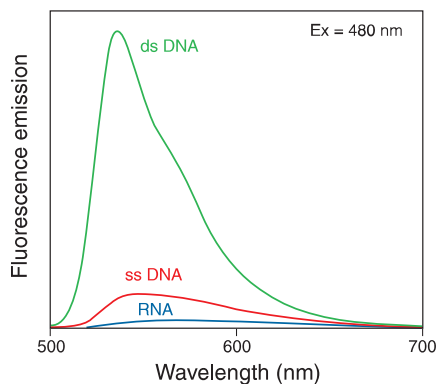


## 8.3 Nucleic Acid Detection and Quantitation in Solution

### Nucleic Acid Detection and Quantitation in Solution



**Figure 8.50** Fluorescence enhancement of the PicoGreen reagent upon binding dsDNA, ssDNA and RNA. Samples containing 500 ng/mL calf thymus DNA, M13 ssDNA or *Escherichia coli* ribosomal RNA were added to cuvettes containing PicoGreen reagent (P-7581, P-7589, P-11495, P-11496) in TE buffer. Samples were excited at 480 nm, and the fluorescence emission spectra were collected using a spectrofluorometer. Emission spectra for samples containing dye and nucleic acids, as well as for dye alone (baseline), are shown.

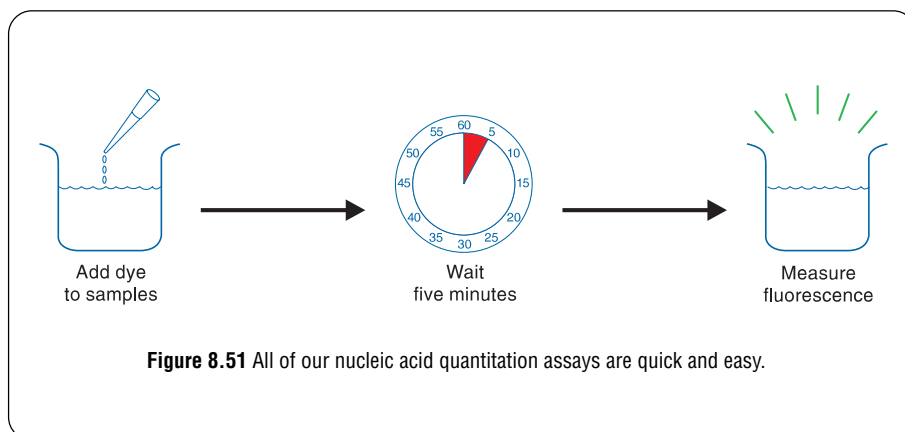
### Add and Read

Many of the premiere reagents and kits that have been developed at Molecular Probes enable researchers to perform homogeneous “add and read” assays with no separation steps required. In most cases this is made possible by a combination of high affinity and selectivity of the dyes for their targets and, in several cases, the reagent being essentially nonfluorescent unless bound to its target. Add and read assays are typically faster and have the capacity for very high throughput and automation. They require much less hands-on time, making them cost-effective. As a bonus, most of these reagents and assays show a linear response to their targets that extends over several orders of magnitude.

Among the best reagents and assays of this type are our PicoGreen, RiboGreen and OliGreen reagents; the SYBR Green and SYBR Gold nucleic acid gel stains (Section 8.4); all of our SYTO and SYTOX dyes and the TOTO and TO-PRO dye series (Section 8.1); the NanoOrange and CBQCA protein quantitation reagents (Section 9.2); the SYPRO Orange, SYPRO Red and SYPRO Tangerine protein gel stains (Section 9.3); and most of our LIVE/DEAD Cell Viability/Cytotoxicity Kits (Section 15.3).

Through our intensive research efforts in both chemical synthesis and bioassay development, Molecular Probes’ scientists have developed rapid and exceptionally sensitive fluorescence-based assays for quantitation of nucleic acids in solution. The PicoGreen dsDNA quantitation reagent, the OliGreen ssDNA quantitation reagent and the RiboGreen RNA quantitation reagent — optimized for double-stranded DNA, oligonucleotides and RNA, respectively — have a high affinity for nucleic acids and an extremely large fluorescence enhancement upon binding, making possible the direct detection of minute amounts of nucleic acids in complex solutions within minutes, usually without interference from other biomolecules. These patented reagents and quantitation assays provide the following advantages:

- **Sensitivity.** The PicoGreen dye-, OliGreen dye- and RiboGreen dye-based fluorescence assays are up to 10,000-fold more sensitive than UV absorbance measurements and at least 400-fold more sensitive than assays that use the Hoechst 33258 dye<sup>1</sup> (H-1398, H-3569; FluoroPure grade, H-21491), requiring much less precious sample for quantitation.
- **Accuracy.** Unlike measurements of UV absorbance, these assays are not affected by the presence of proteins, free nucleotides or very short oligonucleotides, making quantitation of intact oligonucleotides and nucleic acids much more accurate in complex mixtures such as serum or whole blood.
- **Precision.** The average standard deviations of triplicate assays using these reagents are typically less than 5%.
- **Simplicity.** These assays have a very simple protocol that requires no separation steps, making them ideal for automated, high-throughput measurements.
- **Broad dynamic range.** Quantitation is accurate over four orders of magnitude for the PicoGreen and OliGreen assays, with a single dye concentration. The RiboGreen assay is accurate over three orders of magnitude.
- **Instrument compatibility.** Quantitation assays can be performed using a fluorescence-based microplate reader with standard filters optimized for fluorescein-like dyes, a relatively inexpensive filter-based spectrofluorometer or a standard spectrofluorometer.
- **Convenience.** Each reagent is available separately or in a kit that additionally contains a nuclease-free buffer and standards. The PicoGreen Assay is also available in Redi-Plate 96 and RediPlate 384 formats — pre-prepared microplates with standards — for high-throughput applications.



