Impact of RNA quality on reference gene expression stability

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Gene expression quantification methods are important tools in the understanding of the molecular events underlying human diseases and in the identification of diagnostic and therapeutic targets. Generally, the messenger RNA (mRNA) used for these analyses is derived from human biopsies obtained after surgery. As a consequence, several steps during tissue handling have to be carefully controlled in order to preserve the quality and integrity of the RNA material. It is well known that RNA is sensitive to degradation by postmortem processes and inadequate sample handling or storage (1). However, RNA integrity control is often not systematically performed prior to (PCR-based) downstream analyses. While in the past, RNA quality could often not be assessed due to the limited availability of the precious sample (e.g., from microdisected cells or small biopsies), the advent of capillary gel electrophoresis and (sample retention) spectrophotometry technologies (e.g., NanoDrop® ND-1000; NanoDrop Technologies, Wilmington, DE, USA) has addressed this issue, allowing quality estimations using only nanograms (or even picograms) of total RNA (2). In addition, amplification of RNA is now an alternative method to obtain sufficient amounts to conduct gene expression studies when postmortem tissues are scarce; however, assessment of RNA quality based on the 18S and 28S ribosomal RNA bands is often not possible anymore after amplification. Furthermore, it remains to be determined whether the amplified mRNA can faithfully be used to assess RNA quality of the starting material.

Apart from RNA quality, the choice of a proper set of reference genes for accurate normalization is another crucial factor with a profound impact on the reliability of the obtained gene expression levels (3). Reference genes are expressed constitutively in every cell; however, their expression can be regulated with diseases state, during cellular proliferation, due to cellular composition and by mitogenic stimuli (e.g., growth factors) (4,5). Furthermore, it is now known that life styles and genetic make-up of individuals can influence mRNA expression (6). That is why the validation of the expression stability of reference genes remains an important step to ensure the accuracy and reliability of gene expression studies. The objective of this study was to analyze the influence of RNA degradation on the stability and expression pattern of different internal control genes. To this purpose, 10 commonly used reference genes were quantified in both intact and degraded RNA from clinical specimens obtained from ethmoidal and maxillary sinuses collected from patients with nasal polyposis (NP) and chronic rhinosinusitis (CRS).

Sixteen clinical tissue samples (30 mg) were homogenized in Tri-reagent buffer (Sigma, St. Louis, MO, USA) (1 mL/50–100 mg of tissue) in a chilled pestle mortar. Total RNA isolation and cDNA synthesis were performed as described previously (7). RNA

Step*	Degraded RNA (CRS samples)	Intact RNA (CRS samples)	Degraded RNA (NP samples)	Intact RNA (NP samples)
1	HPRT1	GAPD	HPRT1	YWHAZ
2	YWHAZ	YWHAZ	ACTB	B2M
3	B2M	RPL3IA	RPL3IA	RPL3IA
4	TBP	B2M	GAPD	UBC
5	RPL3IA	UBC	TBP	GAPD
6	UBC	HPRT1	YWHAZ	HMBS
7	ACTB	TBP	HMBS	HPRT1
8	GAPD	ACTB	SDHA	SDHA
9	HMBS- SDHA	HMBS- SDHA	B2M- UBc	ACTB- TBP

Table 1: Reference genes ranked in order of their expression stability increasing from top to bottom; the two most stable genes in each sample series; for example ACTB and TBP in intact RNA from nasal polyp tissue, cannot be ranked in order because of the required use of gene ratios for gene-stability. *, the step number at which the least stable gene is excluded, see right panel.

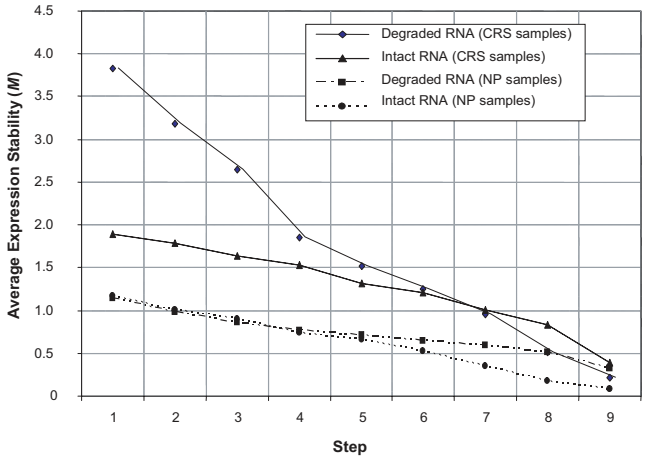

Figure 1. Average expression stability values of remaining control genes. Expression stability (*M*) was analyzed during stepwise exclusion of the least stable control gene in the different tissue samples (the different steps match those in the table). CRS, chronic rhinosinusitis; NP, nasal polyp.

