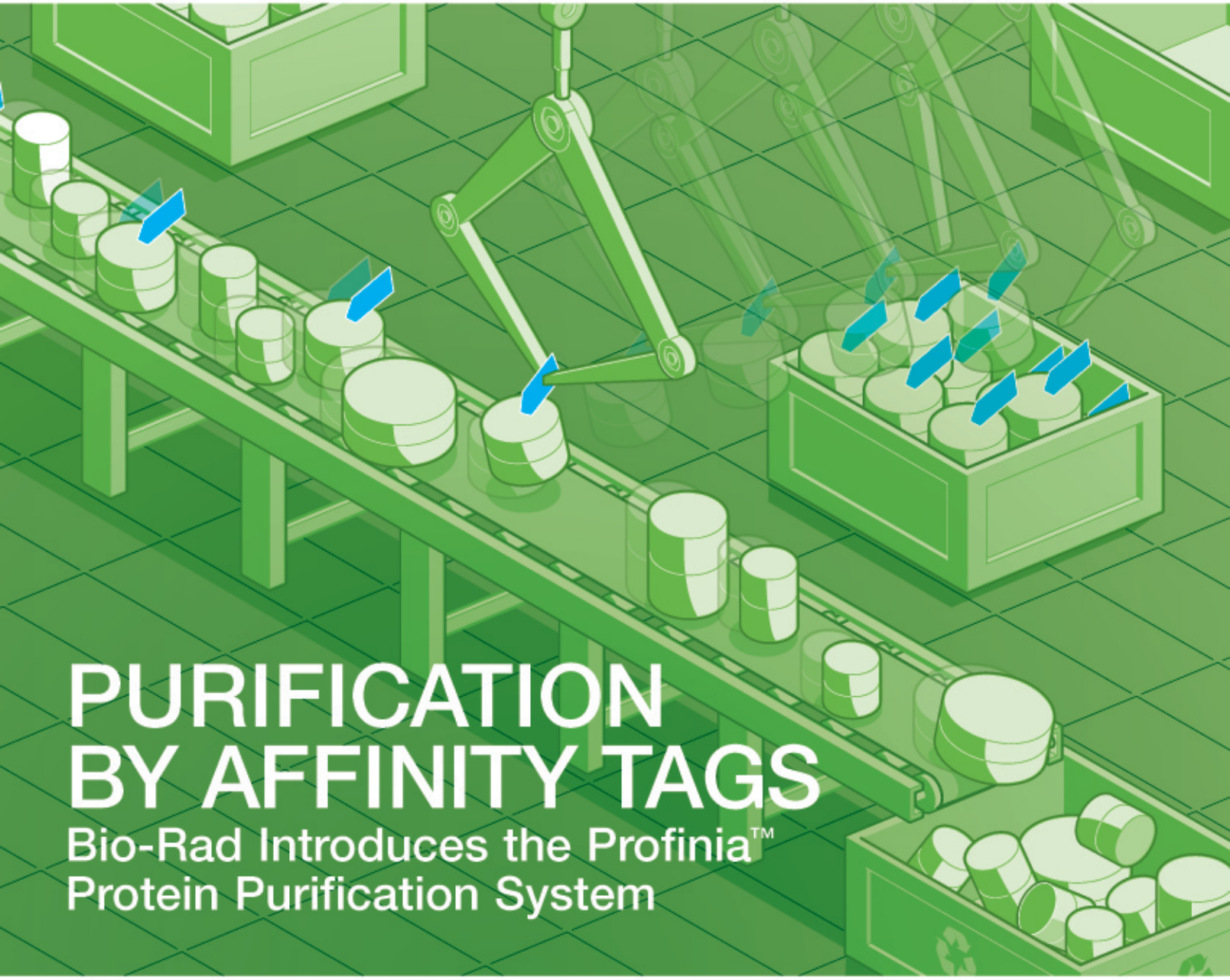
121

BioRadiations

A Resource for Life Science Research



PURIFICATION BY AFFINITY TAGS

Bio-Rad Introduces the Profinia™ Protein Purification System

In this issue:

BIO-RAD

Normalization Methods for qPCR

Real-time PCR has been used for gene expression analysis for over a decade (Heid et al. 1996, Higuchi et al. 1992). The advent of better reagents and better techniques for assay design has increased the accuracy and efficiency of the nucleic acid quantification process, making quantitative PCR (qPCR) an even more powerful tool for gene expression studies.