**Figure 8.51** All of our nucleic acid quantitation assays are quick and easy.

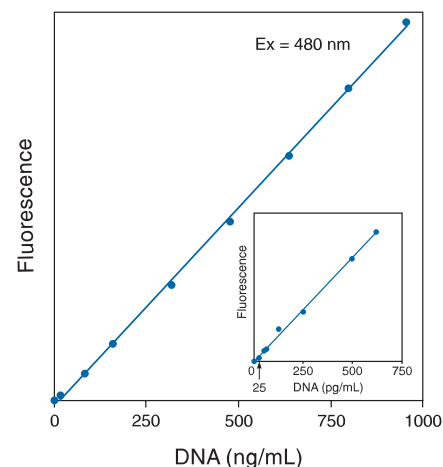
## PicoGreen dsDNA Quantitation Reagent and Kits

The patented PicoGreen reagent (P-7581, P-11495) and Kits (P-7589, P-11496) can accurately quantitate as little as 25 pg/mL of double-stranded DNA (dsDNA) in a fluorometer or 250 pg/mL (typically 50 pg in a 200  $\mu$ L volume) in a fluorescence-based microplate reader. The PicoGreen assay is greater than 10,000 times more sensitive than conventional UV absorbance measurements at 260 nm (an  $A_{260}$  of 0.1 corresponds to a 5  $\mu$ g/mL dsDNA solution), at least 400 times more sensitive than the Hoechst 33258 dye-based assay and even more sensitive than assays based on our YO-PRO-1 and YOYO-1 dyes, which have reported detection limits of approximately 2.5 ng/mL<sup>2</sup> and 0.5 ng/mL,<sup>3</sup> respectively. Although the PicoGreen reagent is not specific for dsDNA, it shows a >1000-fold fluorescence enhancement upon binding to dsDNA, and much less fluorescence enhancement upon binding to single-stranded DNA (ssDNA) or RNA, making it possible to quantitate dsDNA in the presence of equimolar amounts of ssDNA, RNA or proteins<sup>4</sup> (Figure 8.50). The PicoGreen reagent also selectively detects DNA–RNA hybrids in the presence of ssDNA and RNA.<sup>5</sup> Differences in the emission lifetimes of the PicoGreen complexes with dsDNA and ssDNA make it possible to quantitate the relative amounts of each species in solution by time-resolved measurements.<sup>6</sup> By contrast, UV absorbance measurements cannot distinguish between dsDNA, ssDNA and RNA or proteins. Thus, the PicoGreen reagent allows direct quantitation of PCR amplicons without purification from the reaction mixture and makes it possible to detect low levels of DNA contamination in recombinant protein products.<sup>7</sup> In comparison to the Hoechst 33258 dye, which shows significant AT selectivity, the PicoGreen reagent shows little if any AT- or GC-selectivity and is thus accurate for quantitating DNA from almost any source. The PicoGreen reagent can be excited at 488 nm with an argon-ion laser, and is reported to be the best nucleic acid stain for analysis of single DNA molecules in a flow cytometer.<sup>8</sup>

The protocol for the PicoGreen assay is easy to conduct and requires very few steps — the dye is simply added to the sample and incubated for five minutes, then the fluorescence is measured (Figure 8.51). In addition, the fluorescence signal from binding of the PicoGreen reagent to dsDNA is linear over at least four orders of magnitude (Figure 8.52) with a single dye concentration, whereas assays using ethidium bromide, Hoechst 33258 or the YOYO-1 dye exhibit a much more limited linear range.<sup>3,9,10</sup> We have found that this linearity is maintained in the presence of several compounds commonly found in nucleic acid preparations, including salts, urea, ethanol, chloroform, detergents, proteins and agarose.<sup>4</sup> The PicoGreen assay is faster, more precise and much less expensive than dipstick or blot assays.

The PicoGreen assay is useful for quantitating DNA templates for PCR,<sup>11</sup> labeling reactions, electrophoretic mobility-shift (bandshift) assays, DNA-footprinting assays and filter-binding assays, and for measuring yields from PCR reactions,<sup>12,13</sup> DNA minipreps and maxipreps, cDNA synthesis and nuclease protection assays. The simplicity and selectivity of the assay also make it ideal for high-throughput automated quantitation assays used in forensic and genomics research. Furthermore, the PicoGreen reagent has been used for:

- Genotyping by allele-specific PCR<sup>14</sup>
- Quantitating dsDNA samples before and after PCR amplification and after agarose gel electrophoresis<sup>7,15</sup>
- Determining PCR amplification yields before sequencing<sup>12</sup>
- Automating quantitation of DNA isolated from biological samples or obtained from PCR reactions, prior to running DNA typing gels for high-throughput genotyping<sup>16</sup>
- Quantitating DNA from buccal scrapes prior to DNA profiling by short tandem repeat (STR) analysis<sup>17</sup>
- Identifying contaminating DNA in recombinant protein products<sup>18</sup> or purified monoclonal antibody preparations<sup>19,20</sup>
- Quantitating the efficiency of DNA extraction from frozen and formalin-fixed tissue sections<sup>21</sup>
- Detecting mammalian telomerase activity in tumor cells using the PCR-based TRAP assay<sup>22,23</sup>



**Figure 8.52** Linear quantitation of calf thymus DNA from 25 pg/mL to 1000 ng/mL using the PicoGreen dsDNA quantitation reagent (P-7581, P-7589, P-11495, P-11496). Samples in 10 mm  $\times$  10 mm cuvettes were excited at 480 nm. The fluorescence emission intensity was measured at 520 nm using a spectrofluorometer and plotted as a function of DNA concentration. The inset shows an enlargement of the results obtained with DNA concentrations between 0 and 750 pg/mL.

### Custom Synthesis and Bulk Sales

Molecular Probes' custom synthesis services can provide an almost unlimited variety of organic compounds and bioconjugates, including labeled antibodies, immunogens and analogs of biologically active small molecules. We can prepare known compounds using published procedures, or if these are not available we can apply our expertise to design and synthesize reagents. We also offer quantity discounts for multiple-unit purchases of most of our catalog products. Please contact our Custom and Bulk Sales Department to discuss your requirements.

