Video Article

Quantification of dsDNA using the Hitachi F-7000 Fluorescence Spectrophotometer and PicoGreen Dye

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Abstract

Quantification of DNA, especially in small concentrations, is an important task with a wide range of biological applications including standard molecular biology assays such as synthesis and purification of DNA, diagnostic applications such as quantification of DNA amplification products, and detection of DNA molecules in drug preparations. During this video we will demonstrate the capability of the Hitachi F-7000 Fluorescence Spectrophotometer equipped with a Micro Plate Reader accessory to perform dsDNA quantification using Molecular Probes Quant-it PicoGreen dye reagent kit.

The F-7000 Fluorescence Spectrophotometer offers high sensitivity and high speed measurements. It is a highly flexible system capable of measuring fluorescence, luminescence, and phosphorescence. Several measuring modes are available, including wavelength scan, time scan, photometry and 3-D scan measurement. The spectrophotometer has sensitivity in the range of 50 picomoles of fluorescein when using a 300 μL sample volume in the microplate, and is capable of measuring scan speeds of 60,000 nm/minute. It also has a wide dynamic range of up to 5 orders of magnitude which allows for the use of calibration curves over a wide range of concentrations. The optical system uses all reflective optics for maximum energy and sensitivity. The standard wavelength range is 200 to 750 nm, and can be extended to 900 nm when using one of the optional near infrared photomultipliers. The system allows optional temperature control for the plate reader from 5 to 60 degrees Celsius using an optional external temperature controlled liquid circulator. The microplate reader allows for the use of 96 well microplates, and the measuring speed for 96 wells is less than 60 seconds when using the kinetics mode.

Software controls for the F-7000 and Microplate Reader are also highly flexible. Samples may be set in either column or row formats, and any combination of wells may be chosen for sample measurements. This allows for optimal utilization of the microplate. Additionally, the software allows importing micro plate sample configurations created in Excel and saved in comma separated values, or "csv" format. Microplate measuring configurations can be saved and recalled by the software for convenience and increased productivity. Data results can be output to a standard report, to Excel, or to an optional Report Generator Program.

Video Link

The video component of this article can be found at https://www.jove.com/video/2465/

Protocol

1. Instrument Setup

1. Turn on the spectrofluorometer and allow to warm up for 1 hour.
2. Using Excel create a file with the Standards and Sample data, as shown in Figure 1 and save it in comma separated values "csv" format.

	A	B	C	D
1	Standard 1	mg/ml	1000	
2	Standard 2	mg/ml	100	
3	Standard 3	mg/ml	10	
4	Standard 4	mg/ml	1	
5	Standard 5	mg/ml	0	
6	Sample 1	mg/ml		
7	Sample 2	mg/ml		
8	Sample 3	mg/ml		
9	Sample 4	mg/ml		
10	Sample 5	mg/ml		
11	Sample 6	mg/ml		
12	Sample 7	mg/ml		
13	Sample 8	mg/ml		
14	Sample 9	mg/ml		
15	Sample 10	mg/ml		
16	Sample 11	mg/ml		
17	Sample 12	mg/ml		
18	Sample 13	mg/ml		
19	Sample 14	mg/ml		
20	Sample 15	mg/ml		
21	Sample 16	mg/ml		
22	Sample 17	mg/ml		
23	Sample 18	mg/ml		
24	Sample 19	mg/ml		
25				
26				
27				
28				
29				

◄ ◄ ► ►◄ \ Well information /

Figure 1. Standards and samples data.

