0.2 – 0.4 µM each
- Add 2 – 10% DMSO for GC-rich sequences
- Hot start to reduce formation of primer dimers and improve sensitivity

Conclusion

An optimized PCR is a prerequisite for any quantification assay. The better the PCR efficiency of a test system, the more reliable and accurate the results.

4.6 Detecting Regulation of Housekeeping Genes

Expression Profiling Experiments

Expression levels of housekeeping genes should not be affected by different experimental conditions. Before you can gather any reliable quantification data, you must conduct profiling studies to demonstrate that the expression of these genes remains constant under all experimental conditions or, alternatively, to determine whether any observed variations in expression are significant.

Example: For any relative quantification, consider running comparative tests similar to those shown in the examples below. Generally, there are two different methods available for determining the expression levels of reference genes:

1. If the concentration of the sample RNA can be measured and identical amounts of template RNA are to be used in all the assays, use absolute quantification, as in these experiments:
   1.1. **Quick check**: Just obtain the crossing points of various reference genes and select those with Cp-values similar to those of the target (as in figure 5 below).
   Caution: This test is based on the assumption that RNA preparation and yield are reproducible, that the PCR efficiency is the same for all targets and that the Cp values occur within the linear detection range.
   1.2. **Accurate quantification of expression levels**: Prepare an external standard curve. This method gives absolute values and allows direct comparison of reference gene expression *e.g.* in a tissue panel (as in figures 6 – 11 below).
2. If only minimal amounts of sample RNA are available and the use of identical template concentrations in all experiments cannot be guaranteed, use a relative quantification approach. Assay target gene levels in relation to the levels of at least two reference genes; examine the target:reference ratios for evidence of housekeeping gene regulation (as in figures 12 – 13 below).

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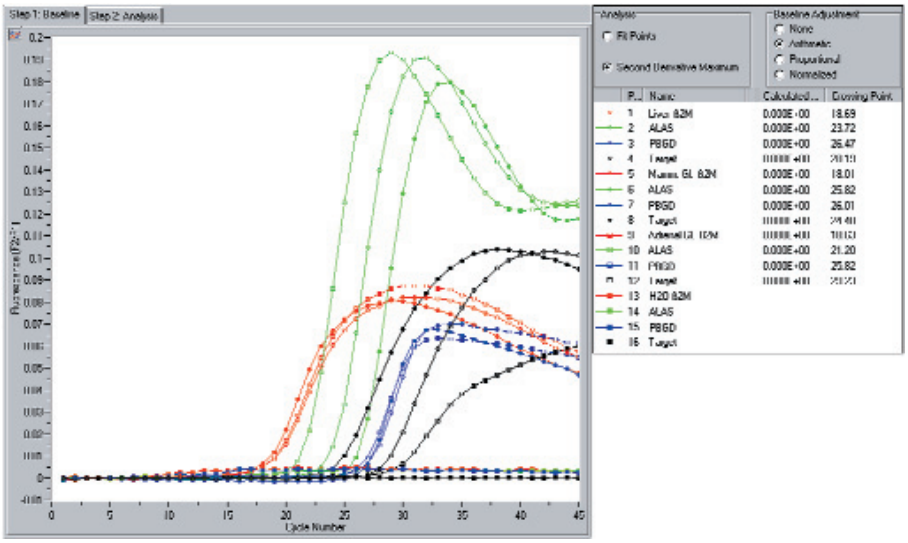
Case 1.1.
Template Amount Known
Quick Check

Figure 5 shows a LightCycler run performed with three human tissue samples (liver, mammary gland and adrenal gland, 25 ng total RNA in each experiment).
Three housekeeping genes (from the Roche LightCycler Housekeeping Gene Selection Set) and a target gene were analyzed in parallel to define the best reference gene for further research.



Quick Check Results

The table below summarizes the crossing point values (Cp) gathered from the LightCycler analysis example in Figure 5.
In the three samples tested, similar Cp values were obtained for both β2M and PBGD, but the Cp values of ALAS varied.

	sample 1 Liver	sample 2 Mammary Gland	sample 3 Adrenal Gland
	Cp	**Cp**	**Cp**
β2M	18.69	18.01	18.63
ALAS	23.72	25.82	21.20
PBGD	26.47	26.01	25.82
target	28.19	24.48	29.23

Conclusion: Not only does PBGD have the most constant Cp value, but it also has expression levels comparable to target levels. PBGD is therefore the best candidate for reference gene in this application.