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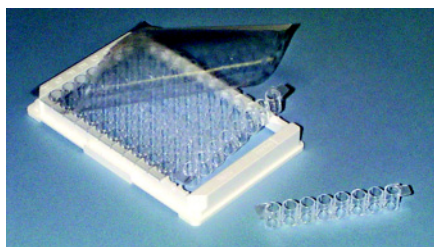
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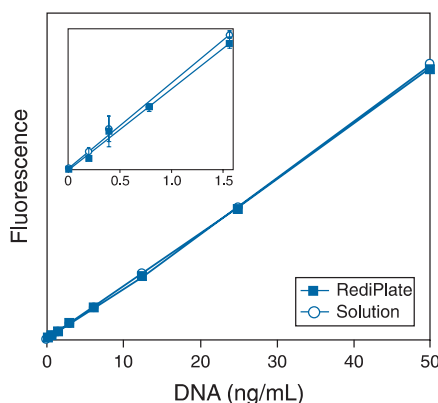
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**Figure 8.53** RediPlate 96 microplate.



**Figure 8.54** Linear quantitation of DNA from zero to 50 ng/mL using the PicoGreen dsDNA quantitation reagent in the standard microplate assay (open circles; P-7581, P-7589, P-11495, P-11496) or in the RediPlate 96 format assay (closed squares; R-21495, R-21496). The fluorescence emission intensity was measured using a fluorescence microplate reader and plotted as a function of DNA concentration. The inset shows an enlargement of the results obtained with DNA concentrations between 0 and 1.5 ng/mL. The assay shows consistently reproducible results, regardless of the format.

- Developing assays for DNA polymerase<sup>5</sup>
- Measuring the activity of reverse transcriptase,<sup>5</sup> or DNase I<sup>24</sup>
- Measuring supercoiled DNA forms in solution, based on their renaturation properties<sup>25</sup>
- Monitoring plasmid production during fermentation and downstream processing<sup>26</sup>
- Quantitating DNA denaturation as a measure for DNA damage in purified DNA preparations, cell lysates or homogenized solid tissues<sup>27–29</sup>

Each vial of the PicoGreen dsDNA quantitation reagent (P-7581) contains a sufficient amount of dye for at least 200 assays using a 2 mL assay volume and a standard fluorometer, or 2000 assays using a 200  $\mu$ L assay volume and a fluorescence microplate reader. The product is accompanied by a simple protocol that ensures linear and reproducible quantitation of dsDNA. We also provide the PicoGreen reagent in a kit (P-7589) that contains:

- The PicoGreen dsDNA quantitation reagent
- A low-fluorescence, nucleic acid- and nuclease-free, concentrated assay buffer, which is essential for the accurate measurement of dsDNA
- A dsDNA standard solution for assay calibration
- A detailed protocol for dsDNA quantitation

This kit provides a sufficient amount of each reagent for 200 assays using a 2 mL assay volume and a standard fluorometer or 2000 assays using a 200  $\mu$ L assay volume and a fluorescence-based microplate reader. Both the standalone reagent and the kit are also available in special packaging, in which the PicoGreen reagent is supplied as 10 vials of 100  $\mu$ L aliquots for added convenience (P-11495; Kit, P-11496). The special packaging reduces thawing times, provides individual aliquots for each person performing the assay, and allows smaller amounts of dye to be taken into the field for analysis of water or other samples.

The PicoGreen Assay is also available in convenient RediPlate 96 or RediPlate 384 PicoGreen dsDNA Assay Kits in which the reagent is dried down into either 96-well or 384-well microplates (R-21495, R-21496). The buffer and sample are added directly to the plates — there is no need to handle the PicoGreen reagent. The microplates used in the RediPlate 96 PicoGreen dsDNA Assay Kit snap apart to permit assays in any multiple of eight (Figure 8.53). The RediPlate 384 PicoGreen dsDNA Assay Kit is useful for high-throughput assays of dsDNA. The RediPlate PicoGreen assays have sensitivity and linearity identical to that of the PicoGreen reagents and kits described above (Figure 8.54). Both RediPlate PicoGreen dsDNA Assay Kits include DNA for generating a standard curve, nuclease-free buffer and detailed instructions. Multiple plates are available at significant discounts.

### OliGreen ssDNA Quantitation Reagent and Kit

For researchers working with ssDNA and oligonucleotides, as well as for companies that synthesize oligonucleotides, Molecular Probes offers our patented OliGreen ssDNA quantitation reagent (O-7582) and Kit (O-11492). Short, synthetic oligonucleotides are used in a number of molecular biology techniques, including DNA sequencing, site-directed mutagenesis, DNA amplification, antisense gene suppression and *in situ* and blot hybridization. The conventional methods for quantitating oligonucleotides are not very sensitive, often requiring highly concentrated samples, and are quite subject to interference from sample contaminants. The most commonly used technique for measuring oligonucleotide and ssDNA concentrations is the determination of absorbance at 260 nm (an  $A_{260}$  of 0.1 corresponds to  $\sim 3 \mu\text{g/mL}$  solution of a synthetic 24-mer M13 sequencing primer).

The OliGreen ssDNA quantitation reagent enables researchers to routinely quantitate as little as 100 pg/mL of ssDNA or oligonucleotide (200 pg in a 2 mL assay volume with a standard fluorometer) or 200 pg in a 200  $\mu$ L assay volume using a fluorescence microplate reader (Figure 8.55). Thus, quantitation with the OliGreen reagent is about 10,000

**Product-Specific Bibliographies and Keyword Searches**

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times more sensitive than quantitation with UV absorbance methods and at least 500 times more sensitive (and far faster, with a greater throughput) than detecting oligonucleotides on electrophoretic gels stained with ethidium bromide. Using an easy-to-follow protocol (Figure 8.51) and fluorescein excitation and emission wavelengths, we have quantitated oligonucleotides that range from 10 to 50 nucleotides in length, as well as several sources of ssDNA, such as M13 and  $\phi$ X174 viral DNA and denatured calf thymus DNA, and obtained similar sensitivity. The OliGreen reagent has also been used to detect phosphodiester and phosphorothioate oligonucleotides.<sup>30</sup>

