Quantification of cDNA generated by reverse transcription of total RNA provides a simple alternative tool for quantitative RT-PCR normalization

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Quantitative reverse transcription PCR (RT-PCR) is typically used to assay transcript abundance (often generalized as "gene expression") by measuring a specific cDNA level. The method is very sensitive and is suitable for a broad range of cDNA concentrations. Its reliability depends on, among other factors, appropriate normalization (for a review, see References 1 and 2).

The preferred method of quantitative RT-PCR normalization uses housekeeping genes with presumably invariant levels of expression as internal controls. Housekeeping gene-based normalization corrects the measured transcript levels for variable starting RNA amounts and for differences in RT efficiency. However, as there are no universally applicable genes with invariant expression, it is necessary to carefully evaluate the expression of candidate reference genes for every particular experimental system. Normalization with suboptimal housekeeping genes may result in different estimated values and lead to erroneous interpretations (3). To avoid a bias caused by the expression fluctuation of a single reference gene, Vandesompele et al. (4) proposed computation of the correction factor from several internal controls. However, this approach may increase the cost and laboriousness of experiments significantly. Another approach derives the correction factor for each sample from the input RNA amount, based either on spectrophotometric (A_{260}), or on fluorometric estimation (5), meaning that there is no need to select a proper reference gene and verify its expression. However, this method relies upon the reproducibility of the RT reaction, which has been shown to be a major source of quantitative RT-PCR variation (6). Several other normalization strategies have been reviewed recently (7), none of them being used frequently.

In this paper, we present a method of quantitative PCR normalization using the total amount of cDNA generated during RT. Our approach controls for the amount of starting material and variation of transcription efficiencies across RT reactions.

Volkov et al. used PicoGreen® for quantifying RNA-DNA hybrid molecules after RT with poly(A)+ RNA added as a template (8). PicoGreen is a fluorescent dye that preferentially binds to double-stranded nucleic acids—the fluorescence of RNA-PicoGreen complexes reaching up to 10% of double-stranded DNA (dsDNA)-

