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Quantification of rRNA in *Escherichia coli* using capillary gel electrophoresis with laser-induced fluorescence detection

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Abstract

Over the past 10 years, sophisticated powerful techniques have been developed for the quantification of messenger RNA (mRNA) and ribosomal RNA (rRNA), enabling researchers in science, industry, and molecular medicine to explore gene expression. These techniques require the (reverse) transcription of analyte RNA, hybridization with synthetic oligonucleotides, and other additional steps that make them costly, time-consuming, and quantitatively difficult to perform. The current work demonstrates how 16S and 23S rRNA can be quantified precisely using capillary gel electrophoresis with laser-induced fluorescence detection (CGE–LIF) directly after the extraction of total RNA without requiring further reactions or calibration. CGE–LIF normally is used for the qualitative examination of RNA preparations. Its quantitative performance could be improved significantly using MS2 bacteriophage RNA as an internal standard. The entire analytical procedure was validated for linearity, repeatability, reproducibility, and recovery. This validation also included total RNA extraction from bacterial cells, an aspect examined for the first time in absolute RNA quantification. Recovery is close to 100%, and the analytical precision was increased 10-fold (CV < 3%), as compared with similar approaches. The demonstrated method is simple and opens up new possibilities for the absolute quantification of not only rRNA but also individual mRNAs.

Keywords: Absolute quantification; Gene expression; Internal standard; Linearity; mRNA; Precision; Recovery; Repeatability; Reproducibility; RNA extraction; rRNA

Scientific and industrial research, as well as molecular medicine, has always relied heavily on tools for gene expression profiling and transcriptome analysis. Most approaches provide semiquantitative data (relative expression levels), which usually are sufficient for a wide range of applications. However, the latest challenge to RNA analysis concerns the requirement for absolute high-precision quantification. Extremely reliable estimates of the concentrations of RNA species, such as messenger RNA (mRNA),[3] ribosomal RNA (rRNA), and transfer RNA (tRNA), are a prerequisite for clinical diagnostics or systems biology when quantitatively studying gene expression kinetics based on mathematical modeling [1,2]. The concentration of mRNA is an important variable for modeling of a wide range of dynamic processes, for example, the behavior of producer

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[3] *Abbreviations used:* mRNA, messenger RNA; rRNA, ribosomal RNA/tRNA, transfer RNA; qPCR, quantitative real-time polymerase chain reaction; IS, internal standard; RPA, ribonuclease protection assay; SAGE, serial analysis of gene expression; CGE, capillary gel electrophoresis (or CE [capillary electrophoresis]); LIF, laser-induced fluorescence; MIAME, minimum information about a microarray experiment; FA, formaldehyde agarose; DEPC, diethyl pyrocarbonate; CV, coefficient of variation.

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strain metabolisms during biotechnological processes [3]. Recent results indicate that the dynamic modeling of the central carbon metabolism and its regulation by gene expression also needs to take growth rate control, which is exerted by the regulation of rRNA expression, into account [3]. For this purpose, ribosome (or rRNA) concentration is another very important variable. High-throughput technologies and automation become necessary when dealing with large sample quantities so that hands-on time can be reduced to a minimum. However, achieving minimal hands-on time for experimental expression profiling is a challenge in itself, particularly because so far only highly sophisticated methods, which produce only reasonably satisfactory results, have been available.