Significant disadvantages of the UV absorbance method for oligonucleotide quantitation include the large relative contribution of free nucleotides to the signal and the interference caused by contaminants commonly found in nucleic acid preparations. By contrast, nucleotides and short oligonucleotides of six bases or less do not interfere with the OliGreen ssDNA quantitation assay. However, the OliGreen ssDNA quantitation reagent does exhibit fluorescence enhancement when bound to dsDNA and RNA. Like the PicoGreen assay, the linear detection range of the OliGreen assay in a standard fluorometer extends over four orders of magnitude — from 100 pg/mL to 1  $\mu$ g/mL — with a single dye concentration (Figure 8.55). The linearity of the OliGreen assay is maintained in the presence of several compounds commonly found to contaminate nucleic acid preparations, including salts, urea, ethanol, chloroform, detergents, proteins, ATP and agarose; however, many of these compounds do affect the signal intensity to some extent, so standard curves should be generated using solutions that closely mimic those of the samples. The OliGreen assay can even be performed using samples as complex as whole blood or serum.<sup>30</sup>

Our experiments with homopolymers have demonstrated that the OliGreen reagent may exhibit significant base selectivity. The OliGreen reagent shows a large fluorescence enhancement when bound to poly(dT) but only a relatively small fluorescence enhancement when bound to poly(dG) and little signal with poly(dA) and poly(dC). Thus, it is important to use an oligonucleotide with similar base composition when generating the standard curve.

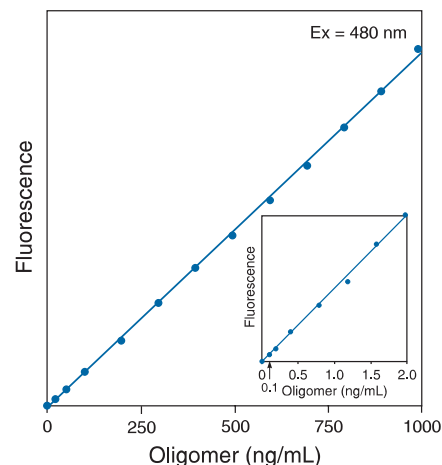
The remarkable properties of our OliGreen ssDNA quantitation reagent make it ideal for the fast and accurate detection and quantitation of:

- Antisense oligonucleotides
- Aptamers
- Genomic DNA isolated under denaturing conditions
- PCR primers
- Phosphorothioate and phosphodiester oligodeoxynucleotides<sup>30</sup>
- Sequencing primers
- Single-stranded phage DNA

Each vial of the OliGreen ssDNA quantitation reagent (O-7582) contains a sufficient amount of dye for at least 200 assays using a 2 mL assay volume and a standard fluorometer, or 2000 assays using a 200  $\mu$ L assay volume and a fluorescence microplate reader. The product is accompanied by a simple protocol that ensures linear and reproducible quantitation of ssDNA. We also provide the OliGreen reagent in a kit form — the OliGreen ssDNA Quantitation Kit (O-11492) — that contains:

- The OliGreen ssDNA quantitation reagent
- A low-fluorescence, nucleic acid- and nuclease-free, concentrated assay buffer, which is essential for the accurate measurement of ssDNA
- An oligonucleotide standard (M13 sequencing primer) solution for assay calibration
- A detailed protocol for ssDNA quantitation

As for the individual reagent, this kit provides sufficient amounts for 200 assays using a 2 mL assay volume and a standard fluorometer or 2000 assays using a 200  $\mu$ L assay volume and a fluorescence microplate reader.

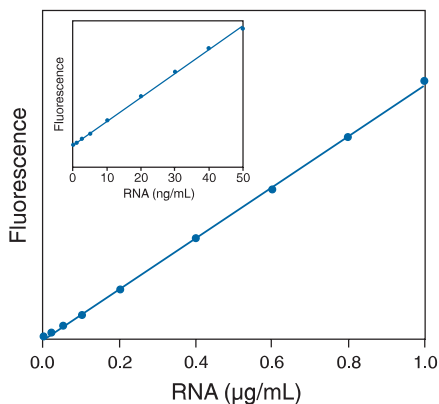


**Figure 8.55** Linear quantitation of a synthetic 24-mer (an M13 sequencing primer) from 0.1 to 1000 ng/mL using the OliGreen ssDNA quantitation reagent (O-7582, O-11492). Samples in 10 mm  $\times$  10 mm cuvettes were excited at 480 nm. The fluorescence emission intensity was measured at 520 nm using a spectrofluorometer and plotted as a function of oligonucleotide concentration. The inset shows an enlargement of the results obtained with oligonucleotide concentrations between 0 and 2.0 ng/mL.

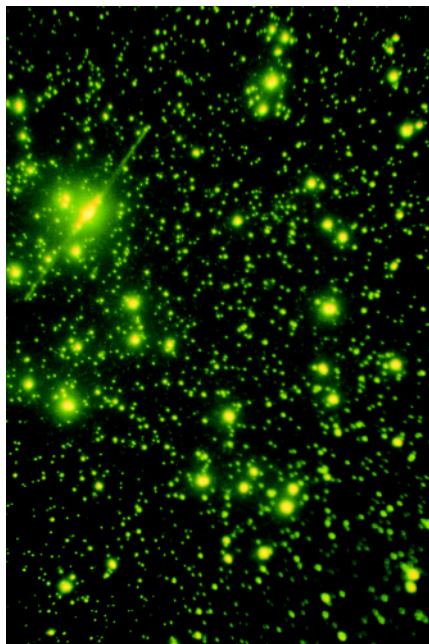
### RediPlate Assay Kits

Molecular Probes' RediPlate 96 and RediPlate 384 Assay Kits provide the ultimate in ease and reliability when using our reagents for high-throughput screening applications. The sensitivity, linearity and dynamic range of the RediPlate versions of these assays are essentially identical to those of the bulk assay kits.

These products contain the required reagents and standards predisposed in either 96-well or 384-well microplates. Some kits also include buffers and additional reagents. The RediPlate 96 Kits include microplates that snap apart in multiples of eight for efficient use of the reagents. Molecular Probes expects to considerably expand the RediPlate Assay Kit product line to include existing and new assays for high-throughput screening. Significant discounts are available for purchase of multiple RediPlate Kits. For more information about these kits, e-mail [RediPlate@probes.com](mailto:RediPlate@probes.com).