Case 1.2.
Template Amount Known
Absolute Expression

To determine the expression level of a reference gene candidate accurately, you must calculate absolute values using an external standard curve (see LightCycler Technical Note No. LC 11/2000 for more information).
Note: All five Roche LightCycler Housekeeping Gene Sets include pre-diluted standards for easy, convenient preparation of a standard curve.

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Absolute Expression: Example 1

Example 1: human porphobilinogen deaminase (h-PBGD)

PBGD is encoded by two distinct mRNA species expressed in a tissue-specific manner from a single gene. One transcript is only expressed in erythroid tissues, while the housekeeping transcript (used in the Roche kits) is expressed in all tissues. PBGD is a low abundance housekeeping gene. So far, no pseudogene analogue of PBGD is known.

Figure 6: Serial dilutions containing 500 – 5 x 10^6 copies of a cloned fragment of PBGD were used to quantify the absolute expression level of PBGD in different types of human tissue.

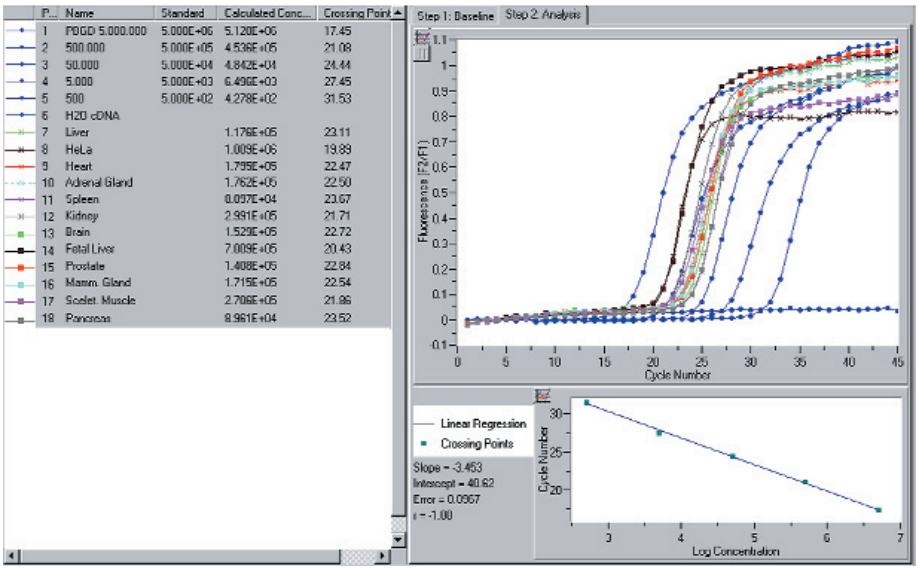
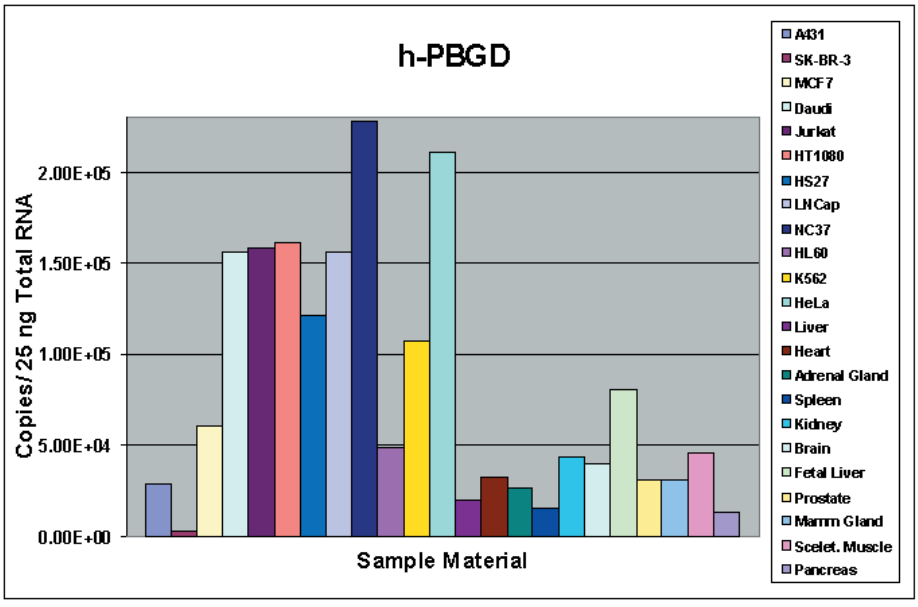


Figure 7 shows the absolute expression levels (copy numbers per 25 ng total RNA) of human PBGD in different tissues and cell lines. Data were derived from the LightCycler run shown in figure 6 above.