3. Set up fluorometer as follows:
    1.
       Click on the **Method** button, this will open the Analysis Method window, shown in Fig 2.
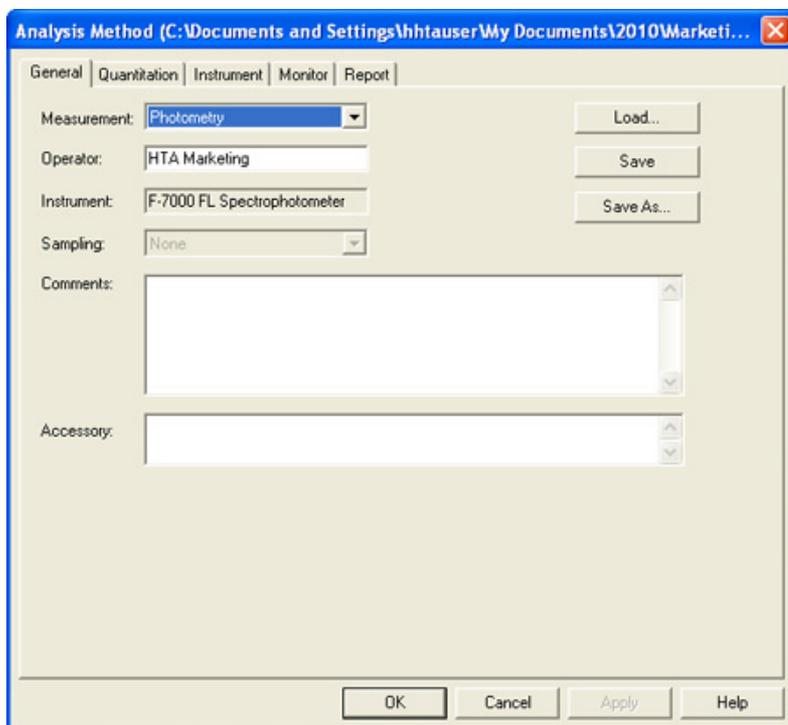
Figure 2. Analysis Method Window, General tab.
Make the following selections:
- In the "Measurement" field, select Photometry from the pull down menu.
- In "Operator" field, enter the appropriate operator's name.
- There is no need to enter information in the "Instrument" field. This is selected automatically by the software.
- The "Sampling" field is inactive when using the microplate reader.
- Enter any comments which you may want included in the report in the "Comments" field.
- In the "Accessory" field, enter information related to the accessories used for this test.
- Note the buttons on the right side of the window.
- The "Load" button allows loading previously saved Analysis Methods.
- The "Save" button allows updating a set of test parameters or Analysis Method previously saved and loaded, using the same name.
- The "Save As" button allows saving a new set of test parameters or Analysis Method under a desired name.

Now click on the "Quantitation" tab, see Figure 3 and make the following selections.
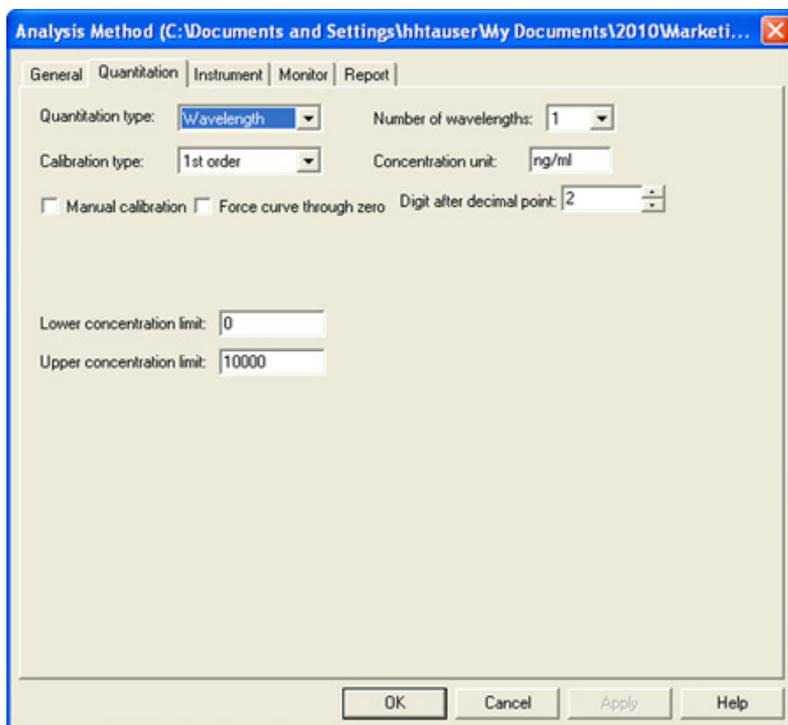
Figure 3. Analysis Method Window, Quantitation tab.