**Figure 8.56** Linear quantitation of ribosomal RNA using the RiboGreen RNA quantitation reagent (R-11491, R-11490). For the high-range assay, the RiboGreen reagent was diluted 200-fold into 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE), and 100 µL of the reagent solution was added to microplate wells containing 100 µL ribosomal RNA in TE. For the low-range assay (see inset), the RiboGreen reagent was diluted 2000-fold into TE, and 100 µL of the reagent solution was added to 100 µL of ribosomal RNA in TE. Samples were excited at  $485 \pm 10$  nm, and the fluorescence emission intensity was measured at  $530 \pm 12.5$  nm using a fluorescence-based microplate reader. Background fluorescence was not subtracted.



**Figure 8.57** An environmental sample of marine viruses (smallest dots), bacteria (larger, brighter dots) and a diatom (long, thin cell with prominent nucleus) stained with our SYBR Green I nucleic acid gel stain (S-7563, S-7567, S-7585). Image contributed by Jed Fuhrman, University of Southern California.

## RiboGreen RNA Quantitation Reagent and Kit

The patented RiboGreen RNA quantitation reagent (R-11491) is our premier stain for quantitation of RNA in solution. Like the PicoGreen dsDNA and OliGreen ssDNA quantitation assays, the RiboGreen RNA quantitation assay relies on a proprietary dye that exhibits a large fluorescence enhancement upon binding to nucleic acids. The extinction coefficient of the RiboGreen reagent, as well as its quantum yield and fluorescence enhancement upon binding RNA, are all significantly greater than those of ethidium bromide, for example (Table 8.11).

The RiboGreen assay allows detection of as little as 1 ng/mL RNA in a standard fluorometer, fluorescence microplate reader or a filter-based fluorometer using standard fluorescein excitation and emission wavelengths (Figure 8.56). This sensitivity is at least 200-fold better than that achieved with ethidium bromide<sup>31</sup> and at least 1000-fold better than that achieved using conventional absorbance measurements at 260 nm (an  $A_{260}$  of 0.1 corresponds to a 4 µg/mL RNA solution). Unlike UV absorbance measurements at 260 nm, the RiboGreen reagent does not detect significant sample contamination by free nucleotides.<sup>32</sup> Thus, the RiboGreen reagent more accurately measures the amount of intact RNA polymers in potentially degraded samples.

Using two different dye concentrations to cover its full dynamic range of three orders of magnitude, we have observed a linear correlation between the RNA concentration and fluorescence for 1.0 ng/mL to 50 ng/mL RNA using a 4000-fold dilution of the RiboGreen dye, and for 20 ng/mL to 1 µg/mL using a 400-fold dilution of the dye (Figure 8.56). Assay linearity is maintained in the presence of several compounds commonly found in nucleic acid preparations, including salts, urea, ethanol, chloroform, detergents, proteins and agarose.<sup>32</sup>

The RiboGreen RNA reagent is not appreciably selective for RNA — the dye also shows significant fluorescence enhancement upon binding to DNA. However, a simple DNase pretreatment of samples removes the contribution of DNA to the signal in the assay. The RiboGreen reagent may also have some base selectivity; it exhibits about 60% less fluorescence when bound to poly(G) homopolymers and virtually no fluorescence when bound to poly(U) or poly(C) homopolymers compared to the fluorescence when bound to poly(A) homopolymers or to rRNA.<sup>32</sup>

Using the RiboGreen RNA quantitation reagent, we have reproducibly quantitated RNA from a wide variety of sources, including ribosomal RNA (rRNA), transfer RNA (tRNA), viral RNA, poly(A)+ fractions and total cellular RNA.<sup>32</sup> We have found the RiboGreen reagent to be ideal for:

- Fast and accurate measurements of RNA yields before generating cDNA
- Determination of RNA yields from *in vitro* transcription
- Accurate measurements of RNA before performing Northern blot analysis,<sup>33</sup> S1 nuclease assays, RNase-protection assays, reverse-transcription PCR and differential-display PCR

Each vial of the RiboGreen RNA quantitation reagent (R-11491) contains a sufficient amount of dye for at least 200 high-range assays or 2000 low-range assays using a 2 mL assay volume and a standard fluorometer. With a fluorescence-based microplate reader and a 96-well microplate, the assay volume is reduced to 200 µL, allowing 2000 high-range assays or 20,000 low-range assays. Included with each vial of the RiboGreen

**Table 8.11** Comparison of the RiboGreen RNA quantitation reagent and ethidium bromide.

Nucleic Acid Stain	Extinction Coefficient (cm <sup>-1</sup> M <sup>-1</sup> ) *	Quantum Yield †	Fluorescence Enhancement ‡
RiboGreen reagent (R-11490, R-11491)	67,000	0.65	>1000-fold
Ethidium bromide (E-1305, E-3565)	5,500	<0.3	<30-fold

\* Extinction coefficients were determined at 482 nm. † Quantum yield for the dye bound to RNA. ‡ Fluorescence enhancement upon binding RNA or DNA.

reagent is a simple protocol (Figure 8.51) that ensures linear and reproducible quantitation of RNA. We also provide the RiboGreen reagent in a kit — the RiboGreen RNA Quantitation Kit (R-11490) — that contains:

- The RiboGreen RNA quantitation reagent
- A low-fluorescence, nucleic acid- and nuclease-free, concentrated assay buffer, which is essential for the accurate measurement of RNA
- A ribosomal RNA standard (16S and 23S rRNA from *Escherichia coli*) solution for assay calibration
- A detailed protocol

As for the standalone product, this kit provides sufficient amounts for 200–2000 assays using a 2 mL assay volume and a standard fluorometer or 2000–20,000 assays using a 200  $\mu$ L assay volume and a fluorescence-based microplate reader. Sufficient assay buffer is supplied for 200 assays using a 2 mL assay volume or 2000 assays using a 200  $\mu$ L assay volume. The RNase-free TE buffer concentrate, the use of which is essential to the success of the assay, is also available separately (T-11493) and can be used to extend the number of low-concentration assays possible with the kit.

