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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

Table 1
Real-time qRT-PCR primer sequences, efficiencies and sources

Gene name (ASAP ID number[a])	Primer name	Primer sequence (5′ to 3′, forward/reverse)	% Efficiency[b]
pelX	P0779	AACAAACGCCGACCTTCC/	105
(ABF-0014783)	P0780	TCCTGATGGGTGACTAAATCC	
(ABF-0019391)	P0781	AAACACCGTCAATTACAG/	86
	P0782	AATTCAGTATCGGAAATCG	
rplU/	P0275	GTTTGACCAGGTTCTGATGGTTGC/	91
(ABF-0046905)	P0276	CCAGCCTGCTTACGGTAGTGTTTA	
gyrA/	P0791	CCACCCGTATCCCGAATC/	90
(ABF-0017293)	P0792	ACAACCGTCAATCACTTCAG	
rpoS/	P0789	CGCTGCTGGATCTGATTG/	109
(ABF-0020446)	P0790	ACGATATGGATGGGTAAACG	
ompA/	P0787	CAGACAGCCACGACAACTC/	87
(ABF-0018822)	P0788	TAGCGTATTCAACACCCACAG	
rpoD/	P0814	GCCATCACCTATCTGTTG/	85
(ABF-0019909)	P0815	TCTTCTTCGTCTTCTTCG	

[a] *Dickeya dadantii* sequences can be obtained (under the former name *Erwinia chrysanthemi* 3937) from the ASAP database at https://asap.ahabs.wisc.edu/asap/logon.php.
[b] Primer efficiencies were determined by the MyiQ Cycler software from a standard dilution curve of target DNA as described in Materials and methods.

chosen on the basis of two criteria: i) primer efficiencies close to 100%; and ii) stable expression within wild-type and mutant samples. Both reference and target primers exhibited comparable efficiencies as determined with a dilution series of DNA derived from *D. dadantii* genomic DNA (Table 1). Two independent 10-fold dilutions of DNA were subjected to real-time RT-PCR using SYBRGreen reaction mix (Bio-Rad Laboratories, Inc.). Master mixes from each dilution series were split into three reactions (25 µl per well) yielding six replicate reactions for each dilution point. Real-time PCR was performed using the MyiQ Real-Time PCR Detection System and analyzed using the MyiQ software package (Bio-Rad Laboratories, Inc.). Stability of reference transcripts was validated using the *BestKeeper* program (Pfaffl et al., 2004).

2.4. Real-time PCR conditions

All reactions were performed in duplicate 25 µl volumes using iQ SYBRGreen Supermix (Bio-Rad Laboratories, Inc.). In all experiments, cDNA from two independent iScript (Bio-Rad Laboratories, Inc.) reactions were analyzed giving a total of four 25 µl reactions for each RNA sample. A master mix was prepared for each primer set containing SYBRGreen and an appropriate volume of each primer to yield a final concentration of 200 nM. For six samples: 29 µl of each 5 µM primer stock solution was added to 365 µl of SYBRGreen Supermix, then 30 µl of the master mix was pipetted into 12 microcentrifuge tubes containing 26 µl of each template cDNA (two cDNA reactions per RNA sample). This yielded a reaction mix containing primers at a 200 nM final concentration and cDNA from 90 ng of total RNA. Reactions were performed with the MyiQ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.) using the default 2-step amplification plus melting curve protocol. The reaction conditions were: enzyme activation and well factor determination at 95 °C for 3 min followed by 40 cycles of 95 °C for 1 min (denaturation) and 55 °C for 45 s (annealing and elongation); the melt curve protocol began immediately after amplification and consisted of 1 min at 55 °C followed by 80–10 s steps with a 0.5 °C increase in temperature at each step. Threshold values for threshold cycle (C_t) determination were generated automatically by the MyiQ software. Lack of variation in PCR products and the absence of primer–dimers were ascertained from the melt curve profile of the PCR products.

2.5. Experimental design and data analysis

Each experiment consisted of three cultures of *D. dadantii* wild-type and the *fliA* mutant grown with agitation in SOBG overnight at 30 °C. Cultures were diluted 1:50 in PEM and incubated for 12 h at 28 °C. Each experiment was repeated twice for a total of two biological replicates with a total of 6 samples.

