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ASSAYWISE LETTER

LIFE SCIENCE RESOURCES AND APPLICATIONS

Tools to unravel the secrets of DNA

Featuring

Low-cost, ultra-sensitive fluorescence detection of DNA by gel electrophoresis using environmentally benign Gelite

Enzymatic and Chemical Labeling Strategies for Oligonucleotides

Sensitive Identification of Newly Synthesized DNA In Situ using click chemistry-based Bucculite

A Practical Guide for the Detection and Analysis of PCR Products

AAT Bioquest®

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Low-cost, Ultra-sensitive Fluorescence

Detection of DNA By Gel Electrophoresis Using Environmentally Benign Gelite

Abstract

Gelite™ Safe DNA Gel Stain has been developed specifically to be a less hazardous alternative to ethidium bromide (EtBr) and SYBR® Green dyes for staining DNA in agarose and polyacrylamide gels, without sacrificing detection sensitivity. The exceptional sensitivity and strong DNA binding affinity of Gelite™ Safe DNA Gel Stain allows for DNA to be stained without any requirement for destaining. In addition to its superior binding properties, Gelite™ Safe DNA Gel Stain is essentially non-fluorescent in the absence of nucleic acids, displaying very low gel matrix background fluorescence. Upon binding to nucleic acids, Gelite™ Safe DNA Gel Stain exhibits a significant fluorescence enhancement, several orders of magnitude greater than that of EtBr. Gelite™ Safe DNA Gel Stain was optimized to be compatible with various gel documentation and imaging instruments, including UV and visible light transilluminators, CCD-camera-based gel documentation systems, and laser scanners. It is the first single formulation that can be imaged either in the green or red emission channel of such devices. Unlike the membrane-permeant SYBR® Green dye, which is highly toxic to cells and the environment, the membrane-impermeant properties of Gelite™ Safe DNA Gel Stain make it a much safer, noncytotoxic alternative, as demonstrated in the WST-8 cytotoxicity assay. Furthermore, Ames testing has confirmed Gelite™ Safe DNA Gel Stain is significantly less mutagenic than EtBr and SYBR® Green stains, even at concentrations well above the working concentration required for gel staining.

Introduction

Ethidium bromide (EtBr) is an intercalating agent commonly employed as a fluorescent nucleic acid stain in biomedical research laboratories for analytical techniques such as agarose gel electrophoresis (1, 2). The health hazards of working with toxic chemicals, such as EtBr, are well recognized, as are the accompanying economic considerations of steeply rising costs that accompany appropriate handling and disposal of mixed waste (1, 2). Conventional DNA staining procedures in agarose and polyacrylamide gels generate significant amounts of toxic waste, creating a need for better reagent alternatives that minimize environmental impact. Furthermore, EtBr is typically visualized using a high energy, 312 nm UV –transilluminator, which itself can represent a hazard to laboratory personnel, potentially leading to sunburn, photokeratitis, photoconjunctivitis, retinal burns, cataracts, and even blindness, if appropriate operating precautions are not heeded (3).

The cyanine dye, Gelite™ Safe DNA Gel Stain, represents the newest addition to AAT Bioquest's growing family of nucleic acid detection reagents. Gelite™ Safe DNA Gel Stain, provides a greatly improved safety profile and uncompromised detection sensitivity, serving as an environmentally benign alternative to conventional DNA stains for gel electrophoresis.

