Normalization of RNA Hybridization Signals by Means of SYBR® Green II-Stained 28S or 18S Ribosomal RNA and a Phosphor Imager

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Northern blot analysis is the most common method for demonstrating altered expression of RNA species in different cells or tissues under different stimulation regimes. Ideally, comparison of transcription levels for a constant number of cell nuclei is accomplished by use of such Northern blots. In loading constant amounts of total RNA onto the gel, one usually assumes that more than 90% of the total RNA is ribosomal RNA (rRNA) and that the stoichiometry of ribosomal RNA to DNA is not likely to vary greatly. For this purpose, it is generally accepted as a prerequisite that equal amounts of total RNA should be used as target for the discrimination of varying transcript signals. One common method is to show the ethidium bromide (EtBr)-stained formaldehyde gel as a demonstration of RNA quantity and integrity. Also, control hybridizations to probes for housekeeping genes can be carried out in many cases where there is equal loading of RNA and successful transfer of that RNA onto membranes. However, in our experience, although (EtBr) staining can demonstrate the integrity of RNA and loading of equal amounts of RNA can be estimated quite accurately from the photograph or videoprint of the gel, the linearity that is demanded for detecting minute transcription changes might not be achieved, especially when small amounts of RNA are analyzed. Secondly, control hybridizations can demonstrate the successful transfer of the RNA from the gel onto the membrane, but steady-state levels of many housekeeping genes, such as β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cannot always be assumed; there can be substantial alterations in levels of expression between tissues and even within a tissue, depending on differentiation status or stimulation protocols used. Consequently, normalization of the amounts of RNA loaded by means of (i) fluorescence staining, (ii) staining of the 28S rRNA (2–5,10) or (iii) by hybridization with a 28S rRNA-specific antisense oligonucleotide probe (1) would appear to represent a more consistent method of verifying equal loading. Some commercially available Northern blots can be obtained that are normalized either to RNA content or to β-actin hybridization signals (i.e., Northern Territory™ Blots from Invitrogen [De Schelp, The Netherlands]).

In our laboratory, we routinely use a phosphor imager to quantify the expression of specific transcripts on Northern blots. Yet the normalization against signals for different housekeeping genes appears neither acceptably reproducible nor consistent results under different experimental conditions. We have now found that measurement of rRNA by a fluorescence-inducing scan of an RNA gel stained with the dye SYBR® Green II (Molecular Probes, Eugene, OR, USA) offers an excellent alternative method of RNA quantification. Several phosphor imagers (e.g., the STORM™ 840 Gel and Blot Imaging System, Molecular Dynamics) at 975 V and a resolution of 256 gray scales to discern the fluorescence between bound and unbound dye. Furthermore, most video imaging systems for photographing EtBr-stained gels only operate with a range of 256 gray scales to discern the fluorescence signals. SYBR Green II was found to be a much more appropriate dye, because when bound to rRNA it exhibits over 1000-fold higher fluorescence than the unbound dye (8) and is about 10× more sensitive than EtBr (9). Also, using the fluorescence module of a phosphor imager has the advantage of quantifying signals over a range of 5 orders of magnitude, and thus offers a wider linear dynamic range for quantification.

Different dilutions of mouse muscle RNA ranging from 25–2.5 μg were dissolved in a mixture of 50% formamide, 7.5% formaldehyde, 10% glycerol, N-morpholinopropanesulfonic acid (1× MOPS, pH 7.0) and 5% saturated bromophenol blue, heated to 65°C for 15 min and then chilled on ice. For one additional sample, containing 10 μg of RNA, 1 μL of EtBr stock solution (10 mg/mL) was added after the heating step. The RNA samples were electrophoresed on 1.3% agarose gels containing 17.5% formaldehyde for 16 h at 1 V/cm. The rate of RNA migration and RNA integrity was checked by measuring the fluorescence of the prestained sample using a conventional UV transilluminator. The gel was then stained in 10× standard saline citrate (SSC, pH 7.0) with 1× SYBR Green II for 1.5 h on a rocking shaker and then scanned with the blue chemiluminescence program of the STORM 840 Gel and Blot Imaging System (Molecular Dynamics) at 975 V and a resolution of 100 μm. Bands were quantified with the ImageQuant™ Software (Molecular Dynamics) using “average lane” background subtraction.

RNA was then transferred to nylon membranes by the upward capillary transfer method in 10× SSC overnight and cross-linked. The membranes were hybridized with a [32P]dCTP-labeled β-actin specific probe, which was generated by the random prime label method (6), in 20% sodium dodecyl sulfate (SDS), 0.25 M sodium phos-

![Figure 1. Comparison of fluorescent 28S and 18S signals with radiolabeled β-actin signals. Fluorescence of SYBR Green II-stained 28S and 18S rRNA of an RNA dilution series after electrophoresis in an agarose/formaldehyde gel and laser-scanning (top). Hybridization signals on phosphor images (bottom).](image)
phosphate buffer, pH 7.2. Three 30-min wash steps followed with (i) 2× SSC, 0.1% SDS at room temperature, (ii) 1× SSC, 0.1% SDS at 65°C and then (iii) 0.1× SSC, 0.1% SDS at 65°C, respectively. Membranes were then exposed to phosphor-storage screens for 24 h and scanned at 825 V and 100-µm resolution with the phosphor-storage program of the STORM 840 imaging system. Bands were quantified with the ImageQuant software, again using average lane background subtraction.

