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Evaluation of isolation methods and RNA integrity for bacterial RNA quantitation

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ABSTRACT

RNA integrity is critical for successful RNA quantitation for mammalian tissues, but the level of integrity required differs among tissues. The level of integrity required for quantitation has not been determined for bacterial RNA. Three RNA isolation methods were evaluated for their ability to produce high quality RNA from *Dickeya dadantii*, a bacterium refractory to RNA isolation. Bacterial lysis with Trizol using standard protocols consistently gave low RNA yields with this organism. Higher yields due to improved bacterial cells lysis was achieved with an added hot SDS incubation step, but RNA quality was low as determined by the RNA Integrity Number (RIN). Contaminating DNA remained a problem with the hot SDS-Trizol method; RNA samples required repeated, rigorous DNase treatments to reduce DNA contamination to levels sufficient for successful real-time qRT-PCR. A hot SDS-hot phenol RNA method gave the highest RNA quality and required only two DNase treatments to remove DNA. The assessment of RNA integrity using the Agilent 2100 BioAnalyzer was critical for obtaining meaningful gene expression data. RIN values below 7.0 resulted in high variation and loss of statistical significance when gene expression was analyzed by real-time qRT-PCR. We found that RNA preparations of different quality yielded drastic differences in relative gene expression ratios and led to major errors in the quantification of transcript levels. This work provides guidelines for RNA isolation and quality assessment that will be valuable for gene expression studies in a wide range of bacteria.
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1. Introduction

Quantitative reverse-transcription PCR (qRT-PCR) has become the standard technology to quantify mRNA for gene expression profiling. Two strategies are commonly employed to enumerate the results obtained by real-time RT-PCR: the standard curve method (absolute quantification) and the comparative threshold method (relative quantification). Absolute quantitation relies on the inclusion of a standard curve on each reaction plate and results in determination of the actual quantity of the target transcript expressed in copy number or concentration. This method has the advantage of correcting differences in primer efficiencies and product fluorescence. The disadvantage of absolute quantitation is the significant reduction in the number of experimental samples that can be run on a single plate. Relative quantification determines changes in steady-state mRNA levels of a gene across multiple samples and biological replicates relative to the mean of control samples that is designated as the calibrator. With this method, target transcript amounts are expressed as a relative expression ratio (Phongsisay et al., 2007) relative to the calibrator. Both methods require the normalization of target gene expression using multiple stably expressed internal control mRNAs. These reference gene mRNAs must be shown to be stable under the experimental conditions being examined and are evaluated using software programs such as *BestKeeper* or *GeNorm* (Pfaffl et al., 2004; Vandesompele et al., 2002).

Intact mRNA must be used with qRT-PCR to accurately quantify mRNA levels; use of low-quality RNA compromises the derived expression results (Imbeaud et al., 2005; Raeymaekers, 1993). Long mRNA molecules are very sensitive to degradation (Bustin, 2002), which occurs through cleavage with RNases during improper handling or through storing the RNA in sub-optimal conditions (Perez-Novo et al., 2005; Schoor et al., 2003). Consequently, determining and confirming the RNA quantity and quality, is a critical step in obtaining meaningful gene expression data using real-time qRT-PCR.

The observed difference in gene expression data between intact and degraded RNA samples has led many authors to propose performing RNA quality control in order to obtain more accurate and reliable results (Auer et al., 2003; Bustin and Nolan, 2004a; Fleige and Pfaffl, 2006; Fleige et al., 2006). RNA purity is normally assessed by measuring its A260/A280 ratio, but the accuracy of this method is questionable because a OD 260/280 ratio of 1.8 corresponds to only 40% RNA in the presence of other contaminants, such as protein (Baelde et al., 2001; Bustin and Nolan, 2004b). Spectrometric methods often fail in sensitivity, are highly variable, and give no input as to the basic integrity of the RNA. Denaturing agarose gel-electrophoresis can also be used, but this method is low throughput and requires larger amounts of RNA than are typically available (Bustin and Nolan, 2004a).

