APPLICATION NOTE

Sensitive fluorescent quantitation of DNA with the Quant-iT PicoGreen dsDNA Assay Kit

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Introduction

Double-stranded DNA is typically quantitated in microplate readers by measuring the absorbance of the DNA solution at 260 nm. However, this method is only able to measure down to about 250 ng/mL on a typical absorbance microplate reader. For biological applications involving small samples, such as next-generation sequencing and quantitation of DNA amplification products, more sensitive methods are needed. The Quant-iT PicoGreen dsDNA Assay Kit from Thermo Fisher Scientific is more specific for DNA and is about 1000 times more sensitive than traditional absorbance methods. The dynamic range of this assay in microplate format, as stated in the product manual, is from 250 pg/mL to 1000 ng/mL with a single dye concentration. Here, we demonstrate that with Molecular Devices SpectraMax® microplate readers and the Quant-iT PicoGreen assay, users can reliably measure concentrations as low as 50 pg/mL of double-stranded DNA.

To maximize sensitivity of the assay, it is necessary to use optimal excitation and emission wavelengths. Unlike filter-based plate readers, the dual monochromators in the SpectraMax iD5 Multi-Mode Microplate Reader and other SpectraMax readers allow the selection of any wavelength within the reader's stated range. It is important to determine the excitation and emission wavelengths that provide the best sensitivity and dynamic range for the assay, since these may differ somewhat from those used with filter-based plate readers, and from wavelengths recommended in the kit manual.

Benefits

- Sensitive fluorescent quantitation of DNA down to 50 pg/mL
- Linear dynamic range spanning over four orders of magnitude
- Easy analysis of results with preconfigured protocol in SoftMax Pro Software

Materials

- Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific cat. #P7589)
- 96-well solid black microplate (Greiner Bio-One cat. #655076)
- Light Safe Black Microtubes (Argos cat. #T7100BK)
- Microplate readers (note: additional SpectraMax readers not listed here have similar performance for the PicoGreen assay)
    - SpectraMax Mini Multi-Mode Microplate Reader (Molecular Devices P/N SMAX MINI AF), with fluorescence filter cube FL-535 (Molecular Devices P/N 5089097)
    - SpectraMax® iD5 Multi-Mode Microplate Reader (Molecular Devices P/N #iD5)
    - SpectraMax® i3x Multi-Mode Microplate Reader (Molecular Devices P/N #i3x)
    - SpectraMax® M5 Multi-Mode Microplate Reader (Molecular Devices P/N #M5)
    - SpectraMax® Gemini™ EM Microplate Reader (Molecular Devices P/N #EM)

Methods

Instrument and protocol setup

- Turn on the microplate reader.
- Launch SoftMax® Pro Software and open the PicoGreen fluorescence protocol from the Protocols dropdown menu. Depending on which SpectraMax reader you are using, you may need to enter the optimized settings for the assay (see Table 1).
- Select Wells to Read and Assay Plate Type by clicking on “Settings” and locating the options on the left side of the screen.
- Click the Template button to open a window where you can assign wells of the microplate to pre-set template groups. Use the dropdown menu to select the appropriate template group. There are preconfigured template groups in the PicoGreen fluorescence protocol including Standards, Unknowns, and Unknowns_NoDiln (use for samples that are undiluted). Assigning wells to preset template groups populates group tables in the protocol with the corresponding data that is acquired when the microplate is read.

Prepare the assay

The method for this assay follows the instructions in the product information sheet for QuantiT PicoGreen dsDNA Reagent and Kits, except the assay volume is proportionately reduced from 2.0 mL to 200 µL to fit a 96-well microplate format.

- Prepare 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) by diluting the concentrated buffer from the kit 20-fold with distilled DNase-free water.
- Prepare an aqueous working solution of Quant-iT PicoGreen reagent by making a 200-fold dilution of the concentrated DMSO solution in TE buffer (prepared above). Preparation of the solution in a plastic container, rather than glass, is recommended, as the reagent may adsorb to glass surfaces. Protect the solution from light by using brown or black tubes, or by covering with foil. This solution should be used within a few hours of its preparation.
- DNA standard curve: Prepare a 2 µg/mL stock solution of dsDNA in TE. The lambda DNA standard provided with the kit can be diluted 50-fold in TE to make the 2 µg/mL solution.