Most gene expression assays are based on the comparison of two or more samples and require uniform sampling conditions for this comparison to be valid. Unfortunately, many factors can contribute to variability in the analysis of samples, making the results difficult to reproduce between experiments.

Variability is most often related to events upstream of the qPCR assay itself — namely, the quantity and quality of the extracted sample and the reverse-transcription efficiency (Fleige and Pfaffl 2006). Not only can the quantity and quality of RNA extracted from multiple samples vary, but even replicates can vary dramatically due to factors such as sample degradation, extraction efficiency, and contamination (Perez-Novo et al. 2005). The reverse-transcription efficiency can vary due to sample concentration, RNA integrity, the reagents used, and the presence of contaminants. Because the amount of starting material may vary greatly from sample to sample, accurate analysis requires that the samples be normalized.

This article describes two methods of sample normalization for accurate comparison of genes of interest: normalizing to input RNA and normalizing to a reference gene that has little variability as a function of treatment or due to the normal life cycle of the organism.

Normalization to Input RNA

Normalization to input RNA implies starting with the same amount and quality of material in each sample. This is typically done by measuring the absorbance at 260 nm (A_{260}) (Sambrook el al. 1989). This method cannot determine the integrity of the RNA molecules, however, because whole RNA and degraded RNA absorb light equally. Therefore, a secondary analysis, typically on a formaldehyde agarose gel, is required to determine whether there is degradation. Microfluidic electrophoresis on the Experion™ system is faster and requires much less RNA for this assessment than traditional agarose gel electrophoresis.

To illustrate the use of normalization in qPCR, we monitored the expression of four genes: annexin A3, CAPZB, cofilin, and destrin, in HeLa cells subjected to different treatments. Two sets of samples were transfected with small interfering RNAs (siRNAs, Act8 and Act9) that targeted different regions of the β-actin gene. A third set was transfected with siRNA targeting Green Fluorescent Protein (GFP) as a nonspecific control that is absent in HeLa cells. Two additional control samples were treated with lipid transfection reagent or buffer only. All analyses were performed using standard curves to determine individual amplification efficiencies.

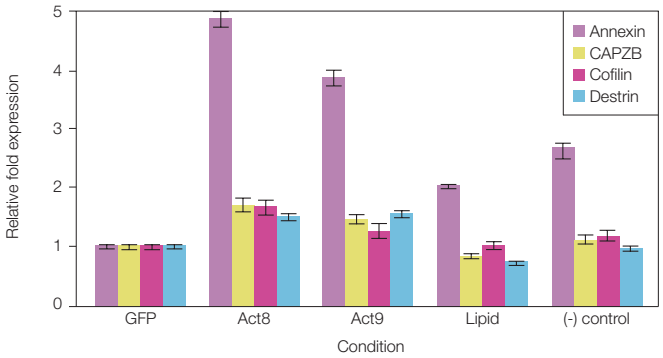


Fig. 1. Gene expression analysis of four genes across five conditions normalized to initial amount of starting RNA. GFP, Act8, Act9, treated with corresponding siRNA; Lipid, treated with lipid reagent; (–), control, treated with buffer only.

The relative expression of the four genes of interest across treatments was normalized to the amount of input RNA (Figure 1). This analysis showed that annexin A3 expression increased approximately 2- to 5-fold depending on the treatment. In addition, CAPZB, cofilin, and destrin displayed changes in expression varying from slightly below normal expression levels to a 50% increase in expression level.

Normalization to a Single Reference Gene

Although normalizing gene expression to the input amount of RNA ensures that equivalent amounts of RNA are compared, it cannot compensate for variations in the efficiency of reverse transcription, which is required to produce cDNA for PCR. Therefore, researchers often normalize expression levels of genes of interest to that of a reference gene. This can remove inaccuracies due to variations in reverse-transcription efficiency because the mRNA of the reference gene is reverse-transcribed along with that of the gene of interest.

Housekeeping genes such as β-actin, tubulin, GAPDH, and 18S ribosomal RNA have often been used as reference genes for normalization, with the assumption that the expression of these genes is constitutively high and that a given treatment will have little effect on the expression level.

To illustrate normalization to a reference gene, the data presented in Figure 1 were analyzed again, this time normalizing to the expression level of 18S RNA (Figure 2). The results of this analysis were qualitatively similar to those of the analysis using input RNA as the normalizer, but the calculated increases in expression were greater. For example, the expression level of annexin A3 in the Act8-treated sample showed a 4.9-fold increase when normalized to input RNA, but showed a 6.3-fold increase when normalized to 18S RNA expression. An obvious question is, which analysis is more accurate?

