

## Quantitative assay of total dsDNA with PicoGreen reagent and real-time fluorescent detection

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**Summary.** - We describe a quantitative assay of dsDNA based on real-time PCR measurement of fluorescence due to the interaction of PicoGreen dye with dsDNA. An aliquot of 1 to 5  $\mu$ l of the sample is mixed with 45  $\mu$ l of diluted PicoGreen reagent within an optical PCR tube. This is placed into the real-time apparatus set to read SYBR Green I dye at the end of three cycles of 94 °C for 30 s and 65 °C for 30 s. The averaged fluorescence value is converted into DNA amount using a calibration curve prepared with  $\lambda$ -DNA standard. The calibration curve has a dynamic linear range from 0.20 to 50 ng and a standard deviation variability below 5.0%. In conclusion, this method allows reliable determinations on minimal amounts of DNA from biological samples and PCR products in clinical applications of molecular biology.

*Key words:* real-time PCR, DNA quantitation, fluorescent dyes.

**Riassunto** (*Analisi quantitativa del DNA a doppio filamento con il reattivo fluorescente PicoGreen e apparecchiatura per real-time PCR*). - Viene descritta l'analisi quantitativa del DNA a doppio filamento con l'utilizzazione della *real-time* PCR per rivelare la fluorescenza prodotta dall'interazione del colorante PicoGreen con il DNA. Brevemente il campione viene mescolato con 45  $\mu$ l di PicoGreen. Il tubo viene posto all'interno dello strumento *real-time* PCR, predisposto per la lettura del colorante SYBR Green; la lettura viene effettuata alla fine di tre cicli di 94 °C per 30 s e 65 °C per 30 s. Il valore medio della fluorescenza viene convertito in quantità di DNA tramite una curva di calibrazione preparata con uno standard di  $\lambda$ -DNA. La curva di calibrazione è lineare tra 0,20 a 50 ng e la determinazione ha un coefficiente di variazione inferiore al 5,0%. Ciò consente determinazioni affidabili su quantità minime di estratti di DNA di prodotti PCR, in applicazioni cliniche della biologia molecolare.

*Parole chiave:* real-time PCR, analisi quantitativa del DNA, reattivi fluorescenti per DNA.

### Introduction

The determination of total dsDNA concentration in extracts from blood, tissues and cells is an important step preliminary to every molecular determination. Most laboratories still use the classical measurement of UV absorbance at 260 nm that is straightforward and cheap, but has some inconveniences. In the first place, the presence of nucleotides, ssDNA, RNA and interfering substances, such as proteins and phenols, may affect the UV readings. Secondly, it is not adequate to small volume and diluted DNA samples.

The fluorescence detection allows to circumvent these limitations, but requires the use of fluorescent dyes and the availability of a spectrofluorometer for either tube or plate readings. Both these requirements posed in the past decades some problem. Fluorescent dyes, such as the ethidium bromide or the Hoechst 33258 stain, were not highly specific for dsDNA; also, the

fluorescent detector response was affected by the base sequence in samples under exam [1-4]. On the other hand, spectrofluorometric apparatuses were expensive items not commonly available in laboratories. The situation changed with the introduction of the very sensitive and selective asymmetrical cyanine dyes, best known as SYBR Green I and PicoGreen [5]. In particular, the former, thanks to its capacity to resist to thermal cycles, became the reagent of choice for Real-time PCR [6-7]; the latter, thanks to its high sensitivity, dynamic range, selectivity for the dsDNA and insensitivity to reagents used for DNA isolation became the most appropriate reagent for spectrofluorometric determinations of dsDNA [8].

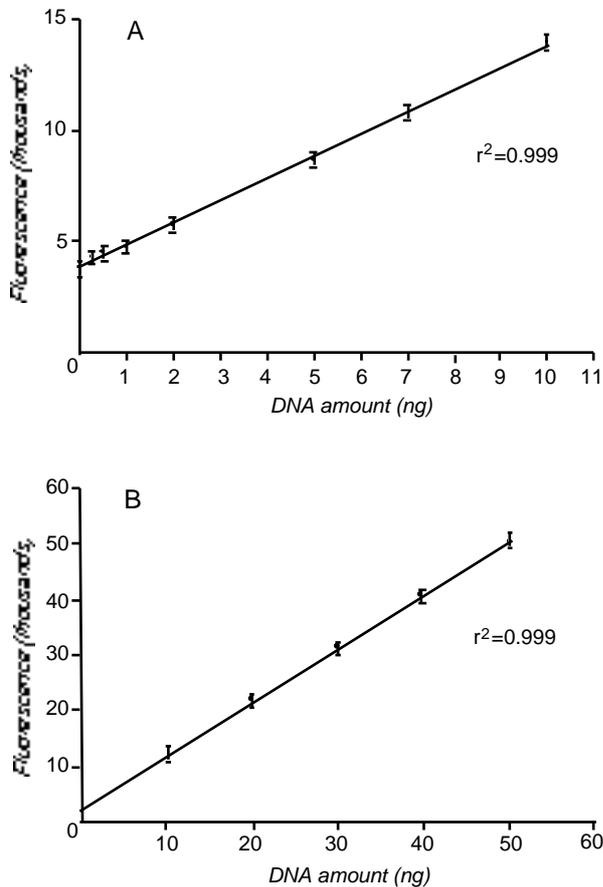
Here, we demonstrate that the PicoGreen quantitation assay kit developed for microplate evaluation of dsDNA works equally well if used with Real-time PCR instruments that are now quite common in molecular biology laboratories.