- In the "Quantitation type" field, select Wavelength. This indicates the fluorescence reading will be made using the selected wavelength.
- In the "Calibration type" field, select 1st order.
- In the "Number of wavelengths" field, select 1.
- In the "Concentration unit" field, enter ng/ mL.
- Leave unselected the boxes for "Manual Calibration" and "Force curve through zero".
- In the "Digit after decimal point" field, select 2.
- In the "Lower concentration limit" field, enter 0.
- In the "Upper concentration limit field, enter 10000.

Now click on the "Instrument" tab and make the following selections.
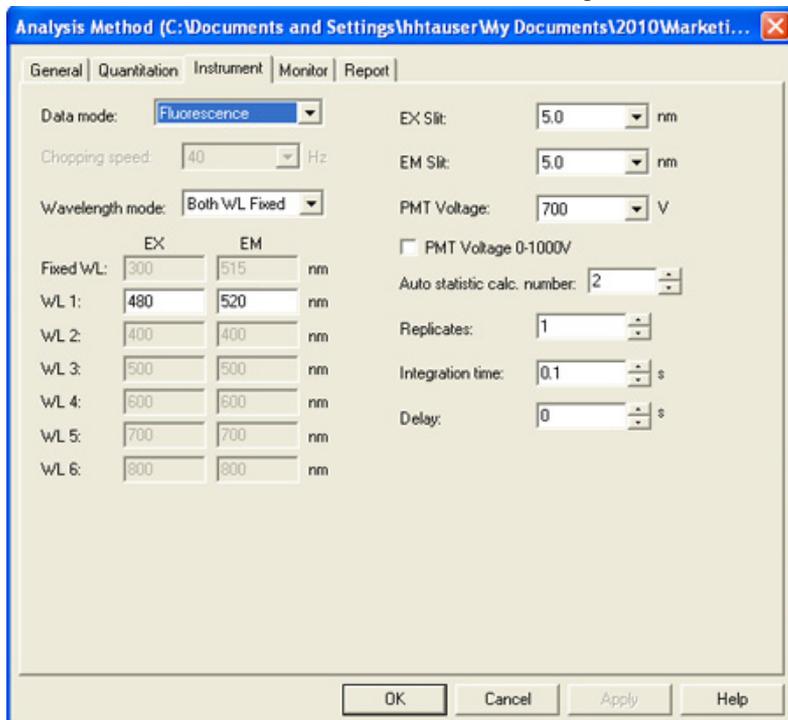
Figure 4. Analysis Method window, Instrument tab
- In the "Data mode:" field, select Fluorescence.
- The "Chopping speed" field is inactive at this time, it is used only for phosphorescence measurements.
- In the "Wavelength mode" field, select Both WL fixed.
- In the "EX / WL1" field enter 480nm.
- In the "EM / WL1" field enter 520nm.
- All the other fields under EX and EM are inactive at this time.
- In the "EX slit" field select 5.0nm from the pull down menu.
- In the "EM slit" field select 5.0nm from the pull down menu.
- Leave unselected the box in front of the "PMT Voltage 1-1000V" field.
- In the "PMT Voltage" field, select 700V from the pull down menu.
- In the "Auto statistic calc. number" field select 2.
- In the "Replicates" field select 1.
- In the "Integration time" field select 0.1s.
- In the "Delay" field enter or select 0s.

