

8.7 Analysis of DNA Structure, DNA Binding and DNA Damage

Nucleic Acid Conformational Analysis

A number of conventional dyes have been used to analyze nucleic acid conformation *in vitro* and *in vivo*:

- Acridine orange (A-1301, A-3568; Section 8.1) is one of the most popular and versatile fluorescent stains for histochemistry and cytochemistry and can provide a wide variety of information about the *in situ* content, molecular structure, conformation and environment of many nucleic acid-containing cell constituents.¹
- Fluorescence photobleaching of DNA that has been photolytically labeled with ethidium monoazide (E-1374, Section 8.1) permits measurement of slow reorientational motions.²
- The fluorescence intensity and binding affinity of the Hoechst dyes appear to be highly dependent on the sequence and conformation of the DNA base pairs.^{3–5} For example, staining by Hoechst 33258 (H-1398, H-3569; FluoroPure grade, H-21491; Section 8.1) can discriminate parallel and antiparallel stem regions in hairpin DNA conformations.⁶
- The fluorescence lifetime of the PicoGreen dye (P-7581, P-11495; Section 8.3) bound to single-stranded DNA is reported to be different when bound to double-stranded DNA.⁷

We also anticipate that several of our cyanine dyes (Section 8.1) — in particular the SYTO dyes (Table 8.3) — may be useful in these applications because many of these stains appear to yield environment-sensitive metachromatic shifts upon binding to nucleic acids. Fluorescence of the TOTO-1, YOYO-1, BOBO-1 and POPO-1 dyes (Table 8.2) is dependent on nucleic acid secondary structure; a shift to longer-wavelength emission and a concomitant drop in quantum yield are observed upon binding of these dyes to single-stranded nucleic acids at high dye:base ratios.⁸ Most of our nonsymmetrical cyanine dyes show this spectral shift, and some show sequence selectivity in their fluorescence intensity as well.

Examining the Behavior of Single Nucleic Acid Molecules

Once bound to nucleic acids, several of the cyanine dyes in Section 8.1 are so bright that they can be used to directly visualize single nucleic acid molecules in the fluorescence microscope (Figure 8.5, Figure 8.129). The YOYO-1 and POPO-3 dyes (Y-3601, P-3584) dyes have also been used to follow the making and breaking of single chemical bonds.^{9,10} A number of laboratories have taken advantage of the high sensitivity of these dyes to detect single nucleic acid molecules and to study biopolymer behavior:

- Video microscopy has been used to observe relaxation of YOYO-1 dye-stained phage lambda DNA multimers, after stretching in a fluid flow,¹¹ on a surface¹² or with optical tweezers.¹³ TOTO-1 dye (T-3600) has also been used in this application.¹⁴