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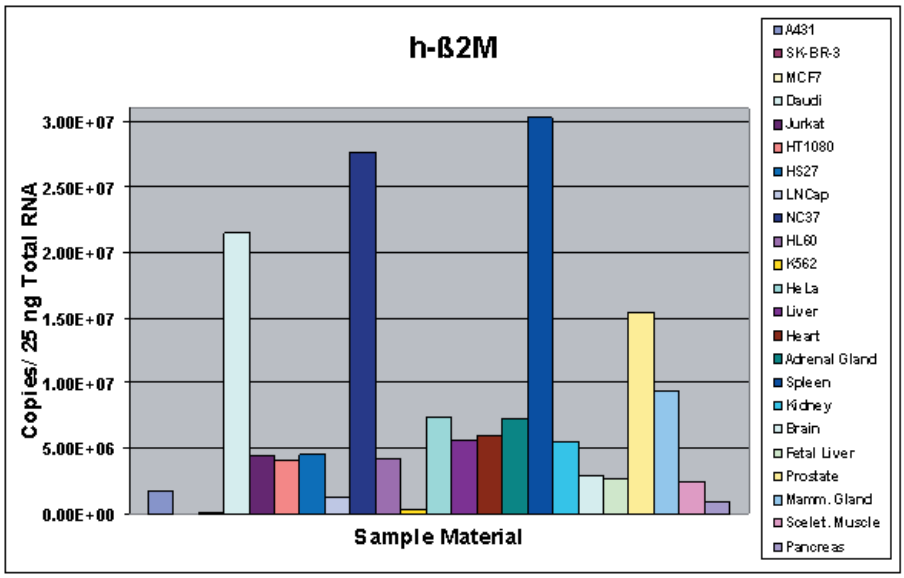
Absolute Expression: Example 2

Example 2: human β2-microglobulin (h-β2M)

β2M is a class I MHC (Major Histocompatibility Complex) protein. This housekeeping gene is detectable in a broad range of tissues, is relatively stably expressed (medium abundance) and has no known pseudogenes.

Note: Experiments have shown that β2-microglobulin mRNA is rapidly degraded in breast cancer cells.

Figure 8: Expression levels of β2M in different tissues and cell lines.



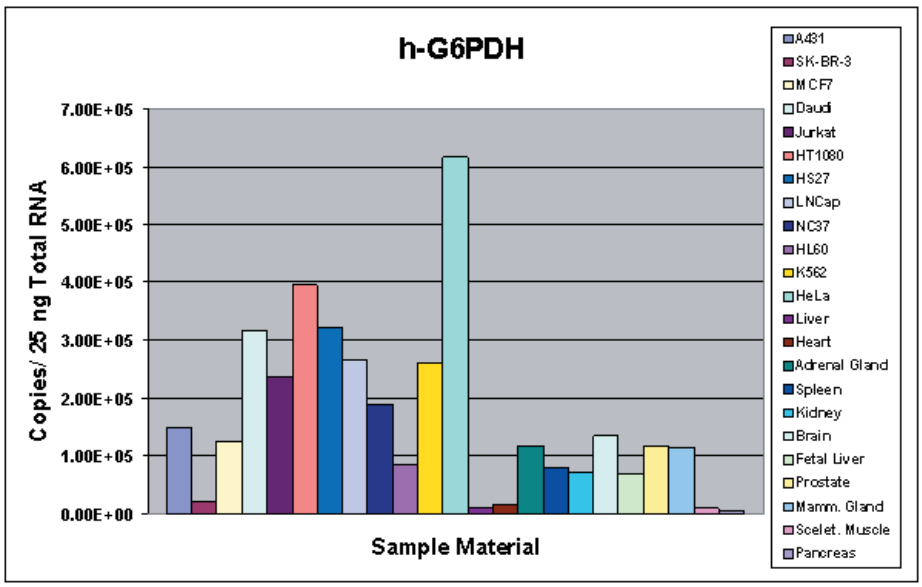
Absolute Expression: Example 3

Example 3: human glucose-6-phosphate dehydrogenase (h-G6PDH)

G6PDH is the first enzyme in the Pentose Phosphate Cycle; it provides the cell with NADPH. G6PDH is a medium abundance housekeeping gene; it has no known pseudogenes.