Click on the "Monitor" tab.
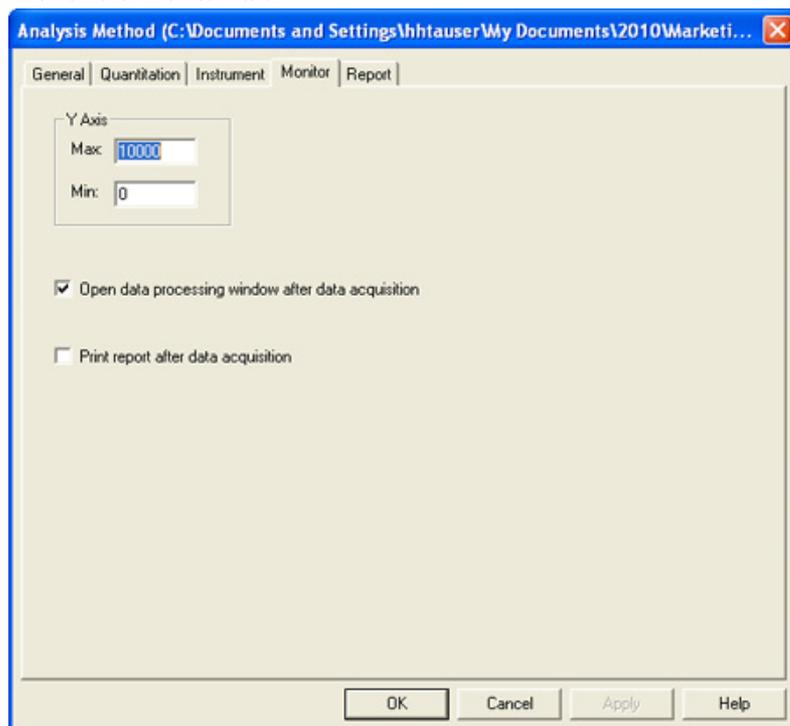
Figure 5. Analysis Method window, Monitor tab.
- In the "Y axis", "Max:" field, select 10000
- In the "Y axis", "Min:" field, select 0.
- Select the box to the left of the "Open data processing after data acquisition".
- Leave unselected the box to the left of the "Print report after data acquisition", unless you want a report to be printed automatically after the readings are completed.

Click on the "Report" tab, see Figure 6.
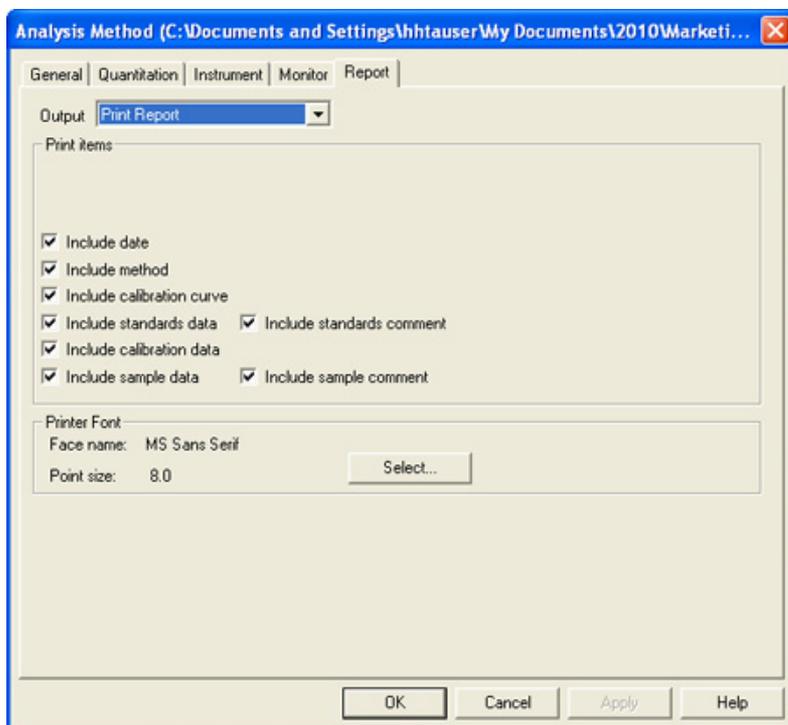
Figure 6. Analysis Method window, Report Tab.

