Biotechnology Advances 27 (2009) 323–333

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

A decade of improvements in quantification of gene expression and internal standard selection

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ARTICLE INFO

Article history:
Received 7 April 2008
Received in revised form 26 January 2009
Accepted 31 January 2009
Available online 8 February 2009

Keywords:
Housekeeping gene
Reference gene selection
Real-Time RT-PCR
mRNA quantification
Software tool
Reference protein
Protein quantification
Non-coding RNA

ABSTRACT

Major improvements have been made in mRNA quantification and internal standard selection over the last decade. Our aim in this paper is to present the main developments that are of interest for practical laboratory work, contrasting the situation as it is now with the one of ten years ago, and presenting some excellent examples of what can be done today. Specifically, we will mainly discuss Real-Time RT-PCR major improvements that have been performed in the following areas: the most commonly used quantification techniques, the mathematical and software tools created to help researchers in their work on internal standard selection, the availability of detection chemistries and technical information and of commercial tools and services. In addition to mRNA quantification, we will also discuss some aspects of non-coding RNA and protein quantification. In addition to technical improvements, the development of international cooperation and the creation of technical databases are likely to represent a major tool for the future in the standardization of gene expression quantification.

Contents

1. Introduction

Quantification of gene expression is one of the most interesting ways to compare experimental or clinical conditions. Completion in the last few years of the sequencing of entire genomes from humans and several other species through the Human Genome Project and related projects has opened up a new era where almost every gene can

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doi:10.1016/j.biotechadv.2009.01.010

be targeted and its expression quantified and analysed. Two types of genes can be found in the genome. They can either be transcripted into messenger ribonucleotidic acids (mRNAs) that will be translated into proteins, or be transcripted into non-coding RNAs. Non-coding RNAs includes the well-known ribosomal RNAs (rRNAs), transfer RNAs (tRNAs) and pseudogene's RNAs, but also small RNAs as microRNAs (miRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs) and small interfering RNAs (siRNAs) (Backofen et al., 2007). Most non-coding RNAs exert regulatory functions. When considering the expression of genes coding for proteins, their quantification can be performed on mRNA or on protein levels. mRNA quantification presents several advantages. For example, this method avoids the need to use suitable antibodies and has the ability to detect very small amounts of target molecule, depending on the technique used. mRNA quantification can be a very powerful and reliable method for investigating gene expression but only if handled thoughtfully. One of the most critical parts of mRNA quantification is the normalization of results using internal standards. Housekeeping genes are genes whose production is present in every single cell of the organism and is necessary for the cell's survival. Therefore, the transcription of these genes into RNA was at first assumed to be constant and some housekeeping genes were selected as internal standards, such as 18S and 28S rRNA or mRNA for reference genes such as glyceraldehydes-3-phosphate dehydrogenase (GAPDH), albumin, β-and γ-actins, α-and β-tubulins, cyclophilin, hypoxantine phosphoribosyltransferase (HRPT) or L32.

Almost 10 years ago, we observed during various mRNA quantification experiments that the expression levels of housekeeping genes we were using as internal standards could fluctuate. At the time, most researchers were routinely considering housekeeping gene expression levels as constant without discussing the issue. Pooling results from several types of experiment, we wrote a short communication presenting and discussing our observations (Thellin et al., 1999). We wrote some recommendations concerning the use and the limitations of housekeeping genes as internal standards.

Today, mRNA quantification remains one of the main ways of investigating cellular expression. At present, the assays most commonly used are Real-Time RT-PCR (reverse transcription polymerase chain reaction) described in details in several reviews (Wong and Medrano, 2005; VanGuilder et al., 2008) and micro-arrays. These powerful but sensitive techniques require a very precise handling when used to validate internal standards. Numerous tools and techniques have been developed to aid the researcher and are now available. This review will first summarize the main improvements from 1999 up to the present day. We will review new mathematical tools created to help researchers to increase the accuracy and precision of Real-Time RT-PCR results and to select and validate their choice of internal standards. We will also briefly list the main chemistries available for signal detection. As mRNA is not always relevant to the real production of biologically active proteins, we will discuss some aspects of protein quantification using reference proteins in addition to standard protein dosage. For the same reason, we will also present recent work in non-coding small RNA quantification. Finally, we will discuss both initiatives created to regroup technical information from protocols into databases and the tool that could emerge out of such work.

