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ScienceDirect
Advances in Space Research 39 (2007) 1050–1055
ADVANCES IN SPACE RESEARCH (a COSPAR publication)
www.elsevier.com/locate/asr

Suitability of commonly used housekeeping genes in gene expression studies for space radiation research

A. Arenz *, N. Stojicic, P. Lau, C.E. Hellweg, C. Baumstark-Khan

DLR, Institut für Luft- und Raumfahrtmedizin, Strahlenbiologie, 51147 Köln, Germany

Received 2 November 2006; received in revised form 26 January 2007; accepted 8 February 2007

Abstract

Research on the effects of ionizing radiation exposure involves the use of real-time reverse transcription polymerase chain reaction (qRT-PCR) for measuring changes in gene expression. Several variables need to be controlled for gene expression analysis, such as different amounts of starting material between the samples, variations in enzymatic efficiencies of the reverse transcription step, and differences in RNA integrity. Normalization of the obtained data to an invariant endogenous control gene (reference gene) is the elementary step in relative quantification strategy. There is a strong correlation between the quality of the normalized data and the stability of the reference gene itself. This is especially relevant when the samples have been obtained after exposure to radiation qualities inducing different amounts and kinds of damage, leading to effects on cell cycle delays or even on cell cycle blocks. In order to determine suitable reference genes as internal controls in qRT-PCR assays after exposure to ionizing radiation, we studied the gene expression levels of nine commonly used reference genes which are constitutively expressed in A549 lung cancer cells. Expression levels obtained for ACTB, B2M, GAPDH, PBGD, 18S rRNA, G6PDH, HPRT, UBC, TFRC and SDHA were determined after exposure to 2 and 6 Gy X-radiation. Gene expression data for *Growth arrest and damage-inducible gene 45* (GADD45α) and *Cyclin-dependent kinase inhibitor 1A* (CDKN1A/p21CIP1) were selected to elucidate the influence of normalization by using appropriate and inappropriate internal control genes. According to these results, we strongly recommend the use of a panel of reference genes instead of only one.

Keywords: Normalization; Reference gene; Real-time quantitative RT-PCR

1. Introduction

The stability of reference gene expression is an elementary prerequisite for internal standardization of target gene expression. In relative expression studies, many so-called housekeeping genes with an assumed stable expression can exhibit gene expression alterations under the chosen experimental conditions. Although housekeeping genes are essential for cell viability and are expressed at a constant level, they may become actively regulated in response to DNA damage, which leads to cell cycle arrest, DNA repair processes or apoptosis. Due to such disturbing effects of radiation related gene expression changes of internal control genes, it is crucial to use more than one housekeeping gene for data normalization.

The goal of this study was to investigate the influence of stability of nine commonly used housekeeping genes in relative quantitative gene expression studies. To demonstrate the significance of suitable reference genes for correct profiling of gene expression data, we measured the time course of transcriptional response of damage-inducible target genes GADD45α (GADD45) and CDKN1A (p21CIP1) in A549 cells after irradiation with 2 and 6 Gy of X-rays. The normalization of target gene expression raw data was performed by using a set of stably expressed reference genes and less stably expressed ones, respectively.

* Corresponding author. Tel.: +49 2203 6012435; fax: +49 2203 61970.
E-mail address: andrea.arenz@dlr.de (A. Arenz).

0273-1177/$30
doi:10.1016/j.asr.2007.02.028

2. Materials and methods

2.1. Cell culture and growth conditions

The A549 human lung carcinoma cell line, expressing wild type p53, was obtained from ATCC (Clone CCL-185). Cells were grown under standard conditions in HAM's F12K medium with 2 mM L-glutamine and 10% Fetal Bovine Serum at 37 °C saturated humidity and 5% CO_2/95% air atmosphere.

2.2. Radiation conditions

A549 cells were seeded into Petri dishes (Ø 3 cm) with cell densities of 6×10^3 cells/cm^2 and irradiated after reaching 70–80% confluence. Irradiation was performed at room temperature with X-rays (150 kV, 19 mA) generated by an X-ray unit (Müller Type MG 150, Germany) and a dose rate of 1.8 Gy/min. Cells were harvested for analysis at indicated time points after irradiation. Control cells were treated similarly but without X-ray exposure.