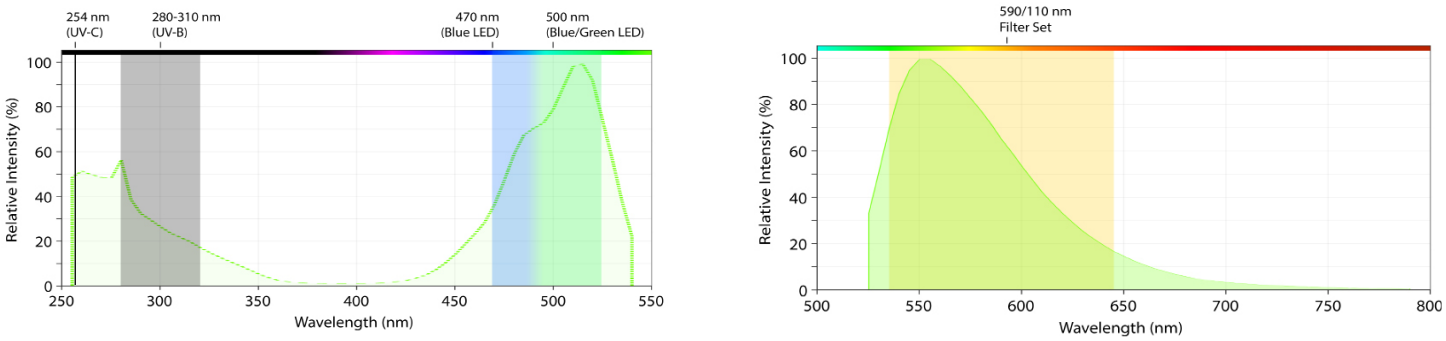

Figure 1. Normalized excitation (left) and emission (right) spectra for Gelite™ Safe DNA Gel Stain bound to DNA in TE buffer. DNA (Calf DNA stock solution: 0.85 mg/mL Sigma D1501) was added at a final concentration of 3 µg/mL. Excitation-emission spectra were measured after addition of the DNA, using a Varian Cary Eclipse fluorescence spectrophotometer (Agilent, Santa Clara, CA).

Upon binding to DNA, the resulting DNA-dye-complex absorbs visible green light with ?max of 512 nm, which is readily detected using Blue or Blue-Green LED systems, but also absorbs significantly in the UV region of the spectrum and is thus readily detectable with a UV light source. The DNA-dye complexes emit in the yellow region of the visible light spectrum, with ?max centered at about 550 nm (Figure 1). The emission peak is relatively broad, facilitating detection of the dye in the green through red regions of the visible light spectrum, thus ensuring compatibility with a wide range of gel documentation and imaging systems.

Human and Environmental Safety

AAT Bioquest is committed to designing our products to be safe to use and environmentally friendly. We endeavor to enable our customers to make the world healthier, cleaner, and safer. Ethidium bromide (EtBr) has been commonly used as a DNA stain for decades. However, EtBr is harmful if ingested and is quite toxic if inhaled. EtBr has been shown to be mutagenic in various tests and is also an aquatic toxin. SYBR® Safe stain was introduced as a safer alternative to EtBr and SYBR® Green dye, but unfortunately, it is much less sensitive than SYBR® Green stain, only displaying sensitivity comparable to EtBr (4).

Gelite™ Safe DNA Gel Stain is a safer alternative to conventional dyes, such as ethidium bromide and SYBR® Green I dye (5, 6). This is readily demonstrated using widely employed in vitro biological assays. Upon testing with the Ames mutagenicity or the WST-8 cytotoxicity assays, Gelite™ Safe DNA Gel Stain exhibits very low mutagenicity and low cytotoxicity (Figure 2). This contrasts markedly with other commonly implemented gel stains such as EtBr, SYBR® Green I, SYBR® Gold, and GelRed® stains (4). Additionally, the DNA stain is stable at room temperature, facilitating long-term storage without consuming valuable refrigeration or freezer space. The non-toxic, environmentally friendly nature of the dye simplifies its disposal, which can be accomplished by discarding into the regular trash.

Detection Sensitivity and Instrument Compatibility

With Gelite™ Safe DNA Gel Stain, there is no need to sacrifice DNA detection sensitivity for safety (Figure 3). While the limit of DNA detection for SYBR® Safe gel stain is approximately 3 ng, Gelite™ Safe DNA Gel Stain detects as little as 0.67 ng DNA. Additionally, the gel stain provides greater sensitivity, with lower background fluorescence, than most conventional DNA gel stains (4). The dye is compatible with denaturing and native agarose and polyacrylamide gels. It can be imaged with various standard imaging systems, such as a 360 nm black light UV epi- or trans-illuminator with a film or digital camera, laser scanner, or Blue-Green LED (490–495 nm) digital gel documentation