At variance of forensic applications of DNA determinations performed by Real-time PCR, that were developed for contaminated or degraded DNA samples [9, 10], the proposed method is meant for fresh samples in clinical applications of molecular biology.

## Materials and methods

### Materials

PicoGreen dsDNA Quantitation kit by Molecular Probes (Leiden, The Netherlands) with its own lambda dsDNA (λ-DNA) calibration standard. DNA samples extracted from blood of healthy subjects by a standard



**Fig. 1.** - Calibration curves obtained with λ-DNA standard in the low (panel A) and high (panel B) detection ranges. Triplicate samples of λ-DNA (1 to 5 μl of either low or high range standards) were added to 45 μl working dilution of PicoGreen reagent, in a total volume of 50 μl. The fluorescence emission (520 nm) was read at the end of each of three thermal cycles (95 °C for 30 s and 65 °C for 30 s) with optical excitation at 480 nm. Each point represents the mean ± sd of triplicate determinations. The linear correlation coefficient ( $r^2$ ) values are reported below each curve.

salting-out technique [11] and quantitated by the UV spectrometry. MX3000P Real-time PCR apparatus (Stratagene, La Jolla, CA) equipped with the QPCR software.

### PicoGreen quantitation standard curve

The λ-DNA standard provided with the kit (100 ng/μl) was diluted with 1x Tris-EDTA (TE buffer) to the concentration of either 2 ng/μl or 10 ng/μl for the low and high range curves respectively.

The PicoGreen dsDNA quantitation reagent concentrate solution in DMSO was diluted on the day of the experiment by making a 200-fold dilution in 1x TE buffer. This working dilution was prepared in a plastic tube protected from light, as suggested by the manufacturer.

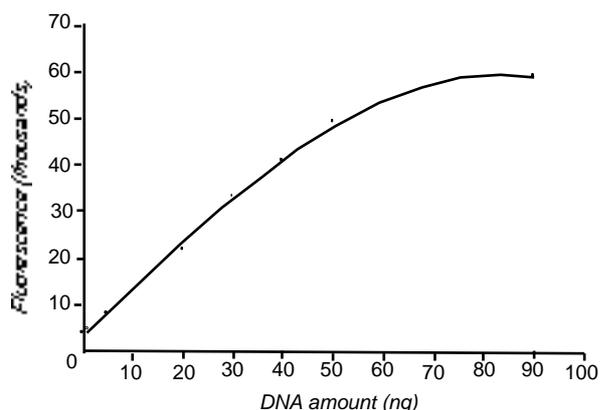
The standard curves were prepared by pipetting increasing volumes from 0.5 to 5 μl (i.e. 1-10 μg or 10-50 ng), of either low range or high range standard solution into optical tubes for Real-time PCR. The volumes were eventually completed to 5 μl with water. Then, we added 45 μl of the working dilution of the Pico-Green reagent. Each curve included a blank prepared with 5 μl of water instead of the sample. Each point was repeated in triplicate.

The tubes were shortly vortexed 3 times and flicked downward to push the liquid to the bottom of the tube. Then, the tubes were put into the MX3000P apparatus set to work with the SYBR Green determination program. The apparatus was set to perform three cycles of 94 °C for 30 s and 65 °C for 30 s with fluorescence reading (set to FAM that allows optical excitation at 480 nm and measurement at 520 nm) at the end of each cycle. The three readings were averaged and used to plot two linear standard curves for low and high range, respectively.