2.3. Total RNA extraction, quantification and cDNA synthesis

Total RNA of irradiated and non-irradiated cells was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) including an on-column DNase-digestion according to the manufacturer's recommendations. RNA integrity and quantification was calculated with the Bioanalyzer 2100 by using the Eukaryotic Total RNA Nano Assay (Agilent Technologies, USA). The obtained RNA integrity numbers (RIN) allow the classification of total RNA in a numbering system from 1 (for the most degraded profile) to 10 (for the most intact one). Only high quality RNA samples (RIN numbers $\geqslant 9$) were used for the gene expression analysis in this study. Purity of the RNA preparation was determined as 260/280 nm absorbance ratio with expected values between 1.8 and 2.0. The RNA was first denatured at 65 °C for 5 min. For the subsequent RT reaction, constant amounts of 500 ng total RNA were converted to double-stranded cDNA using the iScript cDNA synthesis kit, containing a blend of oligo(dT) and random primers, according to the manufacturer's instructions (Bio-Rad Ltd, Munich, Germany). The cDNA was stored at −20 °C until qRT-PCR analysis.

2.4. Oligonucleotide primers and optimization of RT-PCR

Primer pairs were designed using published human nucleic acid cDNA/mRNA sequences (NCBI, RTPrimerDB) with regard to primer dimer formation, self-priming formation and primer melting temperature (Netprimer, PREMIER Biosoft International Palo Alto, CA). Primer sequences were designed to span at least one intron and synthesized commercially (Invitrogen, Karlsruhe, Germany). Primer information and characterization of target- and reference-genes are listed in Table 1. To reduce the possibility, that genes are co-regulated under the chosen conditions, the selected reference genes belong to different functional classes. Conditions for real-time PCR were optimized on a thermal cycler (Biometra, Göttingen, Germany) with regard to annealing temperature and primer concentration and subsequently on an Opticon2 (MJ Research Inc., Waltham, MA) by analyzing the melting curves of the products. Specificity of qRT-PCR products and the absence of pseudogenes (tested by amplification of genomic DNA) were confirmed by electrophoresis on a 2% agarose gel.

2.5. Quantification by real-time qRT-PCR

Real-time PCR using SYBR® Green I technology was performed on the DNA Engine Opticon2 continuous fluorescence detection system with 20 ng reversely transcribed total RNA. Mastermix for each PCR run was performed as follows: 12.5 µl qPCR™ Mastermix for SYBR® Green I (Eurogentec Inc., Cologne, Germany), 9.5 µl water and 0.5 µl (0.2 µM) for each primer set. The following amplification protocol was applied: after 10 min of denaturation at 95 °C to activate the Hot Start DNA polymerase, 40 cycles of amplification were accomplished with (i) 15 s denaturation step at 95 °C, (ii) 30 s annealing step at respective temperature (Table 1) and (iii) 30 s elongation at 60 °C. Subsequently, a melting step was performed with gradual temperature increasement (60–95 °C, 0.2 °C/s), during which SYBR® Green fluorescence was continuously measured. The same gene was always quantified in one run to prevent any inter-run variation. To ensure accuracy and reproducibility, templates were amplified in triplets for each cDNA sample. All experiments contained a negative control (non-template control) and a RT-minus control to detect genomic contamination. PCR efficiency for each gene under investigation was calculated in each run from a pool of all available cDNAs according to the equation $E = 10^{[-1/\text{slope}]}$. The log-transformed data of serially diluted input cDNA concentrations (75–0.024 ng) plotted against their C_t values (Rasmussen, 2001 and Pfaffl, 2001) showed PCR efficiencies consistently over 88%. All samples were analyzed as technical repeat in two independent runs.

2.6. Data analysis and statistics

For the quantitative comparison of amplification rates the *cycle threshold values* (C_t values) were used. This value defines the fluorescent signal point where a background fluorescent is significantly exceeded. Quantitative real time PCR data were processed applying relative quantification method by using the ΔΔCP-method ($2^{\Delta\Delta C_t}$) (Livak and Schnittgen, 2001). Calculation of the relative expression ratios was performed by using the REST-MCS© (*multiple conditions solver*) beta software which compares two differ-