Two iScript reactions were performed on each RNA sample and the SYBR Green reaction mixes for each cDNA sample were run in two wells at 25 µl per well. This results in four replicate real-time PCR reactions for each RNA sample. At each time point, amount of target RNA was determined by first normalizing the target RNA to the internal standard RNA using the formula: $2^{(C_t \text{ internal standard} - C_t \text{ target})}$. To determine the relative expression ratio of the target gene, the normalized target RNA value was divided by the average of the normalized values of six control samples. This average is designated as the "calibrator" since the variation of all samples, including the individual control samples, is determined relative to this value. This method of calculating the RER was derived from the previously published $2^{(-\Delta\Delta C_t)}$ formula (Livak and Schmittgen, 2001; Pfaffl, 2001) as previously described (Rotenberg et al., 2006).

2.6. Statistical analysis

Statistical analysis of RER values was performed with GraphPad Prism software using the unpaired two-tailed *t*-test function (GraphPad Software, Inc).

3. Results

3.1. Extraction of total RNA from D. dadantii

To purify quality RNA from *D. dadantii*, we evaluated several RNA isolation methods in combination with several DNA decontamination methods for RNA purity, quality and quantity. A number of laboratory methods have been reported for the isolation of RNA from both Gram-positive and Gram-negative bacteria but they are time-consuming, laborious and some are costly (Bashyam and Tyagi, 1994; Bernstein et al., 2002; Glisin et al., 1974; Sung et al., 2003; Uyttendaele et al., 1996). For this reason, commercially available kits were initially chosen to isolate *D. dadantii* RNA. We first examined a Trizol (Invitrogen)/RNeasy (Qiagen) hybrid protocol (Wong et al., 2004). This method begins with Trizol homogenization and extraction, after the aqueous phase (containing total RNA) is combined with 70% EtOH, it is then loaded onto an RNeasy column. Purification proceeds as described in Qiagen protocols with the optional steps of DNase treatment on the column. This method has the advantage of being simple and relatively short. It also limits handling of the RNA, perhaps reducing degradation. Yields with this method were low, probably due to poor lysis of bacterial cells before extraction (Table 2). An increase in yield was achieved with enzymatic (either a proteinase k or lysozyme)/hot SDS lysis step inserted before proceeding through the hybrid Trizol/RNeasy method. Although we saw an increase in yield, the RNA quality was poor and a RIN above 5.0 was never achieved (Table 2). A final method that was not kit based gave both high quantity and quality RNA from *D. dadantii*. This method included an enzymatic hot SDS lysis step followed by an acidic phenol extraction. This method had yields that averaged 961 ng/µl with an average RIN of 7.0 (Table 2).

Table 2
Comparison of methods for RNA isolation from *Dickeya dadantii*

Method	Yield (ng/µl)	Quality	Number of DNase treatments
Trizol/RNeasy	40–100	Not tested	5[a]
Hot SDS lysis/Trizol/RNeasy	400–700	RIN 2.7–4.1	5[a]
Hot SDS/hot phenol	600–1200	RIN 4.9–8.4	2[b]

[a] Two on-column DNase treatments with the RNeasy kit followed by three treatments with Turbo DNA-*free* using the rigorous protocol.
[b] Two treatments with Turbo DNA-*free* using the rigorous protocol.