- In the "Output" field, select Print Report from the pull down menu. You could also select "Use Microsoft Excel" if you want the data exported to Excel, or "Use Print Generator Sheet" if using the optional add on program Report Generator which will allow generating custom made reports.
- Click the boxes for the items you want to be included in the report.
- To save all the selected parameters in the different tabs of the "Analysis Method" window, click again on the "General" tab and save them under a file name and in a location where you could find them for future use, and click the "OK" button to close this window.
- This completes setting up the instrument test parameters.
- Now we will set up the microplate reader.
- Click on the [MPR] button, which will bring the MPR Setup Window on top.

Select wells for the Standards and Samples in the MPR Setup Window/Plate tab, as shown in Figure 7.
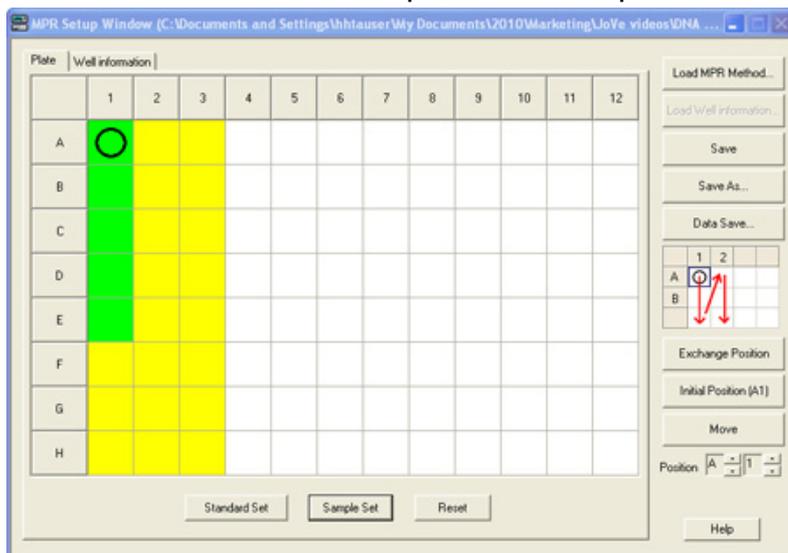
Figure 7. MPR Setup window, Plate tab.

- Click on the A1 position and drag the mouse to E1, then click on the **Standard Set** button. This will select the positions to be used for the standards. Those positions will be highlighted in green color.

- Now we need to select positions F1 to H3 for the samples. Click and drag the mouse starting in position F1, to position H3 and then click on the [Sample Set] button, this will highlight those positions in yellow color.
- Then repeat the same operation for positions A2 to E3, to select those positions for sample measurements.
- Now, let's load the file Comma Separated Variable (csv) file prepared in advance with the Standards and Sample information.

Click on the "Well information" tab of the "MPR Setup Window", as shown in Figure 8.
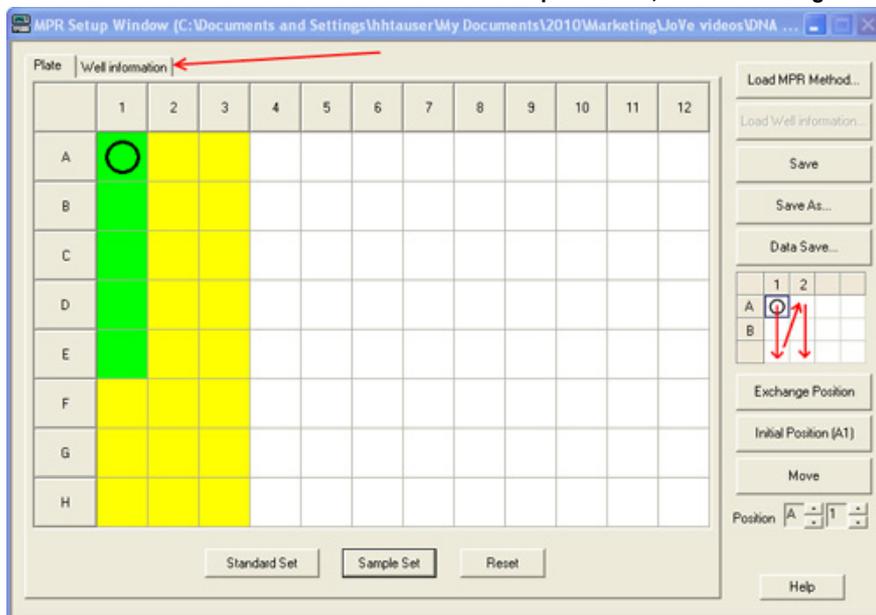
Figure 8. MPR Setup window, Well Information tab.