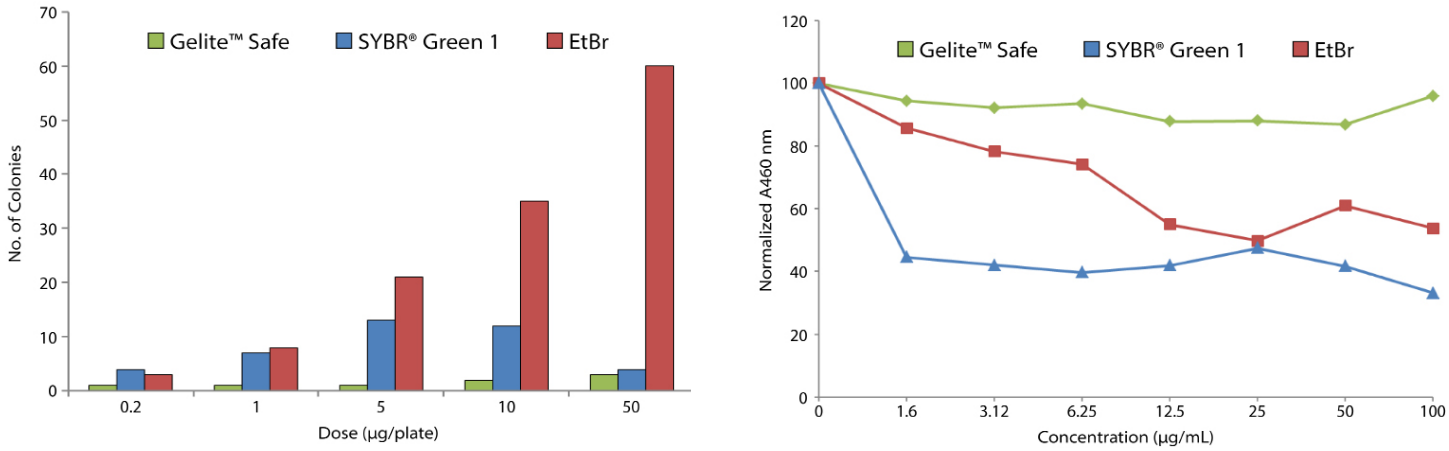

Figure 2. Representative DNA gel stain mutagenicity and cytotoxicity profiles. (A.) Mutagenicity in the Salmonella/mammalian microsome reverse mutation assay (Ames test). (B.) Cytotoxicity in the WST-8 Cytotoxicity Assay. WST-8 is bio-reduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium. The amount of formazan produced is directly proportional to the number of living cells.

systems. Gelite™ Safe dye-stained DNA can readily be visualized using imaging systems equipped with lasers that emit at 473, 488, or 532 nm. Instrument manufacturers can be consulted for optimal filter settings to use in combination with Gelite™ Safe DNA Gel Stain. One important feature of Gelite™ Safe DNA Gel Stain is that the dye maximally absorbs at around 500 nm, allowing it to be excited by visible LED light sources. Visible light illumination avoids personnel exposure to harmful UV irradiation. In addition, cloning efficiency with gel-purified DNA is improved with visible light illuminators, likely because they avoid UV damage to DNA during gel excision (7). Generally speaking, Gelite™ Safe DNA Gel Stain has an excitation/emission profile similar to SYBR® Gold or SYBR® Safe dye and can be imaged and documented with any system that provides filters or settings optimized for these dyes (8).

Key Applications

DNA separation and wdetection by agarose gel electrophoresis is considered one of the most frequently employed techniques in biomedical research laboratories. Traditionally, DNA fragments loaded on agarose gels are stained with ethidium bromide and detected using a UV-transilluminator-based system. Gelite™ Safe DNA Gel Stain is an ultra-sensitive and low-cost dye suitable for DNA detection after denaturing or nondenaturing agarose or polyacrylamide gel electrophoresis. The dye can be used by prior incorporation into the matrix of precast gels or post-electrophoresis gel staining. Loading of the dye in sample wells is not recommended, however, since tight binding of the dye with the DNA can lead to migration anomalies during the electrophoretic separation. This phenomenon also occurs with other commonly used DNA gel stains, such as SYBR® Green and SYBR® Gold stains. Following staining, DNA can be extracted from the gel using either a column-based PCR clean-up system or an ethanol precipitation procedure. Greater than 99% of the stain can be removed from double-stranded DNA simply by ethanol precipitation.

Discussion

DNA is typically detected in agarose gels using fluorescent dyes, such as EtBr, SYBR® Green, or SYBR® Safe stains, or by silver staining procedures (3, 9). The advantages of Gelite™ Safe DNA Gel Stain compared with other commercially available DNA staining technologies has been shown to be high sensitivity detection, combined with low toxicity. Additionally, compared with silver staining, the Gelite™ Safe DNA Gel Staiwn is environmentally friendly and less of a nuisance to laboratory

personnel due to the absence of noxious solvent fumes. Unlike some silver staining methods, heavy metal ions, sodium hydroxide, ammonia, and formaldehyde are not required when using the fluorescent stain (9). Gelite™ Safe DNA Gel Stain allows sensitive detection of DNA using gentle staining conditions and harmless visible light sources that minimize artefactual damage to nucleic acids. Thus, the gentle staining procedure for DNA detection using Gelite™ Safe DNA Gel Stain should have benefits with respect to the elution of DNA from polyacrylamide and agarose gels for cloning and sequencing applications. Staining gels with Gelite™ Safe dye should be particularly attractive in educational settings, especially in combination with a harmless visible light illumination source, such as a Dark Reader? (Clare Research Chemical, Boulder, CO) or FastGene Blue/Green LED Gel Transilluminator (Bulldog Bio, Portsmouth, NH).