Parameter	SpectraMax iD5/iD3	SpectraMax i3x	SpectraMax M5/M5e/ M4/M3/M2/M2e	SpectraMax Gemini EM/XPS	SpectraMax Mini
Read mode	Fluorescence (FL)	Fluorescence (FL)	Fluorescence (FL)	Fluorescence (FL)	Fluorescence (FL)
Read type	Endpoint	Endpoint	Endpoint	Endpoint	Endpoint
Wavelengths	Excitation: 485 nm Emission: 535 nm	Excitation: 490 nm, bandwidth 9 nm Emission: 525 nm, bandwidth 15 nm	Excitation: 490 nm Emission: 525 nm Emission cutoff: 515 nm	Excitation: 490 nm Emission: 525 nm Emission cutoff: 515 nm	Fluorescence filter cube: FL-535 (Excitation 485 nm, Emission 535 nm, Dichroic 508 nm)
Plate type and Read area	Select based on microplate and wells used	Select based on microplate and wells used	Select based on microplate and wells used	Select based on microplate and wells used	Select based on microplate and wells used
PMT and Optics	PMT gain: Automatic Integration time: 200 ms Read height: Optimize for microplate used	PMT gain: N/A Flashes per read: 10 Read height: Optimize for microplate used	PMT gain: Automatic Flashes per read: 10	PMT gain: Automatic Flashes per read: 20	PMT gain: Automatic Integration time: 400 ms Read height: Optimize for microplate used

Table 1. *Instrument settings for SpectraMax readers. For SpectraMax iD5, i3x, and Mini readers, the read height setting should be optimized for the microplate used. Note: Additional readers with similar performance are listed.*

Note: In some cases, it may be preferable to make the standard curve using DNA similar to the type being assayed.

- If desired, a high-range standard curve may be prepared from 1 ng/mL to 1 µg/mL, or a low-range standard curve may be prepared from 25 pg/mL to 25 ng/mL. For high-range or low-range curves, 1:10 dilutions may be used. For the low-range curve, dilute the 2 µg/mL solution 40-fold to yield a 50 ng/mL starting solution.

  For this application note, a series of standards ranging from 50 pg/mL to 1 µg/mL was set up as a 1:3 dilution series.

- Pipette standards into a solid black 96-well microplate at 100 µL per well, preferably in triplicate. Be sure to include a set of buffer blank wells containing TE only (no DNA).
- Add 100 µL of the aqueous working solution of Quant-iT PicoGreen reagent to each well (this results in a 1:2 dilution of the standard already in the wells). Mix well by trituration or plate shaker and incubate for 2 to 5 minutes at room temperature, protected from light.

Read the microplate

- If using a SpectraMax M Series reader, make sure the purple plate adapter is in the microplate reader drawer. Place the microplate in the drawer.
- Click the Read button in SoftMax Pro Software.

Analyze the data

- After the microplate has been read, the relative fluorescence units (RFUs) will be displayed in the Plate section. The data will be analyzed in the Group Tables that were created when the template was set up. For an example of representative data from a Group Table, see Table 2.
- Standards assigned in the Template (and thus displayed in the Standards group table) will be automatically plotted in the Standard Curve section of the protocol. A linear curve fit is applied by default, but a log-log fit may be used when plotting a standard curve over a wide dynamic range. Curve fits are chosen from the dropdown Fit menu in the graph section.

Results

DNA standards ranging from 50 pg/mL to 1 µg/mL were detected using the Quant-iT PicoGreen dsDNA Assay Kit and SpectraMax readers (data from the SpectraMax iD5 reader are shown, but other SpectraMax readers gave similar results). SoftMax Pro Software automatically calculated average RFU, standard deviation, and %CV for each set of standard replicates. A standard curve was plotted using the log-log curve fit in SoftMax Pro Software (Figure 1). Sensitivity down to 50 pg/mL was observed using the 96-well microplate format and standard limit of detection calculation of three times standard deviation of the blank. This is well below the lower limit of 250 pg/mL stated in the Quant-iT PicoGreen assay product insert. Figure 2 shows the high-range (A) and low-range (B) standard curves. Linearity was excellent throughout the standards' range ($r^2 \geq 0.99$ for each curve shown).