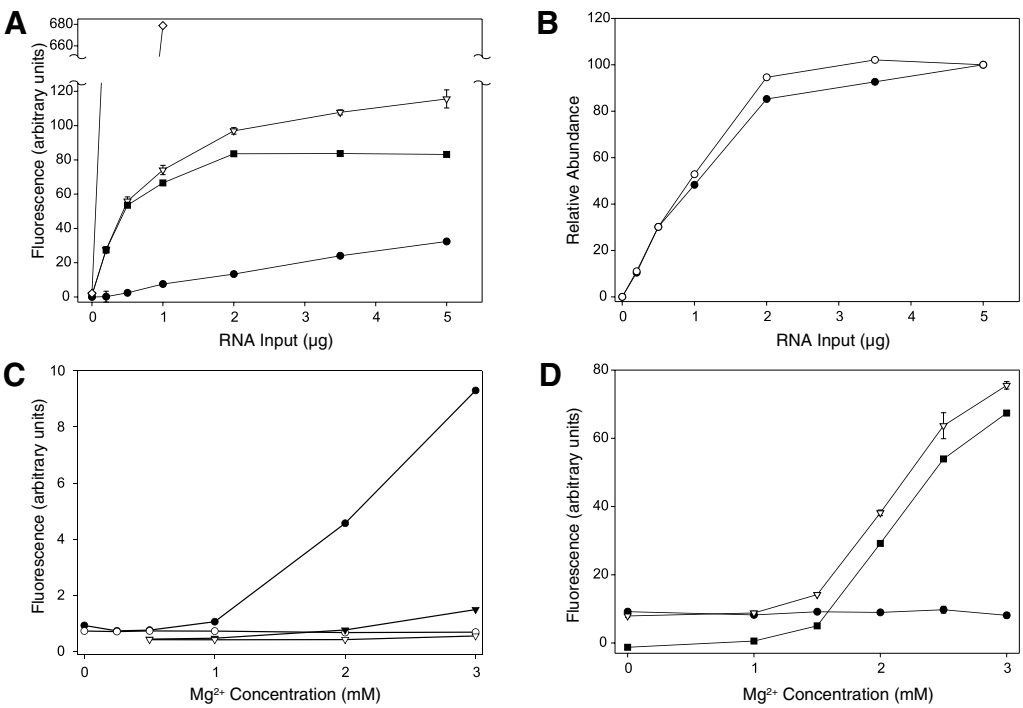

Figure 1. RiboGreen fluorescence as a function of first strand cDNA yield. (A) RiboGreen fluorescence of hydrolyzed samples after reverse transcription (RT;▽), background before RT (●), and the difference between these (■) from reactions at various RNA input. "Mock-hydrolyzed" RT samples in which NaOH was neutralized before adding to the sample (◇) illustrate the efficiency of RNA removal. (B) cDNA survives alkali treatment—relative MPK3 cDNA abundance in the hydrolyzed (●) and mock-hydrolyzed (○) samples from panel A. (C) RiboGreen and PicoGreen staining of identical samples—RiboGreen fluorescence of RT (●) and background (○); PicoGreen fluorescence of RT (▼) and background (▽). (D) RiboGreen fluorescence of reaction mixtures at various Mg^{2+} concentrations—legend is the same as in panel A. In panels A and D, three aliquots of a single RT reaction were independently hydrolyzed, neutralized, and measured; error bars denote the standard deviation.

Protocol 1. Estimation of Reverse Transcription Efficiency by Direct Measurement of cDNA Yield.

Reverse Transcription (RT)

1. Prepare 20 µL RT reaction mixture on ice, with reverse transcriptase added as the last component.
2. Immediately remove 2–5 µL for background measurement. Add EDTA and NaOH to final concentration of 1 mM and 100 mM, respectively, in 20 µL volume. (for example: add 5 µL RT mixture to 15 µL 1.33 mM EDTA and 133 mM NaOH).
3. Complete the RT procedure with the remaining reaction mixture.
4. Take another 2–5 µL and add EDTA and NaOH as in step 2.

Alkaline Hydrolysis of RNA and Generation of Correction Factor

5. Incubate samples resulting from steps 2 and 4 for 20 min at 70°C. Note: the background sample may either be hydrolyzed by performing this heat treatment step immediately after adding the NaOH/EDTA [in which case the sample is left to sit after neutralization (step 6) until the post-RT sample is ready for measurement], or the background sample can be left after addition of NaOH/EDTA (step 2) until
6. the post-RT sample is ready for hydrolysis by heat treatment.
7. Neutralize by adding 6 µL 0.5 M Tris-HCl, pH 6.4. Acidic Tris buffer provides more reproducible neutralization than HCl.
   Mix 5.2 µL neutralized sample (corresponding to 0.4–1 µL RT mixture), 0.2 µL RiboGreen quantitation reagent, and 34.6 µL H_2O (final RiboGreen dilution: 200-fold). We recommend preparing the diluted dye for all samples as a single solution.
8. Incubate samples for 15 min at room temperature and measure fluorescence at 500/520 nm excitation/emission wavelength or at fluorescein/SYBR Green I channel. Although real-time PCR instruments could be used to measure fluorescence, we achieved much more accurate results using a fluorometer (Cary Eclipse; Varian, Palo Alto, CA, USA).
9. Calculate the correction factor for RT-PCR normalization by subtracting the fluorescence of step 2 samples (background) from the fluorescence of step 4 samples (post-RT).
10. Divide transcript abundance calculated from quantitative PCR data by the correction factor of the corresponding sample.

PicoGreen fluorescence at the same concentration (9). However, their method cannot be applied to cDNA reverse transcribed from total RNA. Ribosomal RNA (rRNA), predominant in total RNA, contains many double-stranded motifs that bind PicoGreen, and this RNA-borne signal outweighs the cDNA-associated fluorescence. Because total RNA is employed in quantitative RT-PCR assays much more frequently than poly(A)$^+$ RNA, we have developed a fluorometric method capable of measuring cDNA generated by RT with total RNA. To achieve this we eliminate RNA after performing RT and then estimate fluorescence of single-stranded cDNA using RiboGreen® dye.

Total RNA was isolated from *Arabidopsis thaliana* suspension culture (10). It was treated with 0.1 U RNase-free DNase I (Roche Applied Science, Mannheim, Germany) per µg RNA in 20 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1 mM $MgCl_2$ for 30 min at room temperature. The enzyme was then inactivated by 10 min at 65°C after adding EDTA to a final concentration 1 mM. For the RT reaction, Moloney murine leukemia virus (MoMLV) RNaseH$^-$ point mutant (Promega, Madison, WI, USA) or Transcriptor (Roche Applied Science; the obtained data were similar) with random hexanucleotides (dN_6) or anchored oligo(dT) primer ($dT_{23}dV$) were used according to the manufacturer's instructions.