2. Comparison of the most commonly used mRNA quantification techniques in 1999 and the present day

Our first concern when preparing this article was to explore how researchers' performance of mRNA quantification had evolved. Initially, we did not want to investigate the tools and methods that were available; instead we wished to check which ones had been commonly used in papers utilizing mRNA quantification and how frequently Real-Time RT-PCR had been used as the quantification technique. Therefore, we considered the totality of 114 papers published in 1999 and listed by the NIH Entrez PubMed website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) when using "mRNA quantification" as selection keywords. We first checked how many of them were using Real-Time RT-PCR then we focused on internal standard use. We decided to illustrate them with a collection of 15 papers randomly selected from these 114 articles. In the following chapter, we describe how we undertook the same approach for 2007 and 2008, considering 100 papers out of 916 published in 2007 and 100 papers out of 345 published in 2008, illustrating them with a collection of 20 papers randomly selected from these 1261 publications.

2.1. Techniques most commonly used in 1999

In articles published in 1999, numerous techniques were cited for quantifying mRNA (see examples in Table 1): mainly Northern blotting, semi-quantitative RT-PC, nested-or mimic-based competitive RT-PCR, in situ hybridization (ISH), RT-PCR melting curve analysis, RNAse protection assay (RPA), Real-Time RT-PCR. The techniques most commonly used were firstly end-point RT-PCR techniques, then RPA. Among lesser used techniques, we found Northern blotting, an older and less accurate technique, and Real-Time RT-PCR. Even though Real-Time PCR had been first developed in 1992 (Higuchi et al., 1992), Real-Time RT-PCR use was still very new in 1999 and was still in its test and development phase. This explains why it was used in only 8% of studies, far from being as commonly used as other techniques at the time.

Internal standards typically consisted of ribosomal RNA, housekeeping genes, messenger RNA or total RNA. For the competitive RT-

Table 1
List of techniques and internal standards used in a random selection of 15 papers concerning mRNA quantification, published in 1999.

mRNA quantification techniques	Species and internal standards	References
Northern blot	Mouse: 28s RNA, actin	(Carrier et al., 1999)
Semiquantitative RT-PCR	Human: β2-microglobulin	(Wong et al., 1999)
Semiquantitative competitive mimic RT-PCR	Swine: Added artificial external standard ➔ total RNA	(Nikbakht-Sangari et al., 1999)
Nested competitive RT-PCR	Human: Added artificial external standard ➔ total RNA	(Stoffel-Wagner et al., 1999)
Semiquantitative RT-PCR	Rat: G3PDH	(Charpantier et al., 1999)
In situ hybridization (ISH)	Rat: None	(Huber et al., 1999)
Mimic-based RT-PCR	Human: Added artificial external standard ➔ total RNA	(Lai et al., 1999)
RT-PCR melting curve analysis	Swine: Actin	(Spagnuolo-Weaver et al., 1999)
RNAse protection assay (RPA)	Rat: Total RNA	(Reinhart et al., 1999)
Quantitative RT-PCR	Rat: HPRT	(Tokuyama et al., 1999)
Competitive quantitative RT-PCR	Mouse: Added artificial external standards ➔ total RNA	(Haack et al., 1999)
RPA	Rat: β-actin	(Nag et al., 1999)
RPA	Mouse and rat: β-actin	(Stepan et al., 1999)
Real-Time PCR	Human: G6PD	
Competitive RT-PCR	Added artificial external standards ➔ total RNA	(Emig et al., 1999)
Quantitative RT-PCR	Swine: GAPDH	(Dufour et al., 1999)