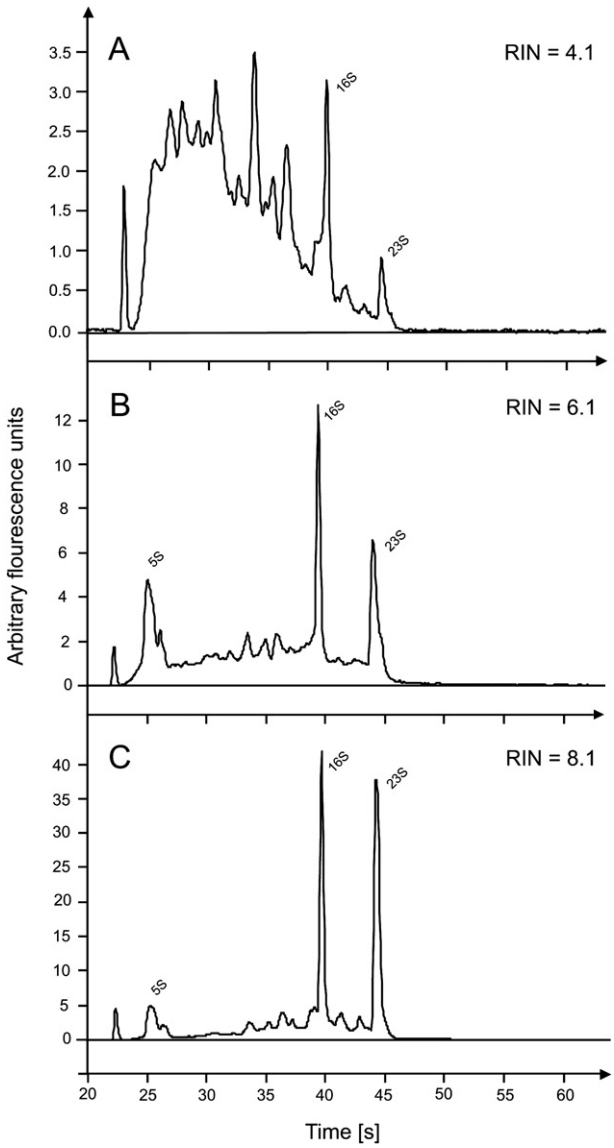

Fig. 1. Typical electropherograms of total RNA extractions using a Trizol/RNeasy or hot SDS/hot phenol. Total RNA was analyzed using an Agilent 2100 Bioanalyzer. (A) RNA obtained using the Trizol/RNeasy method was degraded (RIN=4.1) as indicated by a shift to smaller fragment sizes and a decrease in fluorescence signal. (B) RNA of intermediate quality isolated using the hot SDS/hot phenol method. (C) High quality RNA (RIN=8.1) obtained using the hot SDS/hot phenol method shown by the clearly visible 23/16S rRNA peaks and a small 5S RNA peak. The first peak in each sample corresponds to the Agilent RNA 6000 Nano Marker and the main ribosomal RNA peaks are indicated.

DNA decontamination remained a problem with all methods (Table 2). In some cases RNA samples were treated as many as 5 times with DNase, yet DNA was still present at levels too high for use of the RNA in qRT-PCR experiments (data not shown). With the hot SDS/hot phenol method, the DNase steps could be reduced to only two rigorous treatments with Turbo DNA-*free* (Table 2).

3.2. Selection of suitable reference transcripts for real-time qRT-PCR

Five potential reference gene transcripts based on *D. dadantii* sequences were evaluated for transcript stability under experimental conditions using the *BestKeeper* program (Pfaffl et al., 2004). *BestKeeper* is an Excel-based tool designed to determine the correlation between the raw values of real-time PCR for a particular internal reference gene of interest and the geometric mean (the *BestKeeper Index*) of all of the reference genes tested under various treatments. Ultimately, a strong and significant correlation (*r*-value) between the index and the reference RNA candidate determines its stability. The *BestKeeper Index* values were determined from a data set consisting of C_t values of potential reference transcripts from both wild-type *D. dadantii* and the *fliA* mutant for a total of six RNA samples. Four of the five transcripts were judged to be suitable reference RNAs under our experimental condition: 50S ribosomal protein (*rplU*), DNA gyrase (*gyrA*), the alternative sigma factor σ^S (σ^{38} or *rpoS*), and outer membrane protein A (*ompA*) (Jahn et al., in press). The *rplU*, *gyrA*, and *rpoS* transcripts were deemed very stable and demonstrated a high degree of correlation to the *BestKeeper Index* ($0.986 > r > 0.926$). We used these three transcripts as reference RNA for the subsequent determination of RER. The *rpoD* gene transcript of *D. dadantii* was identified as too variable for use under our experimental conditions.