Unknown samples underwent the same treatment. Generally, we used 1 μl aliquots of sample mixed with 4 μl of water and 45 μl of the working dilution of the PicoGreen reagent. Then, the DNA amount was calculated from averaged fluorescence readings by extrapolating the standard curve. The determination was eventually repeated with a different sample aliquot in order to obtain a fluorescence reading within the linear range. Each determination was repeated in triplicate.

### Analysis of PCR products by microchip electrophoresis

Six samples of genomic DNA extracted from blood of healthy subjects were submitted to PCR amplification of exon 13 of LDL receptor gene (accession NM\_000527) according Hobbs *et al.* [12]. The PCR mix included 200 ng of DNA template, 1U of Taq DNA polymerase (Promega) and 5 pmol each of sense (5'-GTCATCTTCCTTGCTGCCTGTTAG-3') and



**Fig. 2.** - Effect of DNA amount on linearity of fluorescence determination as evaluated with variable amounts of  $\lambda$ -DNA under the same operating conditions used for the preparation of calibration curves.

antisense (5'-GTTTCCACAAGGAGGTTTCAAGGT-3') primers in a total volume of 25  $\mu$ l. The PCR reaction was carried out with 30 cycles (94  $^{\circ}$ C 30"/57  $^{\circ}$ C 30"/72  $^{\circ}$ C 1'), preceded by a 2' denaturation at 95  $^{\circ}$ C and followed by a 7' extension at 72  $^{\circ}$ C. This amplification led to a single product of 207 bp that was analyzed qualitatively and quantitatively using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) equipped with the Agilent DNA500 LabChips. The analysis was performed with 1  $\mu$ l of amplification product without prior purification, according manufacturer's instructions.

### Results and discussion

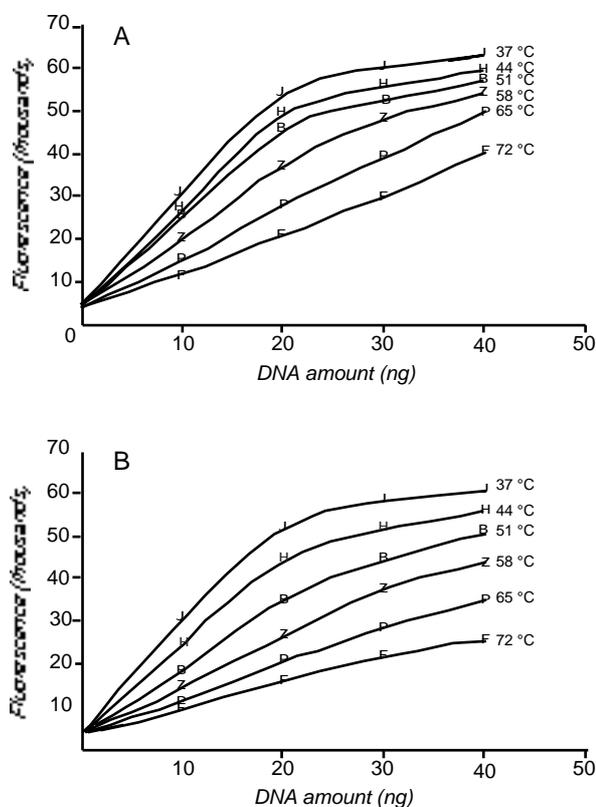
Fig. 1 shows the low range (panel A) and the high range (panel B) calibration curves obtained with the  $\lambda$  DNA ( $\lambda$ -DNA) standard according the above described procedure. Our tests demonstrated that the lower determination limit was in the order of 0.20 ng, while the upper limit was around 50 ng. Over this value the response curve tended to flatten because of the saturation of detector response (Fig. 2) due the fact that, the instrument allowed a maximum reading of 64 000 arbitrary units. Maximum values read with 50 ng were in the order of 52 000 arbitrary units. Therefore, the dynamic linear range of determination (i.e. 0.20-50 ng) covered an interval up to 200 times the minimum determination limit, with  $r^2$  values of 0.999 (Fig. 1). Average standard deviation variations in the five-fold repetition of the same determination increased with sample amounts put into the tube. They were below 4.0% in the lower range values and below 5.0% in the high range values (data not shown).

The comparison of this method with the classic UV determination was performed with five DNA samples extracted by ourselves from human blood. In all these

cases fluorescence determinations were performed in triplicate with 1  $\mu$ l sample amounts that allowed fluorescent readings within the linear tract of the high range curve (results not shown). The UV-derived DNA concentration values were slightly higher (less than 2% average difference) than those calculated by the fluorescence readings, probably because of the higher susceptibility of UV determination to interferences in DNA extracts [8].

