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Research Paper

A PCR-based quantitative assay for the evaluation of mRNA integrity in rat samples

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ABSTRACT

Reverse Transcription quantitative real-time PCR (RT-qPCR) is applied to quantify gene transcript levels in a wide range of investigations. Proper assessment of RNA integrity is essential for reliable assessment of gene expression levels, as RNA molecules are acutely vulnerable to degradation. However, RNA quality control measures are still infrequently reported in rat toxicological studies, which impede proper evaluation of gene expression data reliability. The high operational cost of microfluidic capillary electrophoresis systems along with paucity of alternative methods for the quantitative assessment of rat RNA integrity constitute potential hurdles to the systematic implementation and reporting of RNA integrity assessment in rat studies. This manuscript describes the adaptation of an alternative RT-qPCR-based 3′:5′ assay as an additional option for the quantitative assessment of rat RNA integrity. Two PCR primer sets were designed on the 3′ and 5′ regions of a rat house-keeping gene to evaluate RNA integrity by measuring the relative expression (3′:5′ ratio) of these amplicons. The 3′:5′ ratios were then compared to Agilent Bioanalyzer's RNA integrity number (RIN) for a wide range of RNA samples originating from different tissues, cultured cell lines and rat strains that were prepared freshly, stored for years at −80 °C, purchased commercially or intentionally degraded. The 3′:5′ ratios and RIN values presented similar assessment of RNA integrity status from intact to heavily degraded samples. Based on the LOWESS regression of this large comparison dataset, 3′:5′ ratio threshold criteria equivalent to RIN cut-off values can be proposed for the selection of RNA samples for RT-qPCR analyses. This qPCR-based assay is easy to implement, cost-effective, and provides a reliable quantification of RNA integrity to assist in the selection of rat RNA samples suitable for downstream RT-qPCR gene expression analyses.

1. Introduction

RT-qPCR is widely used to measure relative changes in gene transcript levels in order to assess biological responses associated with disease or toxicant/drug exposure, and to validate high throughput microarray and RNA-seq data [1–3]. RNA samples, the starting material for these studies, are acutely vulnerable to degradation. The use of degraded RNA samples can lead to unreliable gene expression data and hence, proper evaluation of RNA integrity is essential for reproducible results [4–7].

Traditionally, RNA integrity was evaluated qualitatively by inspecting the intensities of the 28S and 18S ribosomal RNA (rRNA) bands following agarose gel electrophoresis. More recently, manufacturers have developed automated microfluidics-based electrophoretic systems that calculate a quantitative RNA quality score based on the analysis of digitalized electropherograms by proprietary algorithms [8]. The Agilent Bioanalyzer system, one of the best known microfluidics-based platforms, assigns RNA Integrity Number (RIN) values ranging from 1 to 10 to categorize the integrity of RNA samples [9]. RIN values above 8.0 indicate intact, high quality RNA samples, between 5.0 and 8.0 moderately degraded samples, and below 5.0 degraded samples [5,6]. The use of RNA samples presenting RIN values above 5.0 is typically recommended to ensure reliable quantification of gene expression by RT-qPCR [5,6].

As most gene expression studies target protein coding genes, RT-qPCR-based methods such as the 3′:5′ assays were proposed to evaluate messenger RNA (mRNA) integrity status [10]. This 3′:5′ approach is based on the measurement of the relative expression of two amplicons located on the 3′ and 5′ regions of a house-keeping gene transcript by RT-qPCR following cDNA synthesis using (anchored) oligo-dT primers [4,10,11]. In theory, reverse transcription should proceed uninterrupted in intact mRNA samples, generating similar levels of 3′ and 5′ amplicons resulting in a 3′:5′ ratio approaching 1.0. In a degraded RNA sample, the interruption of cDNA synthesis from the poly-A tail will

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lead to reduced levels of the cDNA template for the 5′ amplicon, resulting in higher 3′:5′ ratios. The recently published Differential Amplicon Assay (ΔAmp) is another approach to assess mRNA integrity that uses paired qPCR assays producing long and short amplicons from the same region of an mRNA [12].

Rat is a commonly used species for the assessment of chemical toxicity *in vivo* and *in vitro*. A recent literature survey revealed that more than half of rat toxicological studies using RT-qPCR do not describe RNA quality control measures and that only about one in five reported RNA integrity assessment by electrophoretic-based methods (Fig. S1). Although this lack of reporting does not necessarily imply the absence of appropriate RNA quality controls, such widespread omissions nevertheless impede the proper evaluation of the reliability of gene expression data in rat toxicological studies. While agarose gel electrophoresis requires large quantities of RNA and only allows a qualitative evaluation of RNA integrity, the more quantitative microfluidics-based platforms imply further operational costs and require additional equipment that may not be accessible to all laboratories. The development of a simple, affordable and easily implementable alternative method to quantitatively assess rat RNA integrity may facilitate adherence to RNA quality control measures and reporting in rat toxicological studies. The 3′:5′ assay originally developed for human and using probe-based Taqman dye possess many of these attributes and can be adapted to different species and fluorescent detection chemistries [10,11,13]. In order to expand the available options for RNA quality control in studies assessing rat gene expression, we adapted and optimized this 3′:5′ approach for rat RNA samples. Using a wide range of intact to heavily degraded rat RNA samples from different cell and tissue types, we then compared the 3′:5′ ratios obtained to the trusted microfluidic-based RIN values that delineate RNA sample's suitability for down-stream RT-qPCR gene expression analyses.