Note: The gene is ubiquitously expressed, but G6PDH expression is not uniform. Basal activity varies from one tissue to another. In individual cell types (*e.g.* liver, proliferating cells), the cellular level of G6PDH is regulated by a number of stimuli.

Figure 9: Expression levels of G6PDH in different tissues and cell lines.



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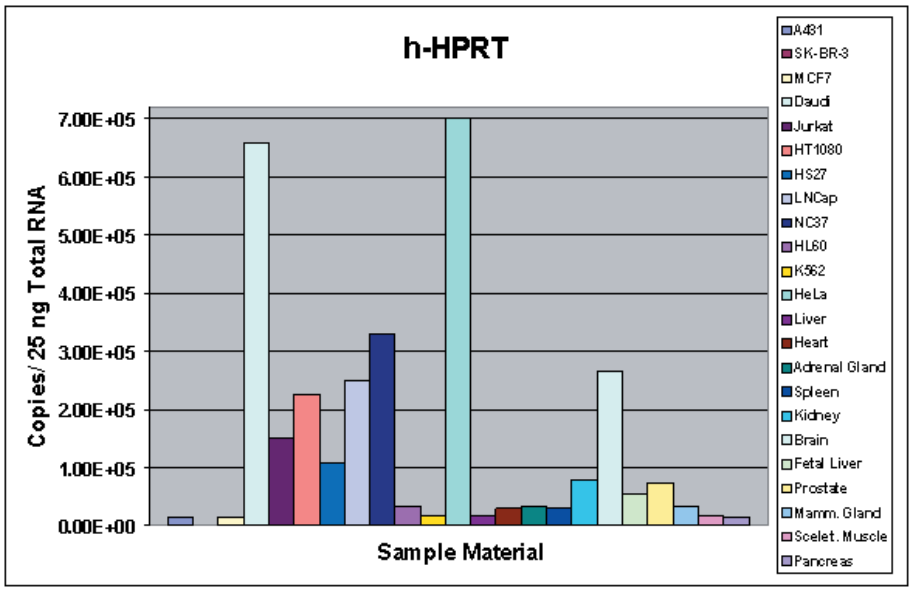
Absolute Expression: Example 4

Example 4: human hypoxanthine-phosphoribosyl-transferase (h-HPRT)

HPRT plays an important role in purine salvage pathways. It is a low abundance gene. The special HPRT-specific primer/probes provided in the Housekeeping Gene Set ensure that known pseudogenes of HPRT will not be amplified.

Note: HPRT RNA levels are higher in growing cells, but reduced levels are observed in cells carrying mutations at the HPRT locus. These mutations can be induced by drugs or x-irradiation.

Figure 10: Expression levels of HPRT in different tissues and cell lines.



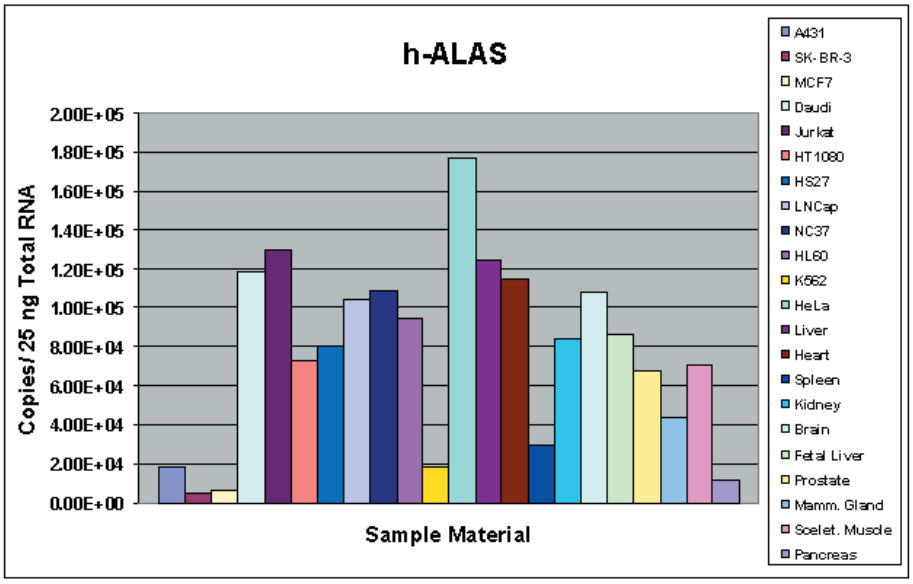
Absolute Expression: Example 5

Example 5: human 5-aminolevulinate synthase (h-ALAS)

This enzyme is the first and rate-limiting enzyme in the mammalian biosynthetic pathway for heme. This medium abundance housekeeping gene has no known pseudogenes.