- Then click on the [Load Well information...] button. A Windows explorer window will open which as shown in Figure 9, which will allow locating the "Well information.csv" file and loading it.
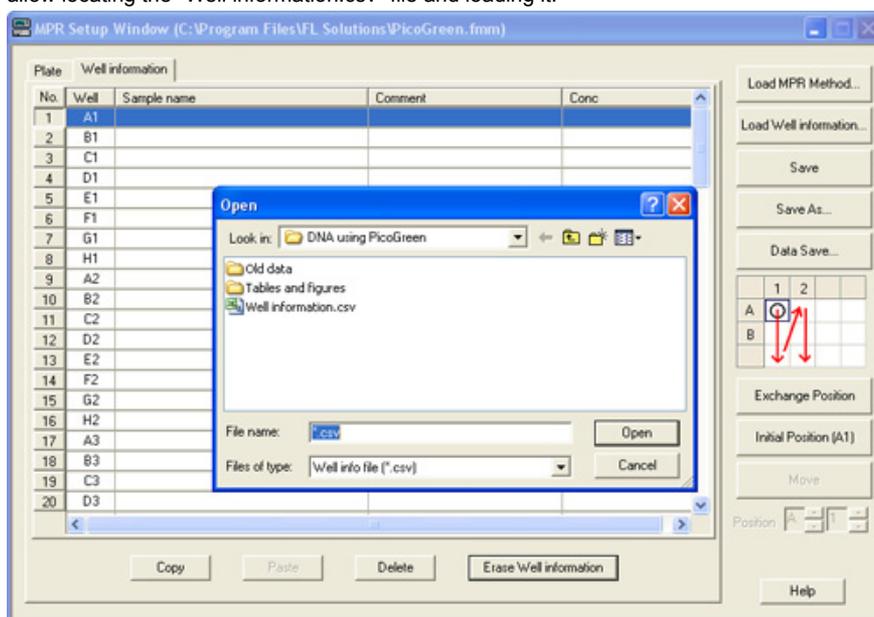
Figure 9. Loading well sample information.
After loading the "Well information" file, the "Well information" will look as shown in Figure 10
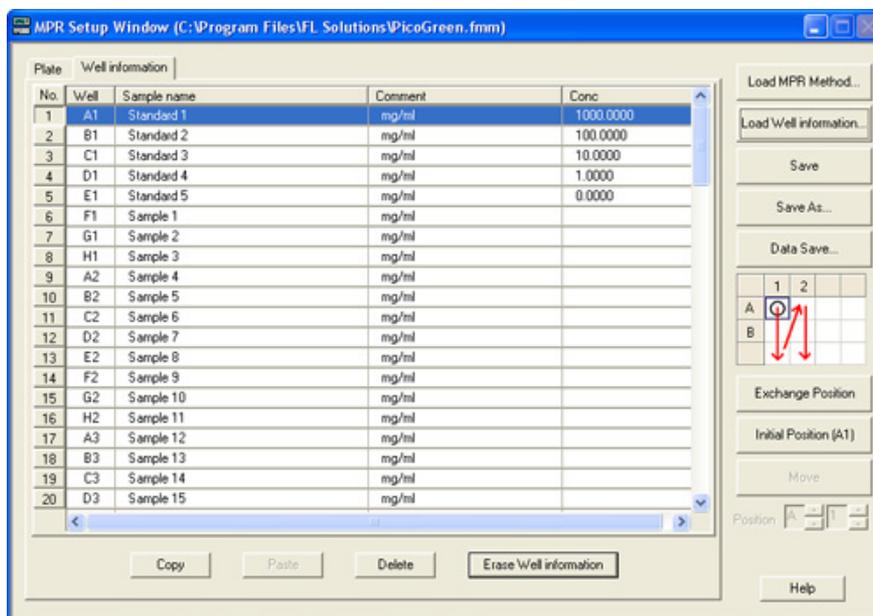
Figure 10. Loaded sample well information.