Figure 1 shows the SYBR Green II-stained 28S and 18S rRNA band after size separation of the RNA in a conventional agarose/formaldehyde gel and the corresponding β-actin hybridization signal after transfer. To determine the linearity of the concentration/fluorescence relationship, total RNA in a dilution from 25–2.5 µg was applied. Within a range from 2.5–20 µg total RNA, a high degree of linearity (r = 0.999) was reproducibly found within the 18S band, while the 28S band exhibited a slight decrease in linearity (r = 0.987; Figure 2A). The linear relationship broke down at amounts exceeding 20 µg total RNA per lane for both rRNA species. This linear concentration/signal relationship is better than reported previously with EtBr staining and two-dimensional scanning laser densitometry (2), where a maximum of 12.5 µg and a linear regression coefficient of r = 0.89 was reported. Figure 2B shows the linear relationship between the fluorescence signal and the corresponding hybridization signal with a β-actin-specific radiolabeled probe. In the range of 2.5–20 µg total RNA, the fluorescence/radioactive signal linearity of r = 0.993 is maintained in the 18S band, while higher amounts of total RNA show a decrease in linearity (data not shown). The 28S band also demonstrated a decrease in linearity.

Note that a high-voltage output of the fluorescence-inducing laser is necessary for measuring small amounts of total RNA (i.e., 2.5 µg). Although the use of voltages lower than 975 V did not influence linearity, low voltages did decrease the sensitivity of gel scanning.

The quantification of the more prominent 28S rRNA band resulted in higher peak-volume values, but the gel band of this higher molecular weight RNA species had a slightly reduced resolution, leading to a small decrease in linearity in the concentration/signal relationship after scanning. This could be because of a decrease in sharpness of the 28S band resulting from increased lateral diffusion in this slower migrating rRNA species during low-voltage electrophoresis. Thus, use of the 28S rRNA band shows a small disadvantage in terms of the quantification of the fluorescence signals. A lower fluorescence of the 23S rRNA from prokaryotes compared with the 16S rRNA when normalized to mass has also been reported elsewhere (7). This could reflect the fact that such intercalating dyes are dependent on the relative extent of double-stranded RNA regions in the rRNA.

In this study, we demonstrate the applicability of the sensitive DNA-binding fluorescent dye SYBR Green II in the normalization of RNA hybridization
signals. The advantages, compared with the previously used dye EtdBr in conjunction with a phosphor imager, are in the higher linearity over a broader range of total RNA loaded onto the gel. This range extends to 20 µg total RNA, which is an amount often used when hybridizing to low-abundance transcripts. The method is applicable for all laboratories that use a phosphor imager for quantifying hybridization signals derived from radioactively labeled probes. The method is also fast enough to use for checking inaccuracies of RNA quantitations done by UV photometry or pipetting measurements done before gel loading.

With the same instrument, signal normalization can be accomplished, eliminating hybridization steps involving less accurate “constitutively” expressed genes thus saving time and avoiding the generation of unnecessary radioactive waste.

REFERENCES


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Procedure for the Investigation of Bacterial Genomes: Random Shot-Gun Cloning, Sample Sequencing and Mutagenesis of Campylobacter jejuni

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With the rapidly growing volume of bacterial sequencing information [20 prokaryote genomes have been sequenced, and the sequencing of 45 further genomes is in progress according to the Multipurpose Automated Genome Project Investigation (MAGPIE) data; http://www-fp.mcs.anl.gov/~gaasterland/genomes.html], the availability of experimental approaches validating gene function prediction is of prime importance. Unfortunately, molecular genetic approaches such as transposon mutagenesis and phage transduction are not available for many bacteria. In this report, we reveal how limited sequencing information resulted from random sequencing of a library, based on a suicide vector, can effectively be used in such bacteria. In addition, such libraries, if used for entire genome sequencing projects, can be applied for systematic mutational analysis of the genomes with limited genetic tools.

Campylobacter jejuni is a Gram-negative microaerophilic bacterium, which is a major cause of human enterocolitis (7). The development of disease prevention and infection control strategies are hindered by a poor understanding of the genetics and pathogenicity of the organism. Methods of genetic analysis used to characterize other enteric pathogens (e.g., transposon mutagenesis and phage transduction) are not available for C. jejuni. One of the most powerful tools for the identification of virulence determinants and characterizing pathogenic mechanisms is the construction of genetically defined mutants. On another hand, the procedure for construction of defined mutants by allelic replacement implies availability of the sequencing data. pUC18 has recently been used in different bacterial genome