quality and percent of degradation were assessed with the Agilent 2100 Bioanalyzer system using the RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA); for representative results, see Supplementary Figure S1, which can be found online at www.BioTechniques.com. Real-time PCR amplifications were performed in an iCycler iQ® real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) in a 25-µL volume containing 20 ng cDNA (total RNA equivalent) of unknown samples, 1× SYBR® Green I Master mix (Bio-Rad Laboratories), and 300 nM specific primer pairs for 10 references genes (*GAPD*, *UBC*, *SDHA*, *HPRT1*, *B2M*, *ACTB*, *YWHAZ*, *HMBS*, *RPLI3A*, and *TBP*); see Supplementary Table S1 for more information. Primer sequences are reported previously (3) and are available in RTPrimerDB, the real-time PCR primer and probe database (medgen.ugent.be/rtprimerdb) (8). The amplification protocol consisted of 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 30 s and at 60°C for 1 min. Gene expression level normalization and stability analysis was performed using the Microsoft® Excel® Visual Basic application geNorm as described previously (3).

Our results show that the stability of reference genes not only is different in CRS and NP ethmoidal and maxillary sinus tissues (as can be expected due to differences in cellular origin), but also varies within the same tissue type according to the degradation status of the samples (Figure 1). The fact that highly stable genes in intact RNA samples rank among the most unstable genes in degraded samples (e.g., *ACTB* in NP tissue) and vice versa (e.g., *GAPD* in CRS and *B2M* in NP) suggests that at least some genes show different sensitivity to RNA degradation. The bottom line is that different reference genes appear to be suitable for normalization in degraded versus intact RNA samples. When one has no prior knowledge of the RNA degradation status, incorrect conclusions could thus be drawn for the selection of proper reference genes. Furthermore, once a suitable set of reference genes has been selected (even based on only intact samples), the use of these genes to normalize mRNA content in (partially) degraded samples could lead to significant errors and misinterpretation of target gene expression levels information.

The purpose of normalization is to remove the sampling differences (such as RNA quantity and cDNA synthesis efficiency) in order to identify real gene-specific variation that, for proper internal control genes, should be minimal. Following the approach outlined in Reference 3, we have calculated the gene-specific variation for each reference gene as the standard deviation of normalized expression levels. To this end, the raw expression values were divided by two different normalization factors, calculated as the geometric mean of the three most stable control genes (as determined by geNorm) in