A step-by-step procedure for determining the RT efficiency correction factor is provided (see accompanying Protocol 1). Removing RNA from the RT reaction mixture prior to quantitation by RiboGreen is a critical step in this method. Using RNaseA (Qiagen, Valencia, CA, USA) resulted in incomplete hydrolysis, even after increasing the reaction temperature to 62°C to reduce secondary structures (11). In addition, a component of the reaction partially inhibited PCR. As an alternative, we employed alkaline hydrolysis of RNA using 0.1 M NaOH for 20 min at 70°C. The RNA-RiboGreen fluorescence dropped about 100-fold (Figure 1A), and neither higher hydroxide concentration nor increased temperature or extended time lowered the signal further (data not shown). The cDNA was not affected as assayed by real-time PCR with primers specific for the gene MPK3, using a DNA Master Kit Plus and LightCycler® instrument (both from Roche Applied Science) in combination with polycarbonate capillaries (Genaxxon, Biberach, Baden-Württemberg, Germany) (see Figure 1B).

Our motivation for using RiboGreen instead of PicoGreen is that, after the alkaline hydrolysis, we expected to obtain single-stranded first-strand cDNA. Since this cannot be measured with PicoGreen, we used RiboGreen, which is capable of forming a fluorescent complex with any nucleic acid (5). To confirm our expectation, we compared the fluorescence of RNA samples hydrolyzed before and after RT using a PicoGreen dsDNA Quantitation Kit and a RiboGreen RNA Quantitation Kit (Molecular Probes™; Invitrogen, Carlsbad, CA, USA). As shown in Figure 1C, only RiboGreen fluorescence significantly increased after RT, which suggests that the hydrolysis-resistant DNA is in fact single-stranded. The PicoGreen/RiboGreen comparison was performed with a range of RT reactions containing different concentrations of Mg^{2+}; since RT requires Mg^{2+} as a cofactor, as the Mg^{2+} concentration decreases (and thus RT efficiency declines), the RiboGreen signal is also expectedly reduced (see Figure 1C).

Figure 1A shows RiboGreen signal as measured with a range of different cDNA yields. As might be expected, the amounts of cDNA produced initially increase with the amount of RNA input, then level out as the reaction becomes saturated. In the experiment shown in Figure 1D, RNA input was held constant (1 µg), and we decreased the free magnesium ion concentration by adding various amounts of EDTA. The consequent reduction in RT efficiency is mirrored by lower RiboGreen fluorescence readings.