3.3. RNA quality affect on real-time qRT-PCR normalization

Intact RNA is thought to be essential for many molecular techniques used in gene expression studies, although there is little experimental data to verify this assertion. We analyzed RNA preparations from various extraction methods using the Agilent 2100 Bioanalyzer RNA (Fig. 1). Intact RNA had high 16S and 23S rRNA peaks as well as a small amount of 5S RNA (Fig. 1C). RNA degradation shifted the RNA size distribution towards smaller fragments and a decrease in fluorescence was also seen (Fig. 1A). RNA degradation can be incomplete and was seen as a decrease of the ribosomal 23S/16S signal with an increase in the baseline signal between the two ribosomal peaks and the 5S peak (Fig. 1B).

Little is known about obtaining meaningful real-time qRT-PCR results from RNA with impaired quality. In previous work, intact RNA samples compared to partially degraded RNA samples that still had visible ribosomal bands exhibited a high degree of similarity (Schoor et al., 2003). Using *BestKeeper*, we compared two internal reference transcripts (*rplU* and *gyrA*) to determine the influence of RNA quality on experimental sample-to-sample variation (Table 3). The standard deviation (SD) of the C_t and the Pearson correlation coefficient to the *BestKeeper Index* were then calculated. A transcript showing a SD exceeding 1.0 is considered as too variable to be used as a reference by the *BestKeeper* software. For the hot SDS lysis Trizol/RNeasy hybrid method (typical quality seen in Fig. 1A), the standard deviation (SD) in the C_t from multiple samples was significantly greater than 1.0 (Table 3). We next investigated RNA isolated using the hot SDS/hot phenol method that had RIN values above 5.0 but below 7.0. The profile of this RNA gave ribosomal bands that were still visible and seemingly distinct (Fig. 1B). This RNA gave SD values that still exceeded 1.0 (Table 3). Only with hot SDS/hot phenol isolated RNA that had RIN values greater then 7.0 (Fig. 1C) were the standard deviations in the range that allowed for meaningful analysis of expression data (Table 3). Using RNA with a RIN

Table 3
BestKeeper comparison of internal reference genes from RNA isolated with different methods and different RIN scores

Extraction method	SD[a] *rplU*	SD[a] *gyrA*	*pelX*		*avrL*	
			RER[b]	p-value[c]	RER[b]	p-value[c]
SDS lysis/Trizol/RNeasy	4.59	3.83	ND[d]	ND	ND	ND
Hot SDS/hot phenol (RIN <7)	1.53	1.73	0.2	0.3529	10.9	0.3143
Hot SDS/hot phenol (RIN >7)	0.67	0.80	2.4	0.0011[e]	2.2	0.0086[e]

[a] SD — standard deviation of C_t values using the reference gene primer sets indicated as determined by the *BestKeeper* software package.
[b] RER — relative expression ratio of the target transcript in the *fliA* mutant using the target transcript abundance in wild-type *Dickeya dadantii* as a calibrator.
[c] Determine by two-tailed *t*-test with a 95% confidence interval using Prism 5.0a software (GraphPad Software, Inc.).
[d] ND — not determined.
[e] A *p*-value <0.05 indicates that the mean RER of the target transcript in the *fliA* mutant was significantly different from the target gene mean RER in wild-type.

Table 4
Long term storage of hot SDS/hot phenol *Dickeya dadantii* RNA samples

Strain[a]	RIN		
	Initial	1 month	5 months
Nx3937 (replicate 1)	7.2	7.3	7.5
Nx3937 (replicate 2)	7.1	7.1	7.1
Nx3937 (replicate 3)	7.3	7.4	7.6
WPP381 (*fliA*::Km) (replicate 1)	7.7	7.5	7.8
WPP381 (*fliA*::Km) (replicate 2)	7.4	7.3	2.6
WPP381 (*fliA*::Km) (replicate 3)	7.2	7.0	7.1

[a] RNA samples were thawed and refrozen a total of eight times over five months.

of >7, we observed an increase in the mean mRNA RER of the periplasmic *exo*-pectate lyase *pelX gene* (Shevchik et al., 1999) (2.4-fold, *p*-value=0.0011) and a gene encoding a T2SS substrate of unknown function, *avrL* (Kazemi-Pour et al., 2004) (2.2-fold, *p*-value=0.0086) (Table 3). This indicates that these two transcripts are increased more than 2-fold in our *fliA* mutant compared to wild-type. This relatively small increase is statistically significant by two-tailed *t*-test with a 95% confidence interval. However, when we used total RNA with a RIN <7 for this analysis, the mean RER was dramatically different (0.29 for the *pelX* transcript and 10.89 for the *avrL RNA*). More importantly, these differences, including the apparent 10-fold increase in *avrL* expression, were not statistically significant with *p*-values of 0.3529 and 0.3143, respectively (Table 3).