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Because of the dramatic decrease in the amount of RNA needed to evaluate integrity (picogram amounts of RNA can now be analyzed), instruments such as the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and the Experion (Bio-Rad Laboratories, Hercules, CA) are becoming the standard for analysis (Livak and Schmittgen, 2001; Mueller et al., 2000). These machines use micro-fluidic capillary electrophoresis, in which the samples are first separated electrophoretically and then detected fluorescently with a laser. The machine determines the quantity of RNA, and also the quality of RNA through the 16S/23S ratio. Agilent has developed a new tool to determine the RNA integrity by using an algorithm to assign a RNA Integrity Number (RIN). The RIN number assigns a score of 1 to 10, where 10 is completely intact RNA and 1 represents highly degraded RNA (Mueller et al., 2000). The RIN number assignment allows for a standardized and objective assessment of RNA quality, and interpretation of RNA integrity facilitates the comparison of samples and insures the reproducibility of experiments.

The extraction method used may affect RNA quality. Many factors can affect reproducibility, and therefore relevance, of gene expression profiling results. These include the source of the RNA (tissue or organism) and the sampling and isolation techniques (Bustin and Nolan, 2004b; Pfaffl, 2004). Reliable isolation techniques must yield intact high quality RNA that is free of RNases, proteins and genomic DNA. The extraction and purification procedures should also be free of RT and PCR inhibitors, as well as devoid of substances like Mg^{2+} and Mn^{2+} that are essential reaction cofactors (Bustin and Nolan, 2004b).

The RNA requirements for downstream gene expression applications have been thoroughly investigated in mammalian systems. With animal cells, specific tissues and cell lines yield different RNA quantities, qualities, and integrities (Fleige and Pfaffl, 2006). For example, the mean RIN associated with bovine heart tissue is 6.03, while bovine white blood cells have a mean RIN of 9.36 (Fleige and Pfaffl, 2006). The understanding, application, and adoption of these methods and tools have yet to be investigated in non-mammalian systems. Here we examine several standard laboratory kits and methods, both alone and in combination, to obtain high quality RNA from *Dickeya dadantii*. The plant pathogen *D. dadantii* (formerly *Erwinia chrysanthemi*) causes wilting and soft rot in a wide range of plants, including ornamental plants and economically important vegetable crops (Ma et al., 2007; Samson et al., 2005). Optimization of bacterial lysis was required to obtain sufficient quantities of RNA from this phytopathogenic member of the Enterobacteriaceae. High quality and high quantity RNA was only obtained from hot SDS/hot phenol isolation techniques. Even with increased yield and quality, analysis of RNA integrity was essential in order to ensure reproducibility with qRT-PCR assays.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Wild-type *D. dadantii* (formerly *E. chrysanthemi* 3937) and the *fliA*::Km derivative were described previously (Jahn et al., in press). Strains were grown in Luria-Bertani (LB) medium, SOBG (salt optimized broth plus 2% glycerol (per liter, 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.4 g of $MgSO_4$, 0.186 g of KCl, and 50 ml of 40% glycerol)) (Yap et al., 2005) or polypectate enrichment medium (PEM) (per liter, 1.5 g sodium polypectate, 0.13 g $(NH_4)_2SO_4$, 0.17 g K_2HPO_4, and 0.06 g $MgSO_4{\cdot}7H_2O$). When required, antibiotics were used at the following concentrations (in micrograms per milliliter): nalidixic acid (50) and kanamycin (50).