The question of which reference gene to use for normalization remains a key issue of debate. While it is agreed that the ideal reference gene is one that does not vary as a function of

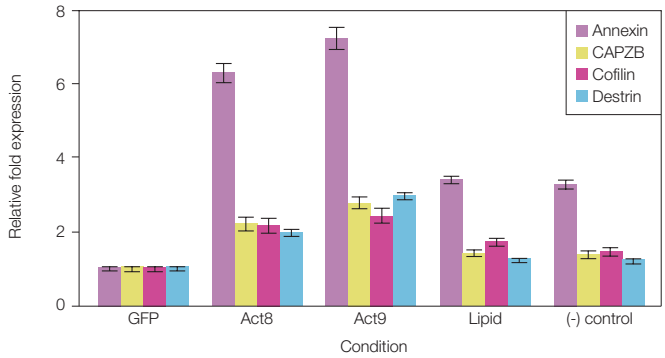


Fig. 2. Gene expression analysis of four genes across five conditions normalized to 18S ribosomal RNA.

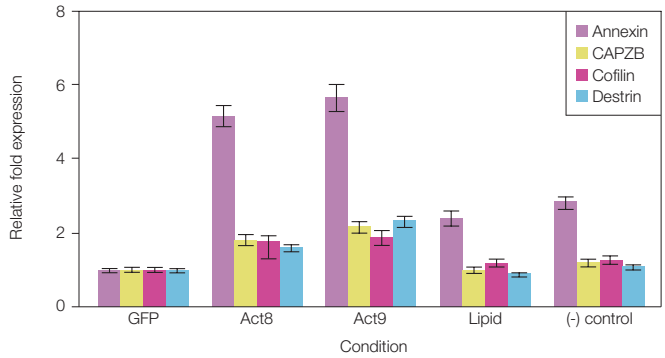


Fig. 4. Gene expression analysis of four genes across five conditions normalized to the geometric average of the three reference genes.

treatment or condition, it is often difficult to identify even a single gene that meets this criterion (Glare et al. 2002, Kamphuis et al. 2005, Schmittgen and Zakrajsek 2000, Thellin et al. 1999). This difficulty is illustrated in Figure 3, which shows an analysis of three commonly used reference genes, 18S, GAPDH, and α-tubulin, from the same input. Although equivalent starting amounts of RNA were used, the expression levels of the three genes varied considerably depending on the treatment. These variations are unlikely to have resulted from errors in the starting amounts of RNA, because some of the genes are expressed at higher levels than the control, while others are expressed at lower levels. Therefore, these data indicate that genes that are generally considered to be housekeeping genes may in fact be expressed at variable levels across treatments or tissues.

Normalization to Multiple Reference Genes

A more accurate strategy for normalization has been proposed by Vandesompele and colleagues (2002). Instead of basing the normalization on a single reference gene that may or may not fluctuate, they propose carefully selecting a set of genes that display minimal variation across the treatment, determine the geometric mean of these genes, and normalize the gene(s) of interest to the geometric mean.

Figure 4 shows the expression levels of the four genes of interest across five treatments, as calculated using the multiple reference gene normalization strategy. This analysis reveals that treatment with either of the two siRNAs targeting β-actin (Act8 or Act9) increased annexin A3 expression levels to about 5.5-fold over basal level, and increased the expression of CABZB, cofilin, and destrin to about 2-fold greater than basal levels. Annexin A3 also showed increased expression with lipid and buffer control treatments, but expression levels of the other three genes were similar in these controls to levels in the sample treated with nonspecific siRNA (GFP).

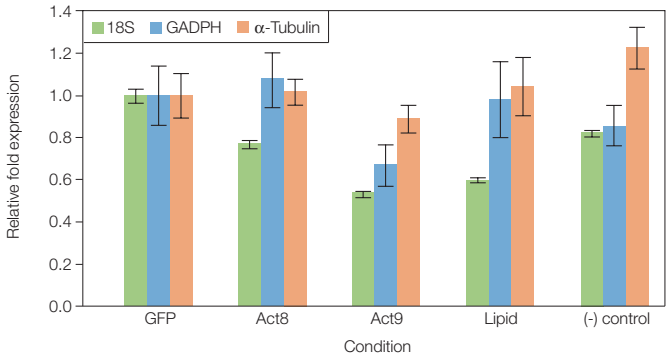


Fig. 3. Gene expression of three reference genes normalized to input amount of RNA.