## Other Stains for Nucleic Acid Quantitation in Solution

### Cyanine Dyes and Phenanthridine Dyes for Nucleic Acid Quantitation in Solution

The dimeric cyanine dyes TOTO-1 and YOYO-1 are also useful for sensitive fluorometric measurement of dsDNA, ssDNA and RNA in solution,<sup>3</sup> although the PicoGreen, OliGreen and RiboGreen reagents generally are faster, have greater sensitivity and show a linear response over a broader range of nucleic acid

concentrations. The linear range of assays that use the TOTO-1 and YOYO-1 dyes for DNA quantitation encompasses about two orders of magnitude, with a sensitivity limit of about 0.5 ng/mL. The TOTO-1, YOYO-1 and YO-PRO-1 nucleic acid stains have been used to quantitate PCR amplification products in a homogeneous human leukocyte antigen (HLA) typing method that requires no transfer or washing steps, thus minimizing the risk of sample contamination.<sup>34,35</sup> Other dyes for nucleic acid quantitation in solution and their applications include:

- YOYO-1 dye (Y-3601, Section 8.1) for solution quantitation of oligonucleotides,<sup>36</sup> PCR products<sup>37</sup> and nuclear run-on assays<sup>38</sup>
- YO-PRO-1 dye (Y-3603, Section 8.1) for quantitating dsDNA in a fluorescence microplate reader, with a reported sensitivity limit of about 2.5 ng/mL<sup>2</sup>
- YO-PRO-1 dye for quantitating RNA isolated from *Xenopus* embryos<sup>39</sup>
- YO-PRO-1 dye and SYBR Green I dye for direct counting of viruses in marine and freshwater environments<sup>40–45</sup> (Figure 8.57)
- PO-PRO-3 dye (P-3585, Section 8.1) for quantitating DNA in a fluorescence microplate reader,<sup>46</sup> with fluorescence measurements reported to be independent of base-pair composition
- Ethidium bromide (E-1305, E-3565; Section 8.1) for measuring the yield of PCR products<sup>47</sup>
- Ethidium bromide and acridine homodimer (A-666, Section 8.1) for quantitating covalently closed, circular DNA and for measuring the activity of polymerases, deoxynucleotidyl transferases, ligases, gyrases, topoisomerases and nucleases<sup>9,48,49</sup>
- YOYO-1 dye for measuring the activity of DNases<sup>50</sup>
- SYBR Gold (S-11494, Section 8.4) for detecting DNA mutations in a simple PCR-based assay<sup>51</sup>
- SYBR Green I dye for quantitation of nucleic acids on plastic wrap or paraffin (see SYBR Green I and SYBR Green II Dyes for Staining Nucleic Acids on Plastic Wrap or Paraffin Sheets)

## TECHNICAL NOTE

### SYBR Green I and SYBR Green II Dyes for Staining Nucleic Acids on Plastic Wrap or Paraffin Sheets

Molecular biologists frequently need a quick estimate of nucleic acid concentration of solutions. We have found that the SYBR Green dyes make excellent detection reagents for DNA and RNA spotted onto plastic wrap or paraffin sheets (e.g., Parafilm brand). Extremely small quantities of the dyes are required for this assay. Using the following simple procedure, we have obtained 10- to 25-fold better sensitivity with SYBR Green dyes than with 1  $\mu$ M ethidium bromide, depending on the nucleic acid tested. The procedure is as follows:

**Step 1.** Dilute an aliquot of the SYBR Green I (S-7563, S-7567, S-7585) or SYBR Green II (S-7564, S-7568, S-7586) DMSO stock solution 1:5000 in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8).

**Step 2.** Mix 5  $\mu$ L of a nucleic acid-containing sample with 5  $\mu$ L of the 1:5000 dilution of SYBR Green I dye or SYBR Green II dye prepared in step 1.

**Step 3.** Spot the mixture onto plastic wrap or a paraffin sheet that is directly placed on a transilluminator.

**Step 4.** Illuminate with 300 nm transillumination and photograph through a SYBR Green/Gold gel stain photographic filter (S-7569) using Polaroid 667 black-and-white print film. Photography is essential for obtaining the highest sensitivity.

**Step 5.** For a semiquantitative assay, repeat steps 2 through 4 with known amounts of nucleic acid and compare the signals with those from experimental samples.

Detection limits per spot of phage  $\lambda$  DNA are approximately 3 ng and 30 ng with SYBR Green I dye and ethidium bromide, respectively. Visual detection limits for ribosomal RNA under the same conditions are approximately 3 ng, 9 ng and 80 ng with SYBR Green II dye, SYBR Green I dye and ethidium bromide, respectively.

Dyes such as the ethidium homodimers and our dimeric cyanine dyes — the TOTO, YOYO, BOBO, POPO, JOJO and LOLO dyes in Table 8.2 — exhibit a high affinity for double-stranded nucleic acids but label small single-stranded oligonucleotides less well. This characteristic of ethidium homodimer-1 (E-1169, Section 8.1) was exploited to assay short self-annealing oligonucleotides for their ability to hybridize.<sup>52</sup> Because our dimeric cyanine dyes and the PicoGreen dsDNA quantitation reagent have extremely low intrinsic fluorescence in the absence of DNA, high fluorescence enhancements upon binding, higher quantum yields and much larger extinction coefficients than ethidium homodimer-1,<sup>2,53</sup> they should prove superior in this application.