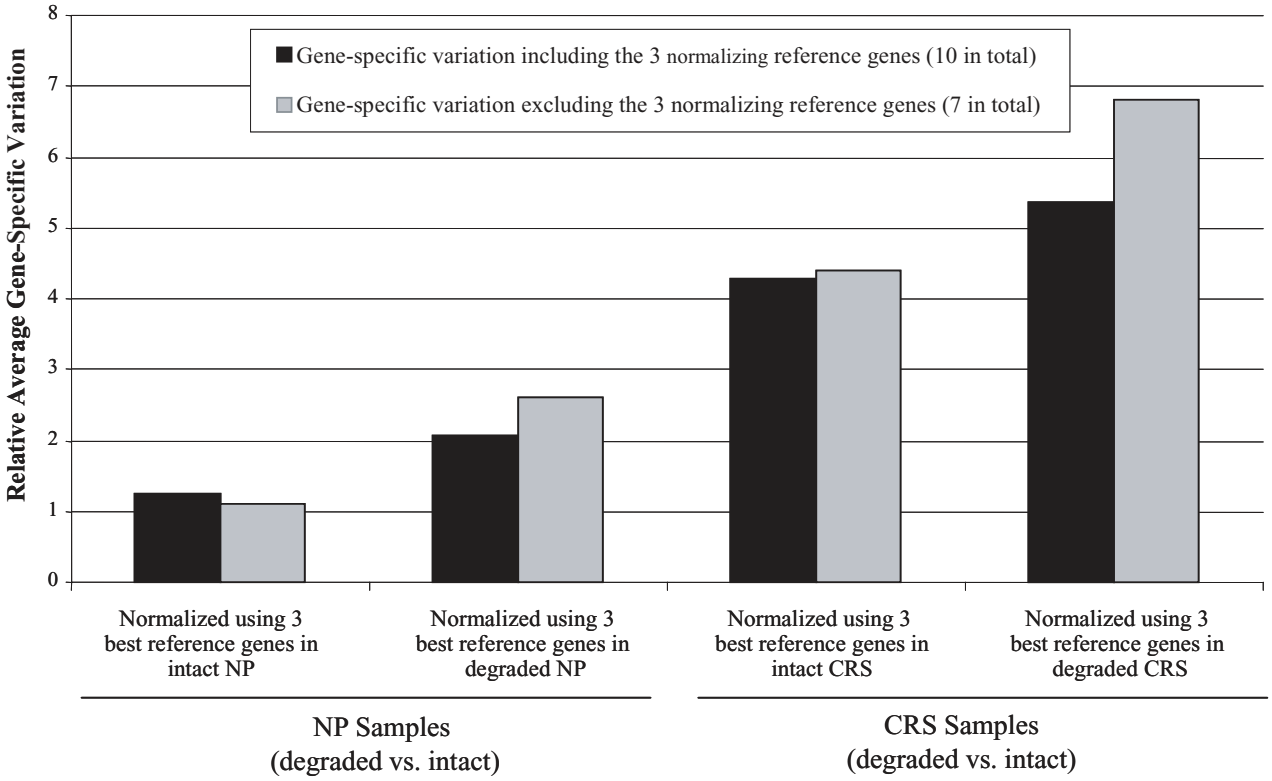

Figure 2. Relative average gene-specific variation in degraded versus intact RNA samples. Gene-specific variation in expression was calculated after normalization using the three most stable reference genes according to geNorm analysis. Bars represent the ratio of the average of standard deviation of normalized gene expression levels in degraded samples versus nondegraded samples. From left to right, nasal polyp (NP) and chronic rhinosinusitis (CRS) tissue samples, normalized using two different normalization factors.

degraded or intact RNA samples. We subsequently determined the average gene-specific variation of all reference genes (either excluding or including the three normalizing reference genes) for each normalization factor, and within each tissue type, and plotted the ratio of the variation in degraded versus intact samples (Figure 2). It is clear that the gene-specific variation is always higher in degraded versus intact samples, with more pronounced differences in CRS compared to NP. Furthermore, normalization using the so-called best reference genes in degraded samples systematically resulted in higher gene-specific variation than using the best reference genes identified on the basis of intact samples. In addition, in degraded samples, relative gene-specific variation was also higher when the three most stable genes (used for normalization) were excluded (Figure 2, grey bars) than when they were included (Figure 2, black bars). In contrast, in nondegraded samples, the inclusion or exclusion of these genes seems not to significantly affect gene-specific variation. Using a normalization factor based on the proper internal reference genes should result in the removal of nonspecific variations. However, unstable reference genes cannot completely remove variation—instead they add more, resulting in larger so-called gene-specific variation for the tested reference genes. This analysis clearly demonstrates that most nonspecific variation was removed when the most stable control genes in intact RNA samples (as determined by geNorm) were used for normalization and that the gene expression variation in degraded RNA samples is inherently higher.

While thorough RNA quality control is routinely being performed prior to microarray-based gene expression profiling, the same quality control is often considered not required or simply not performed for PCR-based quantification methods. Indeed, even on degraded RNA samples, a nice amplification curve can be obtained. However, in view of the observed difference in reference gene expression stability between intact and degraded RNA samples from the same tissue and the higher gene-specific variation in degraded samples, we propose performing RNA quality control prior to downstream quantification assays and discarding degraded samples, especially if one aims to accurately quantify small expression differences. Indeed, as it is of utmost importance to normalize samples using the same set of reference genes, our results suggest that it is inappropriate to compare degraded and intact samples.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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