- Individual YOYO-1 dye–ssDNA molecular complexes have been imaged in solution by fluorescence video microscopy.¹⁵
- Molecular combing, a technique that uses a receding fluid interface to elongate DNA molecules for optical mapping of genetic loci, was developed using the YOYO-1 dye.¹⁶
- Adsorption and desorption of single molecules of YOYO-1 dye-stained phage lambda DNA have been observed on fused-silica and C₁₈ chromatographic surfaces.¹⁷
- The activity of a single RecBCD enzyme, which unwinds and separates the strands of dsDNA, has been studied using YOYO-1 dye-stained dsDNA in conjunction with optical tweezers and epifluorescence microscopy.^{18,19}
- Our YOYO-1 dye (Y-3601) has been used to stain DNA manipulated in solution by changing electronic fields, a technique that could prove valuable in miniaturizing and automating analysis of DNA fragments.²⁰
- Staining with the YOYO-1 dye (Y-3601) was used to observe the interaction of DNA with various liposomes²¹ and to size plasmids in a flowing stream.²²
- The YOYO-1 dye was also used to detect radiation-induced double-strand breaks in individual electrostretched bacterial DNA molecules.²³
- Single-molecule imaging of nucleic acids stained with either YOYO-1 or POPO-3 or a combination of the two dyes through collection of the entire fluorescence spectrum of their complex has been reported.²⁴
- Highly sensitive sheath-flow techniques have also been developed for detecting and discriminating the size of single TOTO-1 dye–DNA molecular complexes.^{25–27}
- Large fragments of DNA stained with our TOTO-1 dye (T-3600) have been sorted by flow cytometry. This extremely rapid analytical method yields a linear relationship between the fluorescence intensity and the fragment size over a 10–50 kilobase pair range.²⁸
- The POPO-1 (P-3580, Section 8.1) and POPO-3 (P-3584) stains have been used to sensitively detect single DNA fragments by flow cytometry using two-photon fluorescence excitation.²⁹
- The POPO-3 dye (P-3584) has been used to study a single chemical reaction with an individual DNA molecule. POPO-3 dye-stained DNA molecules stretched taut on a glass surface relax when a focused laser beam causes fluorescence-related breakage of the DNA backbone, forming a gap that is visible by fluorescence microscopy.⁹
- The TOTO-1 (T-3600), YOYO-1 (Y-3601), POPO-3 (P-3584) and SYBR Green I (S-7563, S-7567, S-7585) dyes have been used to visualize lambda DNA that has been stretched between beads with optical tweezers.^{11,30–32}
- Fragment sizing on single molecules of dsDNA stained with our PicoGreen reagent has also been reported.^{33,34}
- The SYTOX Orange dye (S-11368) is the preferred dye for single-molecule sizing of DNA fragments by flow cytometry in an instrument equipped with a Nd:YAG laser.³⁵
- DAPI (D-1306, D-3571; FluoroPure grade, D-21490) has also been employed to detect a single DNA molecule in solution³⁶ and by fluorescence microscopy³⁷ and to detect femtograms of DNA in single cells and chloroplasts.³⁸

The high affinity and bright fluorescence of other cyanine dimers has allowed researchers to follow stained and transfected plasmids or stained virus particles within a cell.^{39–43}

DNA Binding Assays

Electrophoretic Mobility-Shift (Bandshift) Assays

Bandshift assays to analyze DNA–protein interactions are conventionally performed using radioactively labeled DNA fragments. However, use of our high-sensitivity fluorescent dyes makes these assays much simpler to perform and eliminates radioactive waste issues. For instance, SYBR Green I nucleic acid gel stain (S-7567, S-7563, S-7585) has been used to post-stain gels after electrophoresis and can detect bound and unbound DNA fragments with high sensitivity⁴⁴ (Figure 8.130). The SYBR Gold nucleic acid gel stain (S-11494) is potentially even more useful in bandshift experiments because of its higher sensitivity.

Fluorescent dyes have also been used to stain the DNA fragments or proteins before electrophoresis. For instance, proteins or DNA labeled covalently with a reactive fluorescent dye (Chapter 1, Section 8.2) can be easily tracked during capillary electrophoresis to monitor DNA–protein interactions.⁴⁵ High affinity nucleic acid stains have also been used prior to electrophoresis, although they can potentially interfere with protein binding and alter mobility on the gel. The ethidium homodimer-1 (EthD-1, E-1169; Section 8.1), YOYO-1 and TOTO-1 dyes have been shown by several laboratories to be useful tools for labeling DNA prior to electrophoresis in electrophoretic mobility-shift (bandshift) assays. EthD-1 and TOTO-1 were used to examine interactions between the binding domain of the *Kluyveromyces lactis* heat shock transcription factor and its specific binding site.⁴⁶ YOYO-1 dye has been used to study the association of *E. coli* RNA polymerase with DNA templates⁴⁷ and the binding of a heat-shock transcription factor to its promoter.⁴⁸ All ten of our spectrally distinct (Figure 8.1), high-affinity dimeric cyanine dyes (Table 8.2) and the ethidium homodimers are potentially useful for multicomponent analysis in this application.