Note: Alterations in steady state levels of ALAS RNA have been reported; cAMV stimulates, phorbol esters and insulin inhibit.

Figure 11: Expression level of ALAS in different tissues and cell lines.



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Case 1 Summary: Selection of Reference Gene

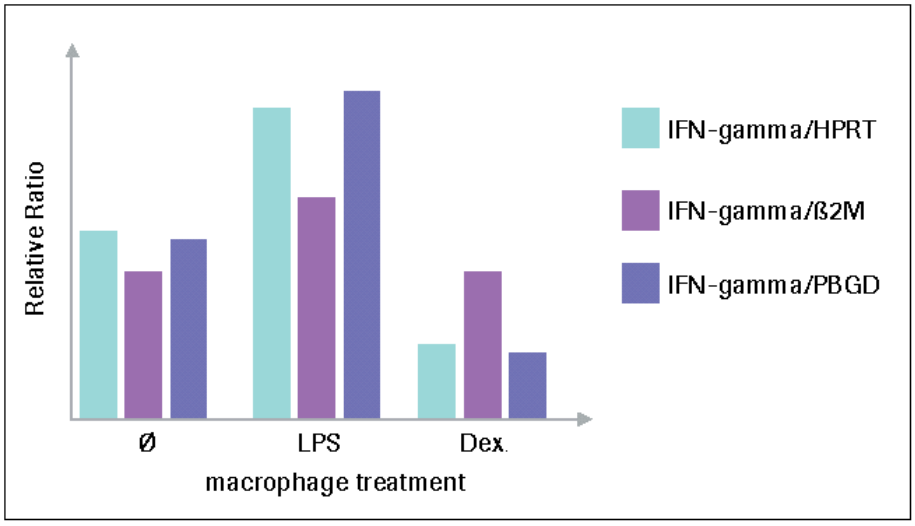
Figures 7 – 11 above demonstrate that housekeeping genes exhibit tissue-specific expression patterns. These may reflect intrinsic differences among the individual tissues, which are probably related to differences in metabolic activity or cytoarchitecture.

Conclusion: For comparative studies involving several different types of tissue, determine the absolute expression levels of various reference genes in all the target tissues, then select the one with the most constant values across all these tissues.

Case 2. Template Amount Not Known: Example 1

In some instances it is impossible to determine the template amount and an absolute quantification approach is not possible, *e.g.* when working with mRNA. In these cases, use a relative quantification approach to detect any regulatory effect of the reference genes. For each experimental condition to be investigated, assay at least two different housekeeping genes (Hk) against the target. Compare the relative ratios (target/Hk1 and target/Hk2) to detect differences caused by the housekeeping genes (as in Figure 12).

Figure 12 illustrates the relative expression level of a target gene (IFN-gamma) normalized to three different housekeeping genes (HPRT, β2M, PBGD). IFN-gamma /HPRT and IFN-gamma/PBGD ratios respond similarly to the experimental treatments [addition of LPS (lipopolysaccharide) for stimulation and Dex. (dexamethasone) for inhibition]. Thus, it is very **unlikely** that either of these two housekeeping genes influences the relative ratio (target:reference). In contrast, the ratios for the IFN-gamma /β2M system clearly behave differently from the other two. Therefore, it is very **likely** that β2M influences the relative expression levels.