3.4. Stability of total RNA in long term storage

A rapid decline was not seen in total RNA quality or quantity after isolation with hot SDS/hot phenol extraction methods after extended RNA storage at −80 °C. RNA samples were thawed, then refrozen seven times over the course of one month as real-time qRT-PCR experiments were completed. The RNA samples were then stored for an additional four months, then thawed once more for RIN analysis. After a total of five months, the RIN of all of the samples, except one, was unchanged (Table 4). One replicate had a significant reduction in RIN only after five months (Table 4).

4. Discussion

The current study demonstrates that isolation of *D. dadantii* RNA requires a lysis step and that high quantity and quality RNA can be obtained using a hot SDS/hot phenol extraction method. This method also drastically reduces the amount of DNA contamination, which results in fewer DNase treatments and reduced RNA handling. Our work is part of mounting evidence that suggests that RNA quality must first be assessed before downstream applications are pursued. Also, an extended storage period at −80 °C does not affect the yield or quality of RNA that was extracted with this method.

Methods for isolating intact RNA from bacteria are problematic. They often include lengthy procedures such as ultra centrifugation, toxic or expensive chemicals, and chemicals such as SDS and phenol in order to inhibit RNases (Bernstein et al., 2002; Glisin et al., 1974; van Keulen et al., 2004). Many commercially available kits that decrease time and cost have also been investigated with varying results (Phongsisay et al., 2007). Previous descriptions of rapid cell lysis using heat and detergents followed by repeated organic extractions with acid phenol have also been described (Majumdar et al., 1991; Sambrook and Russell, 2001). These methods contain longer incubation times, more complex buffers and do not contain steps such as heat treatment or treatment with reducing agents that may be important for organisms with high nuclease activity (Chang and Gallie, 1997). The addition of proteinase K, which is used to degrade RNases, did not enhance the quantity or quality of RNA in the method described in this protocol (data not shown). We found that a bacterial lysis step was required for all methods to achieve larger quantities of RNA and that high quality RNA was only achieved with the hot SDS/hot phenol method. As a control prior to expression profiling, we attempted to PCR-amplify DNA to determine if detectable levels of contaminating DNA were present in the RNA samples. We used primers for *rplU* (the 50S rRNA subunit) in order to detect chromosomal DNA. A total of five DNase treatments were required for RNA samples isolated with the Trizol/RNeasy hybrid method to eliminate DNA contamination. Even with this rigorous treatment, the occasional sample still had detectable genomic DNA contamination. Only the hot SDS/hot phenol method resulted in RNA samples that lacked DNA contamination. DNA contamination may be genus or even strain specific since DNA-*free* RNA can be isolated from *Pectobacterium carotovorum*, a plant pathogen that is closely related to *Dickeya*, with the Trizol/RNeasy hybrid method plus only two treatments of Turbo DNA-*free* without the RNeasy on column DNase clean up step (H.-S. Kim, personal communication). Similar challenges with DNA contamination have been reported with other bacteria. For example, most RNA isolation methods tested for isolation of RNA from seven different strains of *Campylobacter jejuni*, a Gram-negative human pathogen, resulted in RNA samples contaminated with genomic DNA (Phongsisay et al., 2007).

Several methods have been used to determine RNA integrity. One of them is the 23S/16S ratio, which is calculated using the shape of the electropherogram generated from micro-fluidic capillary electrophoresis (Mueller et al., 2000). Several authors have now suggested that this measure should not be used as the standard for assessing RNA integrity because of its high variability (Fleige and Pfaffl, 2006; Imbeaud et al., 2005; Schroeder et al., 2006). In work investigating mRNA quantification models, Fleige et al. (2006) found that there was not a significant correlation between the rRNA ratio and real-time qRT-PCR performance. There was, on the other hand, a significant correlation between RIN values and real-time qRT-PCR results for all samples tested. These results lead the authors to suggest that samples with RIN values higher then 5.0 were appropriate for real-time qRT-PCR. We found that RNA below an average RIN value of approximately 7.0 yielded high variation in reference transcript C_t values when the real-time qRT-PCR data were analyzed by *BestKeeper*.