DNA Binding Assays in Solution

Molecular beacons exploit fluorescence resonance energy transfer (FRET) to simplify detection of nucleic acid hybridization in solution (Section 8.5, Figure 8.101). This method has also proven useful for studying DNA–protein interactions in solution. Binding of a molecular beacon to lactic dehydrogenase separated the fluorophore from the quencher on the two ends of the labeled oligonucleotide, resulting in an increase in fluorescence.⁴⁹ The assay is sufficiently accurate to measure binding constants. A molecular beacon was also used to develop a solution-based binding assay for α -CP₂, which is part of an RNA-binding complex.⁵⁰

Selective Cleavage of Nucleic Acids with a Chemical Nuclease

The thiol-reactive iodoacetamide of 1,10-phenanthroline (P-6879, Section 2.3) is a useful adjunct reagent for bandshift assays. Conjugation to thiol-containing ligands confers the metal-binding properties of this important complexing agent on the ligand. For example, the covalent copper–phenanthroline complex of oligonucleotides or nucleic acid-binding molecules in combination with hydrogen peroxide acts as a chemical nuclease to selectively cleave DNA or RNA.^{51–57} This reagent can also be conjugated to proteins to detect nucleic acid binding and targeted cleavage.⁵⁸

Assessing DNA Damage

Comet (Single-Cell Gel Electrophoresis) Assay to Detect Damaged DNA

The comet assay — or single-cell gel electrophoresis assay — is used for rapid detection and quantitation of DNA damage from single cells.^{59–61} The comet assay is based on the alkaline lysis of labile DNA at sites of damage. Cells are immobilized in a thin agarose matrix on slides and gently lysed. When subjected to electrophoresis, the unwound, relaxed DNA migrates out of the cells. After staining with a nucleic acid stain, cells that

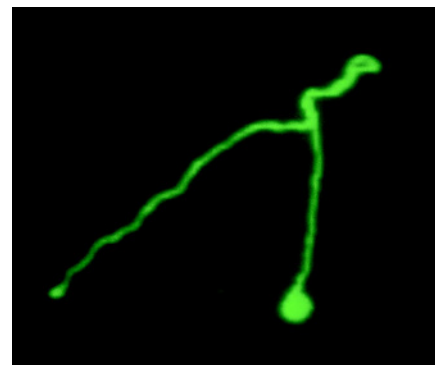


Figure 8.129 T2 phage genomic DNA stained with YOYO-1 (Y-3601), our high-affinity nucleic acid stain. This single 164-kilobase molecule, which appears to be supercoiling, was imaged under rigorously deoxygenated conditions with a cooled CCD camera. This work was performed in collaboration with Sergio Gurrieri and Carlos Bustamante, Institute of Molecular Biology, University of Oregon.

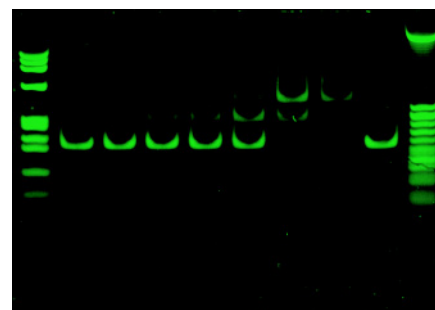


Figure 8.130 Electrophoretic bandshift assays using SYBR Green I nucleic acid gel stain (S-7563, S-7567, S-7585). The association between a 208-bp fragment purified from *Ava*I-digested plasmid p5S208-12 and a mutant restriction endonuclease (*Eco*RI/*Gln*111) was analyzed on a 4% native polyacrylamide gel. Samples containing approximately 50 ng total fragments and various amounts of the mutant enzyme were subjected to electrophoresis and stained with SYBR Green I nucleic acid gel stain. Gel staining was visualized using 254 nm epillumination and then photographed using Polaroid 667 black-and-white print film and a SYBR photographic filter (S-7569). Lane 1 contains *Haell*I-digested ϕ X174 RF DNA markers; lanes 2 through 9 contain 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 0 μ M *Eco*RI/*Gln*111; lane 10 contains *Hha*I-digested plasmid p5S208-12 as a size standard.

Single-molecule detection and multiphoton excitation represent the frontiers of fluorescence applications that are made possible by several of Molecular Probes' products.