2. Picogreen Assay Sample Preparation

1. Prepare 1X TE Buffer (10 mM Tris-HCl, 1 mM EDTA) by adding 1 mL of 20 X TE Buffer to 19 mL dI H_2O.
2. Prepare PicoGreen Reagent Solution by adding 50 µL 200 X PicoGreen Reagent to 9.95 mL 1 X TE Buffer.
3. Prepare standards (Lambda dsDNA) by making serial dilutions of stock dsDNA (100 µg/mL). First prepare a solution of 50 µg/mL dsDNA by adding 25 µL of stock dsDNA (100 µg/mL) and 25 µL 1 x TE Buffer. Then prepare the standard solutions by adding the indicated volumes of dsDNA and buffer as listed in the table below:

Concentration of dsDNA	Volume of dsDNA	Volume of Buffer
1 µg/mL	20 µL of 50 µg/mL dsDNA	980 µL 1 X TE Buffer
0.1 µg/mL	100 µL of 1 µg/mL dsDNA	900 µL 1 X TE Buffer
0.01 µg/mL	100 µL of 0.1 µg/mL dsDNA	900 µL 1 X TE Buffer
0.001 µg/mL	100 µL of 0.01 µg/mL dsDNA	900 µL 1 X TE Buffer

3. Measurement of Standards and Sample Fluorescence

1. Pipette 150 µL of each standard into wells A1-E1 of a 96 well plate, per Figure 7, and according to the following table:

Well	Standard
A1	1 µg/mL
B1	0.1 µg/mL
C1	0.01 µg/mL
D1	0.001 µg/mL
E1	1 X TE Buffer

2. Pipette 150 µL unknown samples into wells F1 to H3, shown in Figure 7
3. Pour PicoGreen solution prepared in step 1.2 into a trough designed for multi-channel pipette use. Use a multi-channel pipette to deliver 150 µL PicoGreen solution to wells A1-H3. Mix solution and standards gently with pipette.
4. Place plate in dark area and wait 2-5 minutes for reaction to develop.

4. Representative Results

A good calibration curve is evaluated using the different parameters provided by the F-7000 software upon measurement of the standards and calculation of the calibration curve by the software.

The determination coefficient indicates the goodness of fit of the measured standards and the calculated calibration curve. The closer this value is to "1", the better the fit of the measured value and calibration curve.

If the value is far from "1", the standards must be re-measured, prepared again, or the fitness of the calibration curve changed.