BestKeeper provides several pieces of data in addition to the standard deviation of the C_t. The program also calculates pair-wise Parson's correlation coefficient for reference genes and calculates a *BestKeeper Index*, which is derived from the geometric means of all the samples. When real-time qRT-PCR data were analyzed for the Trizol/RNeasy samples and the hot SDS/hot phenol samples with RIN values below 7.0, the correlation coefficient of each reference gene in all cases was >0.900 (data not shown), however, the SD of the sample C_t values was greater than 1.0. This indicates that although the reference genes are behaving the same, even in the most degraded RNA samples, the sample-to-sample variation is too great for reliable information on gene expression to be obtained. When samples isolated with the hot SDS/hot phenol (Schoor et al., 2003) protocol that have with RIN values above 7.0 are analyzed, there is a SD of 0.67 (*rplU*) and 0.80 (*gyrA*) (Table 3) with a correlation coefficient to the *BestKeeper Index* of 0.986 (*rplU*) and 0.984 (*gyrA*) and a *p*-value of 0.001 (data not shown), demonstrating that data from these RNA samples were reliable.

Historically, housekeeping genes have been assumed to be constant, minimally regulated, and have been widely used as internal RNA references for RT-PCR analyses. They remain widely used as endogenous controls for real-time qRT-PCR assays, usually without any real investigation as to how invariant their mRNA levels actually are under the experimental conditions being investigated (Bustin and Nolan, 2004a). Without appropriate normalization, the expression profile of a target gene may be misinterpreted so it is desirable to choose more than one internal reference gene transcript (Hamalainen et al., 2001; Tricarico et al., 2002). Under our experimental conditions,

we determined that *rplU*, *gyrA*, *rpoS* and *ompA* are suitable reference transcripts whereas *rpoD* was too variable to use as a reference RNA.

Performing RNA quality assessment prior to downstream application is especially important when quantifying small expression differences (Bustin and Nolan, 2004a; Perez-Novo et al., 2005). In *D. dadantii*, two genes encoding proteins involved in pectate catabolism and one gene encoding a secreted protein are up-regulated in a *fliA* mutant in comparison to wild-type cells (Jahn et al., in press). The RER for *pelX* with RNA RIN values above 7.0 was 2.4 with a *p*-value of 0.0011 and *avrL* had an RER of 2.2 with a *p*-value of 0.0086 (Table 3). With RNA that exhibited an average RIN score lower than 7.0, the RER of *pelX* was 0.23 ($p=0.3529$) and the RER of *avrL* was 10.89 ($p=0.3143$) (Table 3). With both gene transcripts, the lower quality RNA did not show a statistically significant difference in RER compared to wild-type even though the difference in means was greater than 8-fold. This illustrates the need for using high quality RNA for the reliable quantification of gene expression levels.

DNA is exceptionally stable and can be isolated from ancient environmental samples (Nicholls, 2005). It is well understood that RNA is sensitive to degradation with inadequate sample handling or storage (Perez-Novo et al., 2005). The quality and quantity of purified RNA is also variable and after extraction RNA is thought to be unstable during long term storage (Bustin et al., 2005). It is common practice to store RNA at −80 °C to help prevent degradation and some protocols indicate that whole samples can be stored without processing if first flash frozen. In our experience, RNA isolated from bacterial samples that were flash frozen and stored prior to RNA isolation had RIN indicating that the samples were unsuitable for real-time qRT-PCR (data not shown). From work of others, we know that variance in real-time qRT-PCR results is more strongly correlated to RNA extraction method than to storage method (Kim et al., 2007). We found that bacterial RNA extracted with hot SDS/hot phenol was stable for several months when stored at −80 °C, even with repeated cycles of freezing and thawing (Table 4).

Although it is time-consuming and expensive, extraction protocols and reference genes must be empirically assessed and even when methods are taken from closely related organisms, they may be problematic in the organism under study. The results of this study indicate that reproducible transcript profiling requires an investigation into RNA integrity before use and that RIN values acceptable in some biological systems may not work well in all biological systems.

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