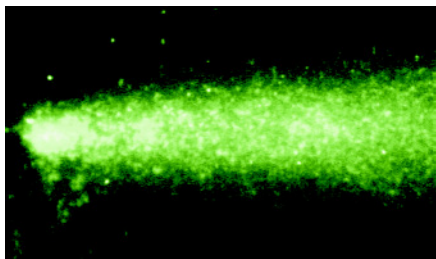


Figure 8.131 Comet assay with SYBR Green I nucleic acid gel stain (S-7563, S-7567, S-7585). DNA fragmentation associated with oxidative DNA damage was visualized using Trevigen's CometAssay kit. HL-60 cells were treated with H₂O₂ and immobilized onto a Trevigen CometSlide for analysis. The cells were gently lysed, washed and treated with endonuclease. Slides were subjected to electrophoresis in alkaline electrophoresis buffer and stained with SYBR Green I stain.

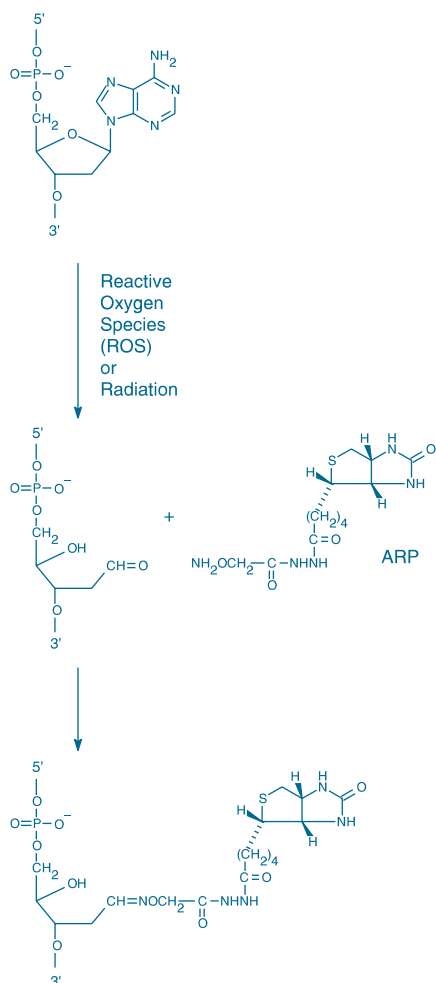


Figure 8.132 Aldehyde-reactive probe used to detect DNA damage. The aldehyde-reactive probe (ARP, A-10550) reacts with aldehyde groups formed when reactive oxygen species depurinate DNA. The reaction forms a covalent bond linking the DNA to biotin. The biotin can then be detected using a fluorophore- or enzyme-linked streptavidin.

have accumulated DNA damage exhibit brightly fluorescent comets, with tails of DNA fragmentation or unwinding (Figure 8.131). In contrast, cells with normal, undamaged DNA appear as round dots, because their intact DNA does not migrate out of the cell. The ease and sensitivity of the comet assay has provided a fast and convenient way to measure damage to human sperm DNA,⁶² monitor the sensitivity of tumor cells to radiation damage⁶³ and to assess the sensitivity of molluscan cells to toxins in the environment.⁶⁴ The comet assay can also be used in combination with FISH to identify specific sequences with damaged DNA.⁶³

Comet assays have traditionally been performed using ethidium bromide (E-1305, E-3565) to stain the DNA;⁶⁰ however, our YOYO-1 dye (Y-3601) increases the sensitivity of the assay eightfold compared to ethidium bromide and the fluorescence background from unbound YOYO-1 dye is negligible.⁶¹ Use of the SYBR Gold and SYBR Green I stains (Section 8.4) further improves the sensitivity of this assay.^{65,66}