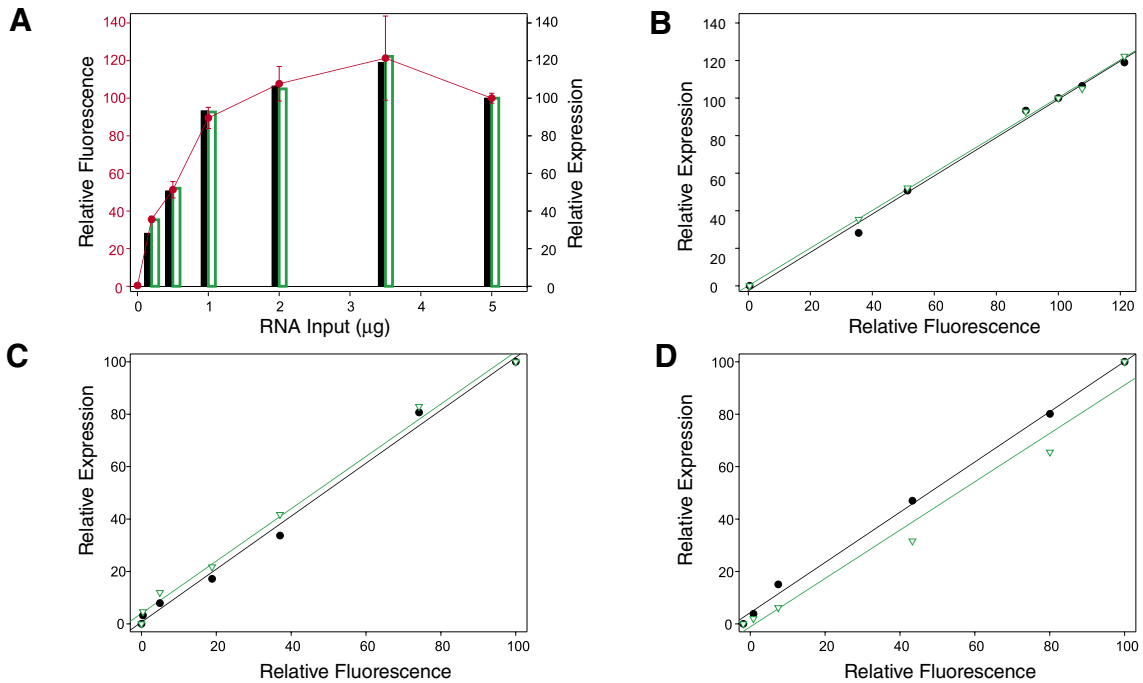

Figure 2. Correspondence of RiboGreen fluorescence to the specific cDNA abundance. (A) Relative abundance of MPK3 (black bars) and UBQ10 (green open bars) compared with relative RiboGreen fluorescence (red circles and line) in dN_6 primed RTs with a range of RNA inputs. The values of 5 µg sample were set to 100. For RiboGreen fluorescence readings, three aliquots of a single RT reaction were independently hydrolyzed, neutralized, and measured; errors bars represent standard deviations. (B) Correlation plot of the data shown in (A); ● = MPK3 and ▽ = UBQ10. (C) Correlation of specific cDNA amount with RiboGreen fluorescence in $dT_{23}dV$ primed RT with a range of RNA inputs. (D) Correlation of specific cDNA amount with RiboGreen fluorescence in dN_6 primed RT with a range of free Mg^{2+} concentration (data from Figure 1D). The values of 3 mM Mg^{2+} sample were set to 100. RT, reverse transcription.

To demonstrate the utility of the proposed method in quantitative RT-PCR, we used primers specific for genes MPK3 (At3g45640, 5′-GAGGATGCGAAAAGATACA-3′ and 5′-CGAACTCAAAAGAGAATGG-3′) and UBQ10 (At4g05320, 5′-GGCCT TGTATAATCCCTGATGAATAAG-3′ and 5′-AAAGAGATAACAGGAACG GAAACATAGT-3′) (12). In calculating the cDNA abundance from the real-time PCR data, we first determined the PCR efficiency from standard curves plotted from cDNA dilutions (values were 0.96 and 1.01 for MPK3 and UBQ10, respectively). We then calculated the relative cDNA abundance of a given sample from its crossing point (Ct) as follows:

$$RA_{SampleX} = 100 \times E_{PCR}^{(Ct_{Standard} - Ct_{SampleX})}$$

where *RA* is the relative cDNA abundance and E_{PCR} is the PCR efficiency.

For comparisons of samples in which RNA input differed, we expressed all values relative to the cDNA abundance measured in the quantitative RT-PCR based on 5 µg input RNA; in the reactions involving different magnesium concentrations, we expressed all values relative to the cDNA abundance measured in the quantitative PCR using the 3 mM Mg^{2+} RT reaction. We then compared the relative percent abundances as measured by quantitative PCR to the relative fluorescence measured by RiboGreen. For different RNA input amounts in RT reactions primed by the dN_6 primer, the relative measures are shown in Figure 2A and the correlation is presented in Figure 2B. Figure 2C shows that the degree of correspondence is equally high in experiments in which RT was performed using the $dT_{23}dV$ primer. Similarly, Figure 2D demonstrates a high level of correspondence between relative fluorescence and relative abundance in reactions performed using dN_6-primed RT with various Mg^{2+} concentrations. The one-to-one correspondence that is observed in these experiments confirms the suitability of the RiboGreen fluorescence reading as a correction factor: if these samples were normalized according to the RiboGreen readings, the resulting abundance values would vary only slightly. They would thereby confirm that MPK3 and UBQ10 expression levels do not fluctuate under the conditions of the experiment (they only appear to differ because of the differences in global RNA input or RT efficiency; this is not surprising as we always used the same RNA stock). It should be noted that some data points correspond to conditions in which the RT reaction is saturated. Saturation occurs if the RNA-to-enzyme activity ratio is too high, due to either excess RNA (e.g., inputs ≥2 µg RNA/50 U MoMLV, dN_6 primed) or RT inhibition (EDTA chelation of Mg^{2+}). Under saturation, normalization to RNA input becomes misleading; however, the fluorescence intensity attributable to cDNA yield correlates with levels of specific cDNA estimated by quantitative PCR whether or not the reaction is saturated.

The method of direct measurement of cDNA amount described in this paper makes possible an independent verification of invariant expression of candidate reference genes. Alternatively, it offers an easy way to normalize messenger RNA (mRNA)

expression levels in the absence of suitable housekeeping genes. As the RiboGreen nucleic acid detection is very sensitive (5) low yield RT reactions may be measured.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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