2.2. RNA isolation and cDNA generation

Several standard laboratory kits and methods, Trizol (Invitrogen), RNeasy (Qiagen), and hot SDS/hot phenol, alone and in combination were tested for RNA extraction from *D. dadantii*. High quality RNA was isolated using a hot SDS/hot phenol method that was modified, from a method described previously (Linchao and Bremer, 1986). Briefly, 12 h cultures were added to a 1/10 volume of 95% ethanol plus 5% saturated phenol to stabilize cellular RNA and the cells were then harvested by 2 min 8200 g centrifugation at 4 °C. The supernatant was aspirated and the pellets frozen in liquid N_2. Pellets were resuspended in 800 µl of lysis buffer. Lysis buffer consisted of TE (10 mM Tris, adjusted to pH 8.0 with HCl, 1 mM EDTA) and 8.3 U/ml Ready-Lyse™ Lysozyme Solution (Epicentre Biotechnologies, Madison, WI). After the pellets were resuspended, 80 µl of a 10% SDS solution (wt:vol) was added and the lysate was mixed and incubated at 64 °C for 2 min. After incubation, 88 µl of 1 M NaOAc (pH 5.2) was mixed with the lysate. To this solution, an equal volume of water saturated phenol was added, mixed and incubated at 64 °C for 6 min inverting the tubes six times every 40 s. The samples were chilled on ice and centrifuged at 21,000 g for 10 min at 4 °C. The aqueous layer was transferred to a Phase Lock Gel (PLG, Heavy) tube (Eppendorf, Hamburg, Germany) with equal volume of chloroform and centrifuged at 21,000 g for 5 min at 4 °C. The aqueous layer was split between two 1.5 ml Eppendorf tubes and ethanol precipitated by adding 1/10 volume of 3 M NaOAc (pH 5.2), 1 mM EDTA and 2 volumes cold EtOH to each sample. The samples were mixed and incubated at −80 °C overnight. The RNA was pelleted by centrifugation at 21,000 g for 25 min at 4 °C. Pellets were washed with ice cold 80% EtOH and centrifuged at 21,000 g for 5 min at 4 °C. The ethanol was carefully removed and the pellets were air dried for 20 min in a fume hood. The pellets from each split sample were resuspended in a total of 100 µl of RNase-free water and combined into one microfuge tube.

Total RNA was quantified using micro-spectrophotometry (NanoDrop Technologies, Inc.). DNA was removed with Turbo DNA-*free* (Ambion, Inc.) using the rigorous protocol. RNA integrity was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). RNA with a RNA Integrity Number (RIN) above 7.0 was used for real-time qRT-PCR. Removal of DNA from the RNA samples was confirmed by performing real-time PCR on 100 ng of total RNA using the *rplU* primer set, but without a reverse transcriptase step (no-cDNA control) and those RNA samples found to yield threshold cycle (C_t) values larger than 32 were judged to be sufficiently free of contaminating DNA for further analysis. Purified RNA was converted to cDNA immediately or stored frozen at −80 °C.

First-strand cDNA synthesis was performed using the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad Laboratories). Briefly, the reaction was performed with 1.0 µg total RNA in 15 µl RNase-free water, 4 µl 5X iScript reaction mix containing a blend of oligo-dT and random hexamer primers, and 1 µl iScript reverse transcriptase. The reaction conditions were performed at 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and the cDNA was diluted to a final volume of 286 µl and stored at 4 °C.

2.3. Real-time RT-PCR primer design

Primers were designed based on sequences obtained from ASAP (Glasner et al., 2003), which were imported into Beacon Designer software (Premier Biosoft International), a program designed to generate primer pairs suitable for real-time PCR. The program setting 'avoid template structure' was chosen to limit primer sequences to regions of little secondary template structure and the SYBR Green module was used. Primers were obtained from IDT (Integrated DNA Technologies) and the sequences, efficiencies, and source sequences are shown in Table 1.

D. dadantii sequences can be obtained (under the former name *E. chrysanthemi* 3937) from the ASAP database at https://asap.ahabs.wisc.edu/asap/logon.php. ASAP is a public database that contains genome sequence, annotations and experimental data for multiple organisms plus an interface for direct community contributions (Glasner et al., 2006). Suitable internal reference gene primers were