### Hoechst 33258 Dye for Quantitating DNA in Solution

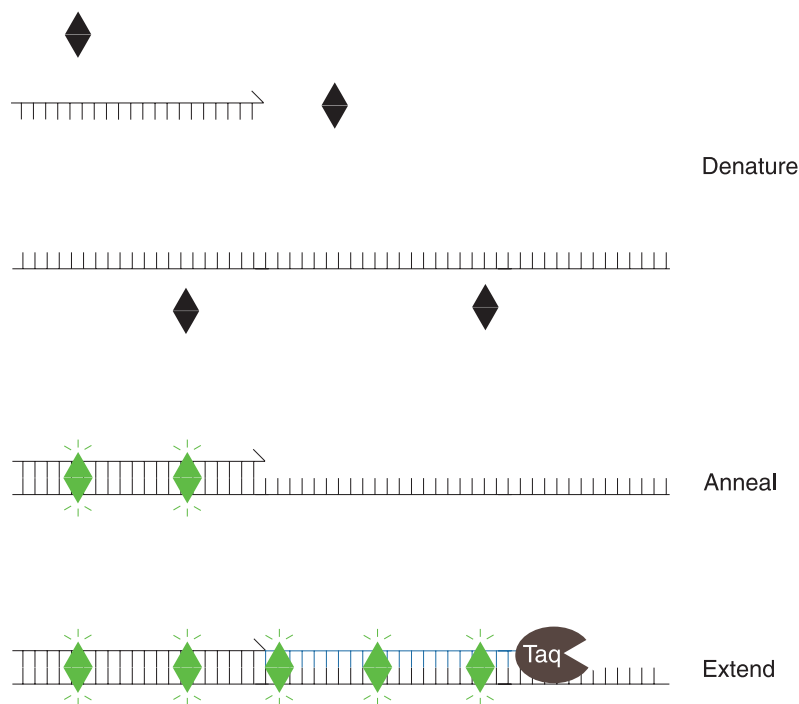
The Hoechst 33258 dye (H-1398, H-3569; FluoroPure grade, H-21491) has been extensively used to quantitate dsDNA in solution. Hoechst 33258 shows a fluorescence increase upon binding nucleic acids and a preference for binding to AT regions. Hoechst 33258 is selective (but not specific) for dsDNA over RNA in high-salt buffers and for dsDNA over ssDNA in low-salt buffers. The Hoechst 33258 dye can quantitatively detect from 10 ng/mL to ~10 µg/mL dsDNA when two different dye concentrations are used.<sup>10</sup> While this assay uses principles that are similar to other fluorescent assays, newer dyes such as the PicoGreen

reagent provide much higher sensitivity, better selectivity and a much broader dynamic range with a single dye concentration.

The FluoReporter Blue Fluorometric dsDNA Quantitation Kit (F-2962) provides the protocols developed by Rago and colleagues<sup>1</sup> for analyzing cellular DNA with the blue-fluorescent Hoechst 33258 nucleic acid stain. The kit enables researchers to detect ~10 ng of isolated calf thymus DNA or ~1000 mouse NIH 3T3 cells in a 200 µL sample (substantially lower levels are detectable using our CyQUANT Cell Proliferation Assay Kit described in Section 15.4). With this kit, quantitation of cellular DNA is rapid, and all manipulations can be carried out in microplate wells. The cells are lysed by freezing them in distilled water, which circumvents the requirement for extraction procedures used in other Hoechst 33258 dye-based protocols.<sup>10,54–57</sup> The diluted dye solution is then added to the lysed cells, and fluorescence is measured. Kit components include:

- Hoechst 33258 in dimethylsulfoxide (DMSO)/H<sub>2</sub>O
- TNE buffer
- A detailed protocol

Each kit provides sufficient reagents for assaying 2000 samples using a fluorescence-based microplate reader.



**Figure 8.58** Schematic representation of real-time PCR with the SYBR Green I dye. SYBR Green I dye (black diamonds) becomes fluorescent (green diamonds) upon binding to double-stranded DNA, providing a direct method for quantitating PCR products in real time.

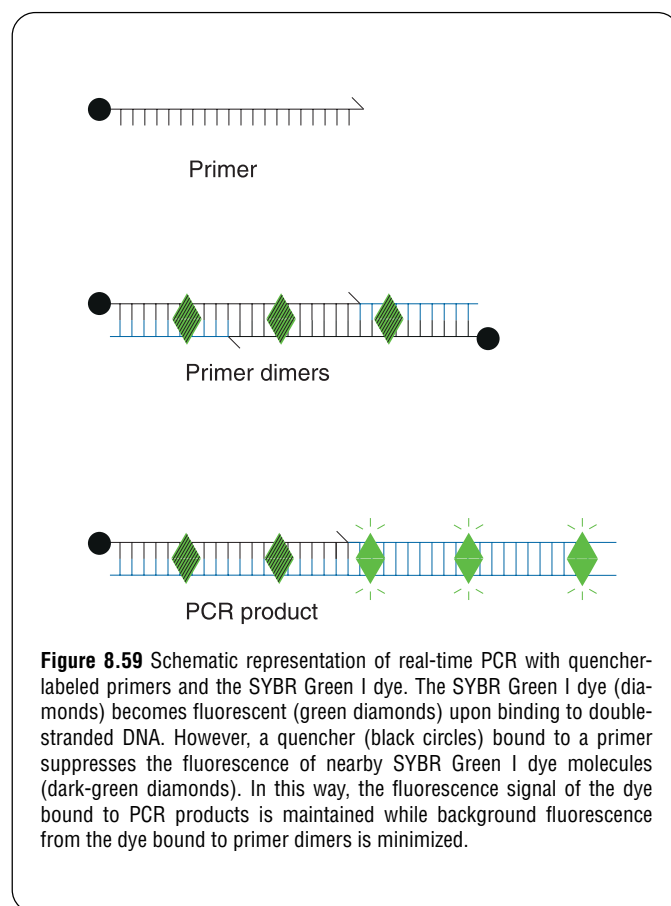
## Real-Time Quantitative PCR Using the SYBR Green I Nucleic Acid Gel Stain

Measurements of PCR products can be taken during the linear portion of the amplification reactions, allowing accurate quantitation of templates. Several methods exist for real-time quantitation of PCR products, including fluorescence resonance energy transfer techniques using fluorescently labeled primers or molecular beacons (Section 8.5). Identification of PCR products during the reaction can also be monitored using the SYBR Green I nucleic acid gel stain (S-7563, S-7567, S-7585); this method has been shown to be more precise than TaqMan assays using labeled oligonucleotide probes.<sup>58</sup> In addition, individual DNA molecules have been detected with on-line capillary PCR coupled with laser-induced fluorescence detection by adding the SYBR Green I stain to the reaction mixture.<sup>59</sup> Our patented SYBR Green I stain binds preferentially to dsDNA and thus can accurately quantitate the amount of double-stranded product in the presence of single-stranded oligonucleotide primers<sup>60</sup> (Figure 8.58). SYBR Green I stain is stable to the extremes of temperature required for PCR reactions and does not interfere with Taq polymerase. More specificity for quantitating desired products can be achieved by using the SYBR Green I stain after the assay to measure the melting temperature of the products.<sup>61–69</sup> Double-stranded DNA with no base mismatches will show a higher melting temperature than the nonspecific templates that contain mismatches. Further specificity may possibly be obtained using PNA probes labeled with one of our reactive SYBR dyes (S-21500, S-21501, S-21502; Section 8.2). Real-time quantitative PCR experiments can be carried out using instruments specialized for the application<sup>70,71</sup> or by quantitating amplification products manually at different time points.<sup>72</sup> Real-time quantitative PCR with the SYBR Green I dye has been used to develop reliable and simple diagnostic assays for detecting genetic mutations, including duplications and deletions in mosquito drug-resistance genes,<sup>73</sup> chromosomal translocations in human disease genes,<sup>65,74</sup> and base substitutions.<sup>62,75,76</sup> It has also been used for the unequivocal identification of viral, bacterial or fungal pathogens.<sup>61,63,64,66,67,77–79</sup> In addition, this method has been used successfully for quantitative reverse-transcription PCR.<sup>58,70</sup>