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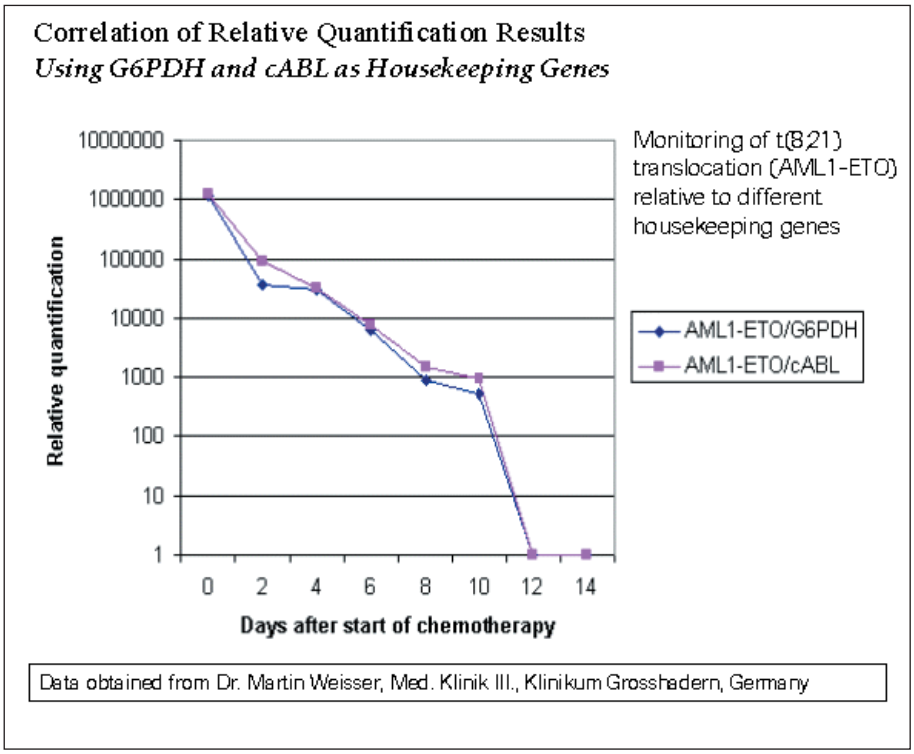
Template Amount Not Known: Example 2

Figure 13: AML-positive sample material was monitored after chemotherapy. In the same experiment, the AML1-ETO target gene was normalized to both G6PDH and cABL. Relative quantification of AML1-ETO/G6PDH and AML1-ETO/cABL revealed virtually identical results at each point of analysis. Thus, both G6PDH and cABL are appropriate reference genes for AML1-ETO quantification in this system.



Case 2 Summary: Significance of Relative Quantification Results

If target RNA values are constant or not statistically different when normalized to several housekeeping genes, you may conclude that any of these housekeeping genes may be used as a reference. But if target values vary according to the housekeeping gene used for normalization, you should continue the search for a "neutral" reference gene.

Note: If no satisfactory reference gene can be identified, consider using two or three types of housekeeping gene transcripts for normalization (as in figure 13 above). (Try, for example, using housekeeping genes that belong to different functional classes, since this significantly reduces the chance that these genes are co-regulated.) The mean value of the two or three target:reference ratios may provide an acceptable final result.

4.7 Setting Up Relative Quantification Analysis

Guidelines for Relative Quantification Analysis

After proper validation of a suitable housekeeping gene, start quantifying target gene expression. Choose one of the two analysis methods available for relative quantification assays:

1. Relative Quantification with external standards

- External standards for the target and/or the housekeeping gene must be available. (Usually cloned fragments or PCR products of known concentration are used.)
- Check efficiencies of both PCRs with serial dilution tests.
- Determine a common linear measurement range for both gene amplification reactions.
- The LightCycler Software automatically constructs the standard curve(s), then determines the experimental values for the target and reference genes; however, the target:reference ratios must be calculated manually.

2. Calibrator normalized relative quantification

- A calibrator must be defined. (The calibrator is usually a typical positive sample that has a constant ratio of target to reference.)
- LightCycler data from amplification of target and reference in both calibrator and samples are downloaded into the LightCycler Relative Quantification Software, which calculates the target:reference ratios automatically.
- For efficiency correction:
  Prepare relative standards (usually made from the calibrator sample material) for both the target and the housekeeping gene to create a *.cof file, which must also be downloaded into the LightCycler Relative Quantification Software.

Note: For more information on these two methods see page 4 of this Note or refer to Technical Note No. LC 13/2001 *Relative Quantification*.

5. Appendix

General Precautions and Limitations of Method

General precautions:

- Pay close attention to each step of the entire procedure: the material, the manipulation, the handling, RT-PCR parameters, and so forth.
- Results from samples that produce very few housekeeping transcripts must be considered less reliable. (*E.g.,* too little sample material, Cp-values outside the linear measurement range.)
- Repeat results at least three or four times for each condition. Try to minimize the variability introduced by the experimental system.

Limitations:

- Perfect synchronization of cell cycles is not always possible; the cell cycle will very likely influence the expression level of target and/or reference.
- Recent reports indicate that inhibitors have variable effects on different housekeeping genes. It is also possible that inhibitors may affect target and housekeeping genes differently.
- Most genes are differentially expressed and therefore dependent on biological context. This phenomenon may also influence the stability of mRNA in vivo. Variations observed during expression studies should be analyzed statistically to determine whether they are significant.

Further Readings

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