Sample	DNA conc (ng/mL)	Average RFU	StdDev	%CV
1	1000.00	54893498	611149.3	1.1
2	333.333	17114392	685401.0	4.0
3	111.111	5884808	249140.5	4.2
4	37.037	1938706	21246.0	1.1
5	12.346	667849	10336.2	1.5
6	4.115	231812	9389.3	4.1
7	1.372	74577	4324.4	5.8
8	0.457	25963	2403.5	9.3
9	0.152	8981	1422.3	15.8
10	0.051	2441	1205.6	49.4

Table 2. *DNA standards.*

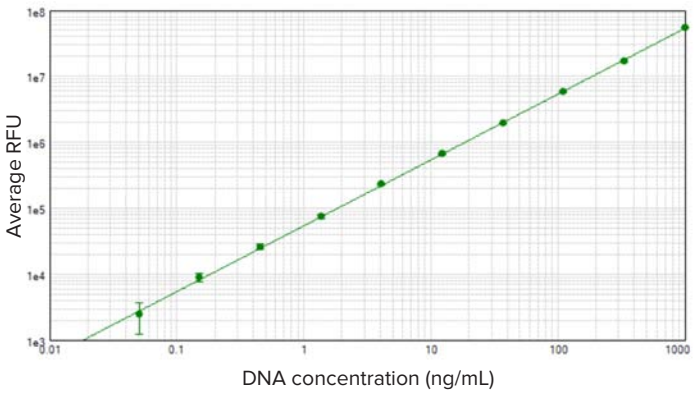

Figure 1. *DNA standard curve. DNA standards ranging from 50 pg/mL to 1000 ng/mL were assayed using the Quant-iT PicoGreen dsDNA Assay Kit on the SpectraMax iD5 reader. The standard curve was plotted using the log-log curve fit in SoftMax Pro Software (r^2 = 1.00).*

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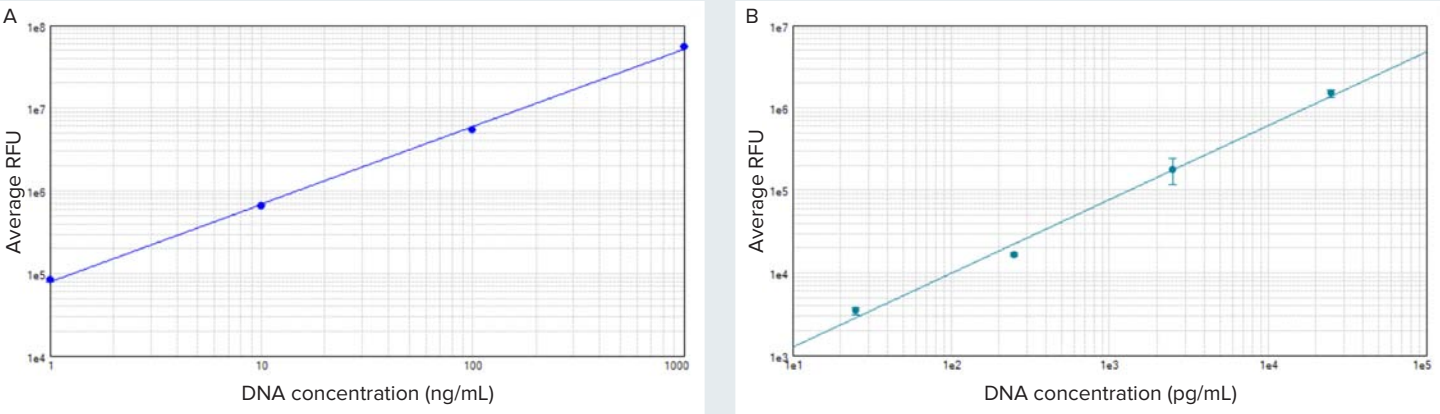

Figure 2. *High-range (A) and low-range (B) standard curves. Curves were plotted using the log-log curve fit in SoftMax Pro Software (both curves, $r^2 = 0.99$).*

Conclusion

The Quant-iT PicoGreen dsDNA Assay Kit, when run on a SpectraMax microplate reader with SoftMax Pro Software, is a quick, sensitive detection method for double-stranded DNA. The analysis capabilities of the software provide quantitation in an easy-to-read, user customizable report format. A preconfigured protocol is available in the software to facilitate rapid assay setup.

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