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Notes & Tips

DNA quantification using EvaGreen and a real-time PCR instrument

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DNA quantification is an important, frequently used technique, and inaccuracies can result in failures with ligation, restriction, polymerase chain reaction (PCR),[1] amplified fragment length polymorphism (AFLP), Southern blotting, and other techniques. DNA is most commonly quantified using absorbance at 260 nm, but because of the existence of many impurities, this can be an imprecise measurement and DNA levels can be more than 10 times overestimated in some cases [1]. Quantification by agarose gel electrophoresis with a known amount of standard DNA [1,2] can provide more accurate data, but the procedures are complicated, the data often still are not accurate enough, and the technique is impractical for routine or high-throughput DNA quantification [3]. Fluorescence spectroscopy using various DNA intercalating dyes is the most widely accepted technique for accurate DNA quantification [4]. However, if the analysis is to be carried out with a fluorescence spectrophotometer, a relatively large assay volume (e.g., 2 ml) is required [5], and this is impractical for small DNA samples and expensive dyes. Fluorescence can also be measured with a smaller volume of DNA sample using other instruments such as fluorescent microplate readers [6], microplate fluorometers [7,8], and transilluminator–microplate–CCD camera systems [9], but the instruments might not be readily available in most molecular biology laboratories.

Real-time PCR instruments are common in molecular biology laboratories and can be used to measure fluorescence. DNA intercalated with a dye emits fluorescence at 520 nm under excitation at 490 nm, and with real-time PCR instrumentation the assay is carried out in PCR tubes, equivalent to spectrophotometer cuvettes used with a fluorescence spectrophotometer. In the former case, the assay volume can be reduced greatly. Here we present a method for quantifying DNA using a real-time PCR instrument without PCR reactions and using EvaGreen, a novel DNA-intercalating dye that is more stable and sensitive than SYBR Green I (according to the manufacturer's product and safety data sheet for EvaGreen).

EvaGreen (20× concentrate) was obtained from Biotium (Hayward, CA, USA), and λDNA and pUC18 DNA were obtained from TaKaRa (Dalian, China). Fluorescence measurements were completed using a Bio-Rad iCycler iQ real-time PCR instrument, with the assay temperature kept at 25 °C and the assay volume kept at 25 μl (containing 1.25 μl of 20× concentrate EvaGreen).

Although the temperature needed for the assay was 25 °C, the default running program of the software for the iCycler iQ real-time PCR instrument includes unchangeable heating steps to 95 °C and then cooling to 60 °C and maintenance at this temperature for 2 min to collect the well factor; this is necessary to compensate for any system or pipetting nonuniformity. Because DNA can be denatured at 95 °C and the annealing process is slow and incomplete, the tubes for collecting the well factor need to be extra tubes (we used extra blank tubes with EvaGreen but without DNA) rather than the experimental tubes themselves. When the temperature reached 25 °C, the well factor tubes were replaced by experimental tubes, including blank ones, and the intensity of fluorescence was measured. The user-defined part of the running program set for the assay was 100 cycles of 25 °C for 5 s, 25 °C for 1 s, and 25 °C for 10 s. Although we used 100 cycles, more cycles could be used if necessary. The program was paused during the first 5 s at 25 °C to replace PCR tubes, and fluorescence was recorded during the final 10 s at 25 °C of each cycle.

We found that the intensity of fluorescence (relative fluorescence units) was proportional to the total amount rather than the concentration of DNA in the tube. For

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[1] *Abbreviations used*: PCR, polymerase chain reaction; AFLP, amplified fragment length polymorphism.