TUNEL Assay for In Situ Detection of Fragmented DNA

To detect fragmented DNA in labeled cells, terminal deoxynucleotidyl transferase (TdT) along with a fluorophore-, biotin-, or hapten-labeled dUTP can be added to cells. TdT adds the labeled nucleotide to all available 3'-ends — the more fragmented the DNA, the more 3'-ends are available and the brighter the fluorescent signal. Direct TUNEL assays using ChromaTide BODIPY FL-14-dUTP (C-7614) to visualize DNA fragment ends are four times more sensitive than TUNEL assays using fluorescein-labeled dUTP⁶⁷⁻⁷⁰ (Figure 15.70). Terminal deoxynucleotidyl transferase (TdT)-catalyzed incorporation of bromo dUTP into nucleic acids of apoptotic cells and detection of the incorporated BrdU with an antibody conjugate is the basis of the APO-BrdU TUNEL Assay Kit (A-23210, Section 15.5). Indirect TUNEL assays using probes such as our ChromaTide biotin-11-dUTP (C-11411, Section 8.2) or ChromaTide DNP-11-dUTP (C-7610, Section 8.2) allow for amplification of the signal with our fluorophore- or enzyme-conjugated streptavidin conjugates (Section 7.6, Table 7.17) or with anti-DNP antibody (Section 7.4). Several additional assays for apoptosis can be found in Section 15.5.

Microplate-Based Assays for DNA Damage

Abasic sites in DNA, generated spontaneously or caused by free radicals, ionizing radiation or mutagens like MMS (methyl methanesulfonate), are one of the most common lesions in DNA and are thought to be important intermediates in mutagenesis. A quick and sensitive microplate assay for abasic sites can be performed using ARP (A-10550, Figure 8.132), a biotinylated hydroxylamine that reacts with the exposed aldehyde group at abasic sites. Biotins bound to the abasic sites can be quantitated with our fluorescent dye- or enzyme-conjugated streptavidin complexes⁷¹⁻⁷³ (Section 7.6, Table 7.17). ARP is permeant to cell membranes, permitting detection of abasic sites in living cells.⁷⁴

The PicoGreen reagent has also been used to simplify denaturation assays for DNA damage. Strand breaks in dsDNA that result from DNA damage can be quantified by measuring the relative amounts of ssDNA and dsDNA in a damaged sample. The relative amounts of dsDNA to ssDNA can be assessed by measuring the increase in absorbance at 260 nm or by separating the two forms of DNA by alkaline sucrose gradient centrifugation,⁷⁵ filters,⁷⁶ or hydroxyapatite chromatography.⁷⁷ However, the absorbance-based technique suffers from low sensitivity and thus requires relatively large sample sizes⁷⁸ and separation of ssDNA from dsDNA is laborious. This assay becomes much simpler and more sensitive using the PicoGreen dsDNA quantitation reagent (P-7581, P-7589, P-11495, P-11496, R-21495, R-21496; Section 8.3), which preferentially detects dsDNA in the presence of ssDNA.⁷⁹⁻⁸¹ The dye can be added directly to the sample and the fluorescence signal read on a fluorescence-based microplate reader. This method makes it possible to screen large numbers of very small samples in a high-throughput setting. The PicoGreen reagent was also used to develop a homogeneous PCR-based genotyping assay.⁸² Because the products do not need to be run on a gel, the assay can be easily adapted for high throughput particularly using the RediPlate 96 and RediPlate 384 versions of the PicoGreen dsDNA assays (R-21495, R21496; Section 8.3).

Assays for Enzymes that Modify Nucleic Acids

Gel-Based Assays for DNase Detection

Our SYBR Green I stain (S-7563, S-7567, S-7585) has been used to develop DNase assays that show up to a 64-fold increase in sensitivity over similar ethidium bromide-based assays and up to 10,000-fold higher sensitivity than the traditional UV hyperchromicity assay. In a fast and simple assay, a single-length fragment of DNA can be incubated with the sample, followed by a short gel electrophoresis. Staining the gel with the SYBR Green I dye permits easy detection of less than 10^{-5} Kunitz units (~ 5 pg) of DNase activity.⁸³ Even greater sensitivity can be achieved using the single radial enzyme diffusion (SRED) method,⁸⁴ in which the SYBR Green I stain is mixed with DNA in melted agarose and the mixture is poured into a 2 mm thick slab. The sample to be tested is poured into 1.5 mm circular wells punched out of the solidified agarose slab. As the sample diffuses through the agarose, the DNase degrades the DNA, creating dark circles around the sample well that do not show staining with the SYBR Green I dye when illuminated with UV light. The radius of these dark circles is proportional to the level of DNase activity. This method allows detection of as little as 2×10^{-7} units (~ 0.1 pg) of DNase I or 2×10^{-6} (~ 0.9 pg) of DNase II. A third DNase assay — called the dried agarose film overlay (DAFO) method — uses the SYBR Green I stain to detect the presence of DNase activity in a polyacrylamide gel, allowing the identification of heterogeneities in DNase species.⁸⁵ This method allows the detection of 4×10^{-6} units (~ 2 pg) DNase I or DNase II.