It is interesting to note that manufacturer's protocol of PicoGreen describes its use at room temperature. Furthermore, researchers involved in the development of this reagent observed that "the dye is not stable to thermocycling when high-temperature melting steps are included" [8]. Therefore, our experiments began with a post-run reading built-in program using temperatures between 25 and 37  $^{\circ}$ C. The observation that small temperature variations affected both detector response and its linearity induced us to deepen the effects of temperature on analytical performances.

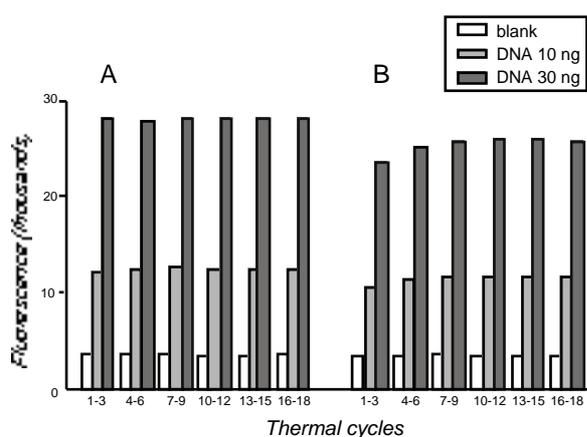
Fig. 3, panel A, shows that the increase of temperature reduced the sensitivity, but increased the linearity of the determination. However, even at 65  $^{\circ}$ C where the linear correlation was optimal ( $r_2 = 0.998$ ),



**Fig. 3.** - Effect of thermal treatments on fluorescence readings. Panel A: determinations performed with  $\lambda$ -DNA amounts ranging from 10 to 40 ng at different temperatures. Panel B: same as above, but preceded by a denaturing step at 94  $^{\circ}$ C for 30 s.

repeated determinations had standard deviation variations up to 9%. We performed also the determinations at different temperatures, but preceded by a short denaturing step at 94 °C. This led to a slight reduction of sensitivity (Fig. 3, panel B), but increased the linearity ( $r^2 = 0.9998$ ) and reduced the standard deviation variation to values below 5.0%.

Taking into account the above mentioned statement on the negative effect of high-temperature melting steps on PicoGreen dye [8], we performed a test where three different -DNA amounts were submitted first to 18 thermal cycles (94 °C for 30 s and 65 °C for 30 s), then were allowed to stand 10 min at room temperature and again submitted to the same 18 thermal cycles. The results of these experiments that are shown in Fig. 4 indicate that thermal cycling induces only slight fluorescence variations on fresh samples while the return to room temperature in samples previously submitted to denaturing steps has more profound effect. Therefore, the thermo-bleaching of the dye occurring during the cycling determines only tiny variations of fluorescence that do not affect the determination. Also, the inclusion of melting step before the determination, notwithstanding a certain thermal sensitivity of the dye, reduces the variability of the determinations probably by inducing a better defined dye/base pair ratio (dbpr) of the complex dye:DNA [13]. On the other hand, the not completely reversible changes in the interaction between dye and DNA that occur when a previously denatured sample is cooled to room temperature exclude the possibility to analyze twice the same aliquot of sample.



**Fig. 4.** - Effect of thermal cycles on fluorescence emission. Panel A: fresh -DNA samples submitted to 18 cycles (94 °C for 30 s and 65 °C for 30 s). Panel B: same samples of panel A, after being thermally cycled are cooled to 37 °C and again submitted to the previously described cycling. Each bar represents the mean of three consecutive cycles.

Finally, we compared the quantitative results obtained with microchip electrophoresis by the Agilent 2100 Bioanalyzer and this method while analyzing 1  $\mu$ l aliquots of six PCR amplification products of exon 13 of LDLR gene (see “Materials and methods”) using both the methods. There was a substantial agreement between the two determinations, apart a negligible overvaluation (less than 1% differences) of the fluorescence over the microchip determination probably due to oligos or other interfering compounds in the PCR mix.

In conclusion, the use of fluorescence readings at temperatures much above the room temperature following a denaturing step allowed, notwithstanding the detrimental effect of temperature on the sensitivity of the method, repeatable evaluations of both genomic DNA extracts and PCR products.

#### Acknowledgments

The research was partially supported by the project ISS-C3BO “Studio clinico e sperimentale di alterazioni biochimiche causa di dislipidemie e loro sequele patologiche” and Ministry of Health - ALZ/8 “Ruolo della Nicastrina nell’etiopatogenesi dell’Alzheimer familiare”. We gratefully acknowledge the skillful collaboration of Mr. Rocco Careri.

Received on 8 February 2005.

Accepted on 16 March 2005.

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