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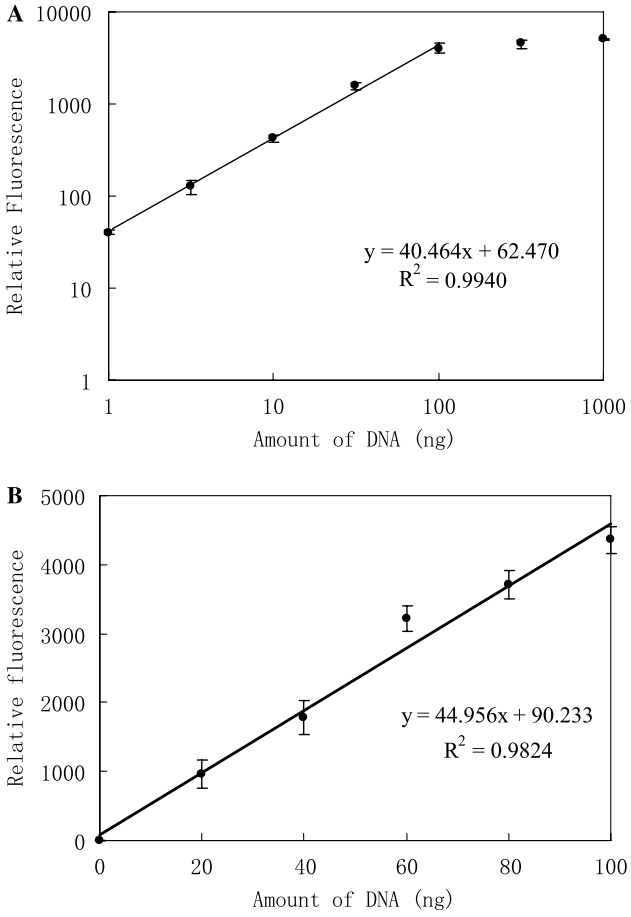

Fig. 1. Relationships between fluorescence intensity and the amount of DNA per tube. Triplicate samples of λDNA in the range of 1–1000 ng (A) or 0–100 ng, (B) were added to PCR tubes containing 1.25 μl of EvaGreen (20× concentrate), and water was added to make a final volume of 25 μl. The fluorescence was recorded at 520 nm under excitation at 490 nm.

example, 40 μl of the assay mixture containing 40 ng of DNA produced twice the fluorescence intensity of 20 μl of an assay mixture containing 20 ng of DNA, although the DNA concentration was the same. Therefore, relationships between the intensity of fluorescence and the amount of DNA per tube rather than the concentration of DNA were analyzed.

To identify the linear part of the DNA curve for the protocol, a range of 1–1000 ng of λDNA was used. A strong linear relationship (data from three replicates) was observed when the amount of DNA was less than 100 ng (Fig. 1A), and this was very reproducible (Fig. 1B). As little as 0.1 ng DNA per tube could be detected by this protocol; however, quantification of DNA less than 1 ng is not recommended because linearity in this region was not reliable.

The linearity of the DNA curve at amounts up to 100 ng was repeated in more than 10 independent experiments. The slope of the curve in these experiments averaged approximately 40 relative fluorescence units per nanogram DNA, with up to 20% variation. When we used a different stock DNA (pUC18 DNA), the relationship between fluorescence and DNA amount was consistently linear, with an average slope of the curve within the range quoted above (data not shown). The protocol has been applied successfully in quantifying DNA from young citrus leaves extracted according to Kim and coworkers [10], except that the temperature for extraction was 65 °C rather than room temperature. The data obtained were very similar (less than one-fold difference) to those obtained from quantification by agarose gel electrophoresis [1]. Quantification of DNA in crude cell or organelle extracts by this method has not been tested, and interference from ssDNA, RNA, and other unknown cell components needs to be evaluated.

The protocol described here requires only small amounts of DNA and dye; therefore, it is especially useful when small DNA samples and expensive dyes are involved. The analysis can be completed in a short time, and for a large number of samples the PCR tubes can be replaced by a PCR plate to further increase the analytical efficiency. In addition, the protocol was found to be suitable for quantification of RNA. A good linear relationship was observed with up to 300 ng of RNA (RNA Marker RL6000, TaKaRa) per tube when EvaGreen was replaced by SYBR Green II (diluted 10,000 times in the final assay mixture, Sigma, St. Louis, MO, USA).

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