References

1. Ge Z, Sun T, Xing J, Fan X. "Efficient removal of ethidium bromide from aqueous solution by using DNA-loaded Fe3O4 nanoparticles." Environ Sci Pollut Res Int. 2019 Jan; 26(3):2387-2396.
2. Li Z, Chang PH, Jiang WT, Liu Y. "Enhanced removal of ethidium bromide (EtBr) from aqueous solution using rectorite." J Hazard Mater. 2020 Feb 15; 384:121254.
3. Harper, C, Emery, R J, Casserly, DM "An assessment of occupational exposures to ultraviolet radiation from transilluminator light boxes in the course of biomedical research procedures." J. Chem. Health Saf. 2008, 15, 2, 16–22.
4. Haines AM, Tobe SS, Kobus HJ, Linacre A. "Properties of nucleic acid staining dyes used in gel electrophoresis." Electrophoresis. 2015 Mar;36(6):941-4.
5. Singer VL, Lawlor TE, Yue S. "Comparison of SYBR Green I nucleic acid gel stain mutagenicity and ethidium bromide mutagenicity in the Salmonella/mammalian microsome reverse mutation assay (Ames test)." Mutat Res. 1999 Feb 2; 439 (1):37-47.
6. Ohta T, Tokishita S, Yamagata H. "Ethidium bromide and SYBR Green I enhance the genotoxicity of UV-irradiation and chemical mutagens in E. coli."Mutat Res. 2001 May 31; 492 (1-2):91-7.
7. Gründemann, D, Schömig, E. "Protection of DNA during preparative agarose gel electrophoresis against damage induced by ultraviolet light." BioTechniques 1996, 21 (5), 898.
8. Tuma RS, Beaudet MP, Jin X, Jones LJ, Cheung CY, Yue S, Singer VL. "Characterization of SYBR Gold nucleic acid gel stain: a dye optimized for use with 300-nm ultraviolet transilluminators." Anal Biochem. 1999 Mar 15; 268 (2): 278-88.
9. Liu W, Li R, Ayalew H, Xia Y, Bai G, Yan G, Siddique KHM, Guo P. "Development of a simple and effective silver staining protocol for detection of DNA fragments." Electrophoresis. 2017 Apr;38 (8):1175-1178.

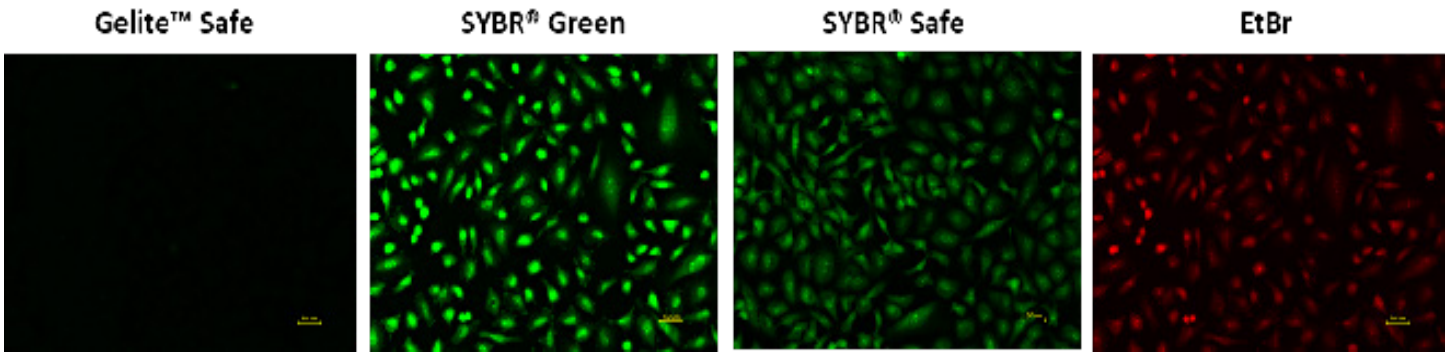

Figure 3. Gelite™ Safe DNA Gel Stain does not readily penetrate eukaryotic cells. HeLa cells were incubated for 30 minutes at 37°C with 1X DNA gel stain. EtBr, SYBR® Green I and SYBR® Safe dyes rapidly bound to DNA in living cells, resulting in bright nuclear staining. Gelite™ Safe DNA Gel Stain failed to penetrate living cells and thus was unable to bind to their DNA, as shown by an absence of fluorescence.