For the quantification of rRNA, radioactive labeling of stable RNA (rRNA and tRNA) and uracil starvation [4] and direct quantification of the ribosome concentration using several extremely time-consuming ultracentrifugation steps with or without density gradients [5,6] have been used previously but proved to be inappropriate for precise and reasonable high-throughput quantification. The latest developments for this purpose are in the field of diagnostic studies and ecological research, where either quantitative real-time polymerase chain reaction (qPCR) assay or DNA microarray technology is employed [7–9]. The latter are also the most frequently used commercially available automated methods for quantifying mRNA expression. Microarray technology [10,11] is unbeatable in the parallel determination of thousands of different mRNA molecules on the genome level in a single run [12–14]. Although precise semiquantitative determination is possible, the generation of precise quantitative data (absolute transcript copy numbers per cell) is quite difficult. Even when internal standards [ISs] are used, it is still only possible to provide estimations of mRNA concentrations [15–20]. Quantitative real-time PCR is the state-of-the-art method for the quantitative determination of specific mRNA molecules [21–23]. However, reproducible quantification depends on careful experimental design, application, and validation [21,24,25]. Furthermore, due to technical limitations, only a restricted number of mRNA molecules can be detected in parallel [21]. Numerous other biochemical approaches, such as Northern blot analysis, ribonuclease protection assay (RPA), and serial analysis of gene expression (SAGE), can also be used for quantification [26,27]. All of the commonly used biochemical approaches deal with samples that contain thousands of individual RNA molecules. The analyses are done in parallel and need to be selective for all individual transcripts, making the investigation procedure labor- and cost-intensive, with oligonucleotide synthesis, hybridization reactions, cloning, and in vitro transcription steps being required. Second, the quantification of RNA usually relies both on a nonlinear signal-to-response behavior, which necessitates complicated calibration procedures, and on statistical data analysis. Thus, a high level of experimental expertise is mandatory, and the downstream data analysis is also very technical and involves a great deal of work [11,21,28].

In more recent, fundamentally different approaches, the analyte RNA molecules are first separated by capillary gel electrophoresis (CGE or CE [capillary electrophoresis]) [29] and subsequently detected using spectrometry. Spectrometric detection, such as using laser-induced fluorescence (LIF), exhibits linear signal-to-response behavior and a large dynamic range. Both costs and the time requirement are very low, and automated microfluidic high-throughput CE–LIF technology is already available [29,30]. CGE–LIF is routinely applied for qualitative controlling of RNA integrity prior to microarray or qPCR analyses [22,24]. Quantitative CE-based approaches employ either unspecific intercalating dyes or specific spectrometric detection after hybridization with fluorescence-labeled synthetic oligonucleotides [31,32]. Because current CE methods are not yet able to resolve all mRNA molecules, hybridization becomes necessary so as to identify specific RNA molecules in total RNA samples. Unspecific dyes greatly simplify the procedure but have only been applied for the estimation of relative total RNA and rRNA concentrations [29,33]. Although current CE systems enable the convenient estimation of RNA concentrations, detailed validation of the degree of precision has not been published so far. This may be attributed to technical limitations in CE analysis that reduce the analytical precision such as the generally observed injection bias [34], the changing physicochemical constitution of samples, and/or the polymer matrix [35], even though the technique is improving constantly [29,30].

A generally disregarded issue, but very important for the absolute quantification of RNA, is the sampling and RNA extraction procedure [18,24,25]. In general, equal samples of total RNA amounts are used, although many techniques have been developed for quantitative RNA analysis [22]. Therefore, the routinely used extraction procedures do not need to be quantitative. Although recommended by the minimum information about a microarray experiment (MIAME) standard, in many cases modifications of standard protocols are not described adequately [36]. Nevertheless, relating transcript numbers to the total RNA content may be critical because total RNA content can vary considerably when cells with different physiological states are being examined [22]. For instance, in microorganisms such as *Escherichia coli,* the total RNA content and the proportions of mRNA and rRNA depend on the growth rate [37,38]. Consequently, a procedure that guarantees the quantitative extraction of total RNA and the normalization of expression data to biomass (or best possible to the cell volume [3]) is mandatory.

The current work uses 16S and 23S rRNA from bacterial cell extracts to show that specific RNA molecules can be quantified precisely using CGE–LIF. Technical limitations inherent to CE can be circumvented by adding an IS to all of the samples. The method reported in this work allows the high-precision quantification of rRNA molecules and does not need oligonucleotide synthesis, hybridization reactions, and calibration procedures, thereby considerably reducing material costs and working time.