2. Materials and methods

2.1. Rat C6, PC12 and CGC cell culture and tissue samples

All cells were grown at 37 °C in a humidified incubator containing 5% CO_2 in cell culture media supplemented with 100 IU/ml penicillin + 100 μg/ml streptomycin. C6 glial cells from American Type Cell Culture (ATCC, Rockville, MD, USA), were cultured in F-12K medium containing 2.5% (v/v) foetal bovine serum (FBS) and 15% (v/v) horse serum. They were grown to confluence before RNA isolation. PC12 pheochromocytoma cells from ATCC were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% (v/v) FBS, 10% (v/v) horse serum and 2 mM L-glutamine. Upon exposure to nerve growth factor (NGF), dividing PC12 cells differentiate by developing axon-like projections [14]. Freshly seeded PC12 cells were allowed to grow for one day and differentiation was initiated by the addition of 50 ng NGF/ml. Total RNA was isolated from dividing and differentiating PC12 cells one day after NGF treatment. Frozen primary Cerebellar Granule Cells (CGCs) from post-natal day 7 (PND7) rat brain purchased from QBM Cell Science (Ottawa, Ontario, Canada) were thawed and seeded at approximately 500,000 cells/well in polyD lysine coated six-well plates. CGCs were grown in Neurobasal A and B27 culture media (20 mM potassium chloride and 1 mM L-glutamine). The cell culture media was replaced by fresh media after one day in culture and total RNA was extracted on the fourth day of culture.

Developing rat brains were harvested from PND14 and PND21 Sprague-Dawley pups following decapitation without anesthesia. The hippocampi were dissected immediately from the brains, flash-frozen in liquid nitrogen and stored at −80 °C until RNA isolation. Further details about rat perinatal exposures, tissue harvesting and RNA extraction can be found in the original developmental neurotoxicity study [15]. Animals were handled following the Canadian Council on Animal Care guidelines and the experimental procedures were approved by Health Canada's Institutional Animal Care Committee.

2.2. RNA extraction

C6, PC12 and CGC cultures were washed with 1× Phosphate-Buffered Saline (PBS) prior to RNA isolation. The cells were lysed directly on the culture dish using the lysis buffer provided in Qiagen's RNeasy Mini Plus kit for total RNA isolation, and genomic DNA was removed using Qiagen's gDNA Eliminator columns following the manufacturer's protocols (Qiagen, Toronto, ON, Canada). Total RNA from juvenile rat hippocampus was extracted using TRIzol (Invitrogen, Burlington, ON, Canada), and further purified using Qiagen's RNeasy Mini Plus kit and gDNA Eliminator columns following the manufacturer's protocols. Total RNA samples from various juvenile and adult tissues and different rat strains (n = 34) were purchased from Zyagen (San Diego, CA, USA), see Table S2.

2.3. Evaluation of RNA purity and integrity

A Nanodrop 1000 spectrometer (Thermo Fisher Scientific, Ottawa, ON, Canada) was used to measure absorbance at 260 nm (A260) to evaluate RNA concentration. RNA purity was estimated using the A260/A280 ratio and only samples presenting a ratio greater than 1.8 were kept for further analyses. RNA integrity was assessed by an Agilent 2100 Bioanalyzer, using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Mississauga, ON, Canada). The Solaris RNA Spike Control kit (Thermo Fisher Scientific, Cat# K-002200-C1-100) was used to assess the presence of inhibitors in a subset of RNA samples (Fig. S2). A PCR-based approach developed in-house [16] was used to assess gDNA contamination in RNA samples purchased from a commercial supplier. All the samples tested proved to be free from inhibitors and gDNA contamination.

2.4. PCR primer design

The ubiquitously expressed housekeeping gene *Phosphoglycerate kinase 1* (*Pgk1*, NM_053291) is well-suited for this 3′:5′ assay. The *Pgk1* gene possesses few pseudogenes and produces a relatively long transcript that presents a well-characterized exon-intron structure. While the low number of pseudogenes and exon-spanning primers will limit the potential interference from inadvertent genomic DNA contamination [16], the lengthy RNA sequence between the two amplified regions will likely contribute to the assay's sensitivity to mRNA degradation. Two PCR primer sets spanning exon junctions and targeting the 3′ and 5′ regions of the *Pgk1* gene were designed (Fig. 1a, Table 1) using the web-based Primer3 software (www.bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm). Primer–BLAST searches were conducted to check the specificity of these primer sets (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). *In silico* PCR analyses of these primer sets at rat UCSC genome browser (genome assembly RGSC 6.0/rn6 at https://genome.ucsc.edu/cgi-bin/hgPcr) were performed to assess cross-match to any non-target sequence such as pseudogenes. The expected amplicon sequences were used to query the rat ENSEMBL database (http://useast.ensembl.org/Rattus_norvegicus/Info/Index) to ensure the absence of Single Nucleotide Polymorphisms (SNPs) at primer binding sites that would impair quantitative PCR efficiency [17]. The potential formation of secondary structures at the primer-template hybridization site that may interfere with PCR amplification was assessed by m-fold (http://mfold.rna.albany.edu/?q=mfold) [18]. The primers were synthesized at Eurofins genomics (Louisville, KY, USA).

2.5. cDNA synthesis, RT-PCR and RT-qPCR

The reverse transcription reactions were performed in a total volume of 20 μl using 0.5–2 μg of total RNA, 500 ng/μl Anchored Oligo-dT primers (Thermo Fisher Scientific), and 200 U Superscript III™ reverse transcriptase (Invitrogen) following manufacturer's first-strand cDNA synthesis protocol. All cDNA samples were diluted in 9 volumes of