Solution-Based Assays for Nuclease Detection

Contaminating DNases are often responsible for poor resolution of DNA fragments, degradation of samples and nicking of supercoiled plasmids. Conventional DNase assays detect DNase activity by monitoring the increase in UV absorbance that occurs when the base pairs unstack as the DNA is degraded. This absorbance method, however, is intrinsically insensitive as it requires large sample volumes and relies on small changes in absorbance. In contrast, our dyes for nucleic acid detection show a tremendous fluorescence increase upon binding to nucleic acids, but their fluorescence is not affected by the presence of a large excess of a nucleotide or very short oligonucleotides. Thus, nuclease activity can be easily and accurately measured by the decrease in fluorescence in the presence of one of these dyes. For instance, the YOYO-1 nucleic acid stain (Y-3601) has been used in a fluorescence-based microplate assay for nuclease activity.⁸⁶ This assay takes advantage of the large fluorescence enhancement of the YOYO-1 dye upon binding to nucleic acids and corresponding lack of fluorescence in the presence of released nucleotides and very small nucleic acid fragments. Other dyes — in particular our PicoGreen dsDNA quantitation reagent (P-7581, P-11495; Section 8.3) — are likely to be more suitable for this assay. Similarly, use of the RiboGreen RNA quantitation reagent (R-11490, R-11491; Section 8.3) should allow ultrasensitive detection of ribonuclease (RNase) activity.

Using a design similar to that of molecular beacons (Section 8.5), the stem sequence in an oligonucleotide hairpin loop can be modified to be a substrate for specific DNA cleavage agents, including nucleases. Dubbed a “break light,” this substrate shows increased fluorescence as the cleavage agent breaks the DNA strand, separating the fluorophore from the quencher.^{87,88}

An Assay for Reverse Transcriptase Activity

The EnzChek Reverse Transcriptase Assay Kit (E-22064) is a convenient, efficient and inexpensive assay for measuring reverse transcriptase activity (Figure 8.133). The key to this method is our PicoGreen dsDNA quantitation reagent, which preferentially detects dsDNA or RNA–DNA heteroduplexes over single-stranded nucleic acids or free nucleotides. In the assay, the sample to be measured is added to a mixture of a long poly(A) template, an oligo(dT) primer and dTTP. Reverse transcriptase activity in the sample results in the formation of long RNA–DNA heteroduplexes, which are detected by the PicoGreen reagent at the end of the assay. In less than an hour, samples can be read in a fluorometer or microplate reader with filter sets appropriate for fluorescein (FITC). The assay is sensitive, detecting as little as 0.02 units of HIV reverse transcriptase, and has about a 50-fold linear range (Figure 8.134). Because it is much more rapid and less expensive than standard isotopic assay or immunoassays, it is suitable for testing large numbers of biological samples. The assay’s simplicity also makes it useful

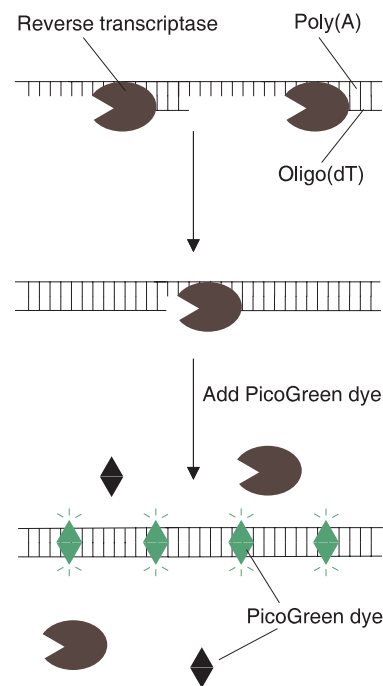


Figure 8.133 Schematic diagram of the mechanism used in the EnzChek Reverse Transcriptase Assay Kit. A poly(A) substrate and oligo(dT) primers are provided in the EnzChek Reverse Transcriptase Assay Kit; after hybridization, the primers can be elongated in the presence of reverse transcriptase and dTTP. The resulting DNA–RNA hybrid is detected using the PicoGreen dsDNA detection reagent, which becomes fluorescent upon binding to DNA–RNA hybrids.