Figure 11 shows an example of a calibration curve with a calculated determination coefficient= 0.99993, obtained for quantification of dsDNA using PicoGreen dye.
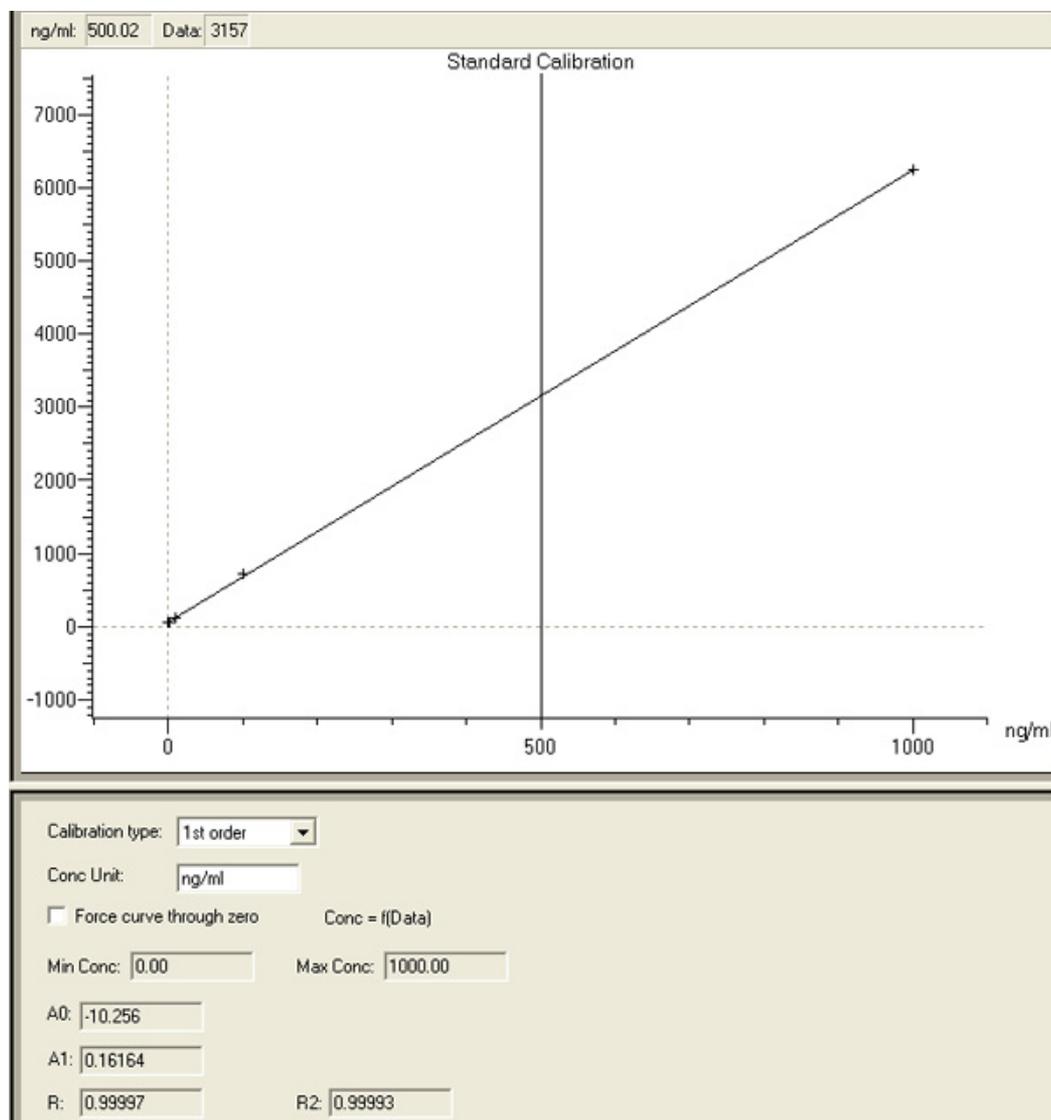
Figure 11. Example of dsDNA calibration curve.

Discussion

Careful pipetting during sample preparation, as well as using a stable and sensitive fluorescence spectrophotometer is essential for obtaining good and reproducible results.

In the case of the fluorescence spectrophotometer used for this test, it is suggested using 300 µL final sample volume in the microplate reader. Lower volume decreases the sensitivity, and a larger volume may cause cross contamination between wells.

Disclosures

Luis A. Moreno and Kendra L. Cox are employed by Hitachi High Technologies America that produces the instrument used in this article.

References

1. Hitachi High Technologies, Application Note FL-080004. Molecular Probes Quant-It PicoGreen dsDNA Reagent and Kits insert. *Analytical Biochemistry*, 249 (2), 228-238 (1997).