Summary

Proper normalization is essential for obtaining accurate gene expression studies. There are many strategies available for normalization, and with proper controls and replicates, all can be valid. The most comprehensive strategy uses a normalization factor calculated from the geometric mean of multiple reference genes.

To simplify data analysis, iQ™5 and MyiQ™ real-time PCR detection systems come with analysis software that permits normalization to a standardized input amount, to a single reference gene, or to the geometric mean of multiple reference genes. Additionally, the software can take individual assay efficiencies into consideration, as well as combine multiple data sets to generate a complete gene study.

References

Fleige S and Pfaffl MW, RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med 27, 126–139 (2006)

Glare EM et al., β-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels, Thorax 57, 765–770 (2002)

Heid CA et al., Real time quantitative PCR, Genome Res 6, 986–994 (1996)

Higuchi R et al., Kinetic PCR analysis: real-time monitoring of DNA amplification reactions, Biotechnology 11, 1026–1030 (1993)

Kamphuis W et al., Circadian expression of clock genes and clock-controlled genes in the rat retina, Biochem Biophys Res Commun 330, 18–26 (2005)

Perez-Novo CA et al., Impact of RNA quality on reference gene expression stability, Biotechniques 39, 52–56 (2005)

Sambrook J et al., Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, NY (1989)

Schmittgen TD and Zakrajsek BA, Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR, J Biochem Biophys Methods 46, 69–81 (2000)

Thellin O et al., Housekeeping genes as internal standards: use and limits, J Biotechnol 75, 291–295 (1999)

Vandesompele J et al., Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol 3, RESEARCH0034, Epub Jun 18 (2002)

Chromatography

- Profinia™ instrument product information sheet (bulletin 5541)
- Automated desalting of proteins with the Profinia protein purification system: comparison to manual desalting by dialysis (bulletin 5539)
- How CHT™ ceramic hydroxyapatite works (bulletin 5518)
- Buffers for use with UNOSphere™ S cation exchange media (bulletin 5515)
- Automated purification of a His-tagged protein with Profinia system: comparison with another low-pressure chromatography system (bulletin 5514)
- Automated purification of a GST-tagged protein with the Profinia protein purification system: comparison to manual protein purification (bulletin 5513)
- Profinia protein purification system brochure (bulletin 5510)
- Rapid, efficient purification and evaluation of His-tagged proteins (bulletin 5501)

Electrophoresis

- Mini-PROTEAN® Tetra cell brochure (bulletin 5535)
- Experion™ DNA 1K and 12K analysis kits product information sheet (bulletin 5520)
- MicroRotofor™ lysis kit product information sheet (bulletin 5517)
- Monitoring development of chromatographic methods with the Experion automated electrophoresis system (bulletin 5506)
- Application of the Experion automated electrophoresis system to glycoprotein visualization and analysis (bulletin 5453)
- Effect of RNA degradation on data quality in quantitative PCR and microarray experiments (bulletin 5452)

Imaging

- Molecular Imager® VersaDoc™ system citations (PDF and fax only; bulletin 5508)
- Molecular Imager system folder and inserts (bulletin 5339)

Protein Interaction Analysis

- Applications of the ProteOn™ NLC sensor chip: antibody-antigen, DNA-protein, and protein-protein interactions (bulletin 5449)

SELDI Technology

- ProteinChip® SELDI system features and components product information sheet (bulletin 5529)
- ProteinChip SELDI system product information sheet (bulletin 5525)

Multiplex Suspension Array Technology

- Bio-Plex Pro™ human isotyping panel flier (bulletin 5521)
- Bio-Plex® suspension array system ordering information (bulletin 5507)
- Bio-Plex PDF literature library CD (bulletin 5406)
- Bio-Plex suspension array system brochure (bulletin 5405)

Microarray Products

- How to print protein microarrays with the BioOdyssey™ Calligrapher™ miniarrayer (bulletin 5512)
- BioOdyssey MCP pins flier (bulletin 5498)

Gene Transfer

- Electroporation systems brochure (bulletin 5542)
- Dicer-substrate siRNA technology (bulletin 5519)
- Biolistic delivery systems brochure (bulletin 5443A)

Amplification

- MyiQ™ and iQ™5 systems brochure (bulletin 5462)

Food Science

- Evaluation of RAPID'*Staph*™ medium for enumeration of coagulase-positive *Staphylococcus aureus* in selected foods (bulletin 5533)

Biotechnology Education

- Biotechnology Explorer™ catalog (bulletin 2112)