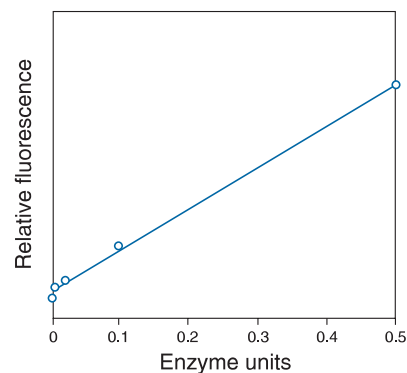


Figure 8.134 Detection of HIV reverse transcription using the EnzChek Reverse Transcriptase Assay Kit (E-22064), showing detection of 0.02 to 0.5 units of the enzyme. One unit of activity is defined as incorporation of 1 nmol of dTTP in 10 minutes at 37°C, using poly(A) and oligo(dT) as the template and primer, respectively.

Our extensive assortment of fluorescent enzyme substrates is described in Chapter 10.

for automated high-throughput screening of reverse transcriptase inhibitors.

The EnzChek Reverse Transcriptase Assay Kit (E-22064) contains:

- The PicoGreen dsDNA quantitation reagent
- A lambda DNA standard
- A poly(A) ribonucleotide template
- An oligo(dT)16 primer
- TE buffer, polymerization buffer and an EDTA solution
- A detailed protocol

Sufficient amounts of reagents are provided for approximately 1000 fluorescence microplate assays.

Telomerase

In a gel-based assay for detection of telomerase activity (the telomeric repeat amplification protocol or TRAP) in human cells and tumors, SYBR Green I dye staining was found to be more sensitive than silver staining and gave results comparable to those achieved with a radioisotope-based TRAP assay.^{89–91} Moreover, unlike the silver stains, the SYBR Green I stain did not label proteins carried over from the reaction mixture. The SYBR Gold stain was also shown to be more sensitive than silver staining in the TRAP assay, and much easier to use.⁹² The SYBR Green I stain (S-7567, S-7563, S-7585) has also been used to develop high sensitivity assays to detect topoisomerase activity.⁹³

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Product List — 8.7 Analysis of DNA Structure, DNA Binding and DNA Damage

Cat #	Product Name	Unit Size
A-10550	<i>N</i> -(aminooxyacetyl)- <i>N'</i> -(<i>o</i> -biotinoyl) hydrazine, trifluoroacetic acid salt (ARP)	10 mg
C-7614	ChromaTide™ BODIPY® FL-14-dUTP *1 mM in TE buffer*	25 µL
D-1306	4',6-diamidino-2-phenylindole, dihydrochloride (DAPI)	10 mg
D-21490	4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) *FluoroPure™ grade*	10 mg
D-3571	4',6-diamidino-2-phenylindole, dilactate (DAPI, dilactate)	10 mg
E-22064	EnzChek® Reverse Transcriptase Assay Kit *1000 assays*	1 kit
E-1305	ethidium bromide	1 g
E-3565	ethidium bromide *10 mg/mL solution in water*	10 mL
P-3580	POPO™-1 iodide (434/456) *1 mM solution in DMSO*	200 µL
P-3584	POPO™-3 iodide (534/570) *1 mM solution in DMF*	200 µL
S-11494	SYBR® Gold nucleic acid gel stain *10,000X concentrate in DMSO*	500 µL
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
S-11368	SYTOX® Orange nucleic acid stain *5 mM solution in DMSO*	250 µL
T-3600	TOTO®-1 iodide (514/533) *1 mM solution in DMSO*	200 µL
Y-3601	YOYO®-1 iodide (491/509) *1 mM solution in DMSO*	200 µL