Product	Unit Size	Cat No.
Gelite™ Safe DNA Gel Stain *10,000X DMSO Solution*	100 µL	17704
Gelite™ Safe DNA Gel Stain *10,000X DMSO Solution*	500 µL	17705
Gelite™ Safe DNA Gel Stain *10,000X DMSO Solution*	1 mL	17706
Gelite™ Safe DNA Gel Stain *10,000X DMSO Solution*	10 mL	17707
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	100 µL	17700
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	500 µL	17701
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	1 mL	17702
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	10 mL	17703

Enzymatic and Chemical Labeling Strategies

for Oligonucleotides

Abstract

Labeling strategies for oligonucleotides (oligos) differ somewhat from those for other commonly encountered biopolymers, such as proteins and carbohydrates, since oligos are exceptionally inert to common bioconjugation reagents under standard reaction conditions. While reagents are available that can modify oligo base units, such base modification usually adversely impact the binding capacity of the oligo for its target sequence. Further complicating matters, many haptens and fluorophores cannot withstand the final base cleavage conditions employed in solid-phase oligo synthesis, thus requiring that the bioconjugation step be performed after the oligo has been released from the resin. However, certain small molecules can often be incorporated directly during the oligo synthesis process. Oligos are usually first modified at either their 5′ or 3′ end during synthesis, using suitable functional groups for bioconjugation such as phosphate, thiol or amine moieties. Then, functional group-reactive probes are introduced to label the targeted site. Oligos may also incorporate modified bases, such as modified CPGs or phosphoramidites, directly during solid-phase oligo synthesis.

Introduction

DNA is composed of a 2′-deoxyribose 5′-phosphate backbone and four heterocyclic bases: adenine (A), guanine (G), thymine (T), and cytosine (C) linked through an N-glycosidic bond, while RNA contains a ribose 5′-phosphate backbone and the same bases, except thymine is replaced with uracil (U). Oligonucleotides (oligos) are short-chain nucleic acid polymers containing ~10 to 100 monomer building blocks. While proteins typically contain inherently fluorescent amino acids like tryptophan and tyrosine, the intrinsic fluorescence emission of the five most common nucleotides (A, G, T, C, U) is too weak to be of analytical utility in detecting oligos for most biological applications. Advances in organic chemistry and in polymerase engineering have broadened the applications of nucleic acids well beyond their native roles in living organisms. To that end, a wide range of chemical and enzymatic methods have been devised to facilitate the detection of oligos. Radioactive phosphate (32P, 33P) is commonly inserted at the 5′ or 3′ end of an oligo post-synthesis using enzyme-catalyzed reactions. In recent decades, labeling with haptens and fluorophores has gained in popularity relative to incorporating such radioisotopes, due to the inherent hazards and high disposal costs associated with handling radioactivity. However, alternative probes, such as fluorescent dyes and small molecule haptens, may adversely influence the conformation and binding properties of an oligo, reducing the specificity of hybridization with its target. Thus, the challenge is to develop nucleic acid analogs and labeling procedures for different applications that do not substantially perturb hybridization. In this context, pre- and post-synthetic routes, as well as automated solid phase-based syntheses and enzymatic labeling methods, have been devised, as summarized

Table 1. Phosphorothiolate-reactive maleimides potentially suitable for 5′-end labeling of RNA or DNA oligos.

Probe	Superior Alternative to	Ex max (nm)	Catalog Number
Tide Fluor™ 1 Maleimide [TF1 Maleimide]	EDANS	341	2242
Tide Fluor™ 2 Maleimide [TF2 Maleimide]	Fluoresceins (FAM and FITC)	503	2247
Tide Fluor™ 2WS Maleimide [TF2WS Maleimide]	Fluoresceins (FAM and FITC)	503	2350
Tide Fluor™ 3 Maleimide [TF3 Maleimide]	Cy3®	554	2270
Tide Fluor™ 3WS Maleimide [TF3WS Maleimide]	Cy3®	551	2344
Tide Fluor™ 4 Maleimide [TF4 Maleimide]	ROX/Texas Red®	578	2287
Tide Fluor™ 5WS Maleimide [TF5WS Maleimide]	Cy5®	649	