### A Useful Technique to Reduce Background Fluorescence in Nucleic Acid Detection

Researchers commonly use SYBR Green I nucleic acid gel stain to detect polymerase chain reaction (PCR) or other nucleic acid amplification products in real time.<sup>60,64,65,68,69,71,74,78,80</sup> The PicoGreen reagent (P-7581, P-11495) can also be used to detect such products after amplification<sup>7,12</sup> and to detect products of chain elongation by telomerase,<sup>23</sup> reverse transcriptase and DNA polymerase<sup>5</sup> (Section 8.7). One drawback of these methods, however, is that both primers and primer dimers can contribute to the fluorescence signal, limiting sensitivity. To solve this problem, we have developed a patented technique that reduces the

contribution of primers and primer dimers to the fluorescence signal. A QSY 7 or QSY 9 dye attached to the 5'-terminus of an oligonucleotide primer effectively quenches the fluorescence of nucleic acid stain molecules that bind to the primer or its dimers. As the chain elongates, the nucleic acid stain molecules that bind at sites sufficiently remote from the quencher exhibit fluorescence (Figure 8.59). Thus, the signal in PCR measurements more accurately indicates initial target numbers, and the background fluorescence in reverse transcription, telomerase or DNA polymerization assays is reduced. The preferred QSY dyes for these conjugations are the succinimidyl esters of QSY 7 carboxylic acid (Q-10193) or more water soluble QSY 9 carboxylic acid (Q-20131), which are readily conjugated to amine-derivatized oligonucleotides. The QSY 7 and QSY 9 dyes have broad visible absorption similar to that of tetramethylrhodamine (Figure 8.47). Our QSY 21 carboxylic acid succinimidyl ester (Q-20132) has even longer wavelength absorption (near 660 nm, Figure 1.66) and is a highly efficient quencher for red-fluorescent dyes such as the Alexa Fluor 633, Alexa Fluor 647, TOTO-3 and TO-PRO-3 dyes (Table 1.8). QSY 35 acetic acid, succinimidyl ester (Q-20133) is an excellent fluorescence acceptor from blue-fluorescent dyes such as the Hoechst and DAPI nucleic acid stains (Figure 1.66).



## References

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## Product List — 8.3 Nucleic Acid Detection and Quantitation in Solution

Cat #	Product Name	Unit Size
F-2962	FluoReporter® Blue Fluorometric dsDNA Quantitation Kit *200–2000 assays*	1 kit
H-3569	Hoechst 33258 (bis-benzimide) *10 mg/mL solution in water*	10 mL
H-1398	Hoechst 33258, pentahydrate (bis-benzimide)	100 mg
H-21491	Hoechst 33258, pentahydrate (bis-benzimide) *FluoroPure™ grade*	100 mg
O-11492	OliGreen® ssDNA Quantitation Kit *200–2000 assays*	1 kit
O-7582	OliGreen® ssDNA quantitation reagent *200–2000 assays*	1 mL
P-7589	PicoGreen® dsDNA Quantitation Kit *200–2000 assays*	1 kit
P-11496	PicoGreen® dsDNA Quantitation Kit *200–2000 assays* *special packaging*	1 kit
P-7581	PicoGreen® dsDNA quantitation reagent *200–2000 assays*	1 mL
P-11495	PicoGreen® dsDNA quantitation reagent *200–2000 assays* *special packaging*	10 x 100 µL
Q-10193	QSY® 7 carboxylic acid, succinimidyl ester	5 mg
Q-20131	QSY® 9 carboxylic acid, succinimidyl ester	5 mg
Q-20132	QSY® 21 carboxylic acid, succinimidyl ester	5 mg
Q-20133	QSY® 35 acetic acid, succinimidyl ester	5 mg
R-21495	RediPlate™ 96 PicoGreen® dsDNA Assay Kit *one 96-well microplate*	1 kit
R-21496	RediPlate™ 384 PicoGreen® dsDNA Assay Kit *one 384-well microplate*	1 kit
R-11490	RiboGreen® RNA Quantitation Kit *200–2000 assays*	1 kit
R-11491	RiboGreen® RNA quantitation reagent *200–20,000 assays*	1 mL
S-7563	SYBR® Green I nucleic acid gel stain *10,000X concentrate in DMSO*	500 µL
S-7567	SYBR® Green I nucleic acid gel stain *10,000X concentrate in DMSO*	1 mL
S-7585	SYBR® Green I nucleic acid gel stain *10,000X concentrate in DMSO* *special packaging*	20 x 50 µL
T-11493	20X TE buffer *RNase free*	100 mL

## Career Opportunities at Molecular Probes

Molecular Probes is a rapidly expanding and financially stable biotechnology company with research, development and production facilities in Eugene, Oregon, and additional customer service activities in Leiden, The Netherlands. We are the leading source of fluorescence detection technology as applied to cell biology, neurochemistry, proteomics and genomics. Molecular Probes provides

products, expertise and services to many companies in the areas of reagents, instrumentation and high-throughput screening. Our laboratories are equipped with advanced instrumentation. Our continued growth frequently provides employment opportunities for scientists and others. For more information, see our Web site ([www.probes.com/about/jobs](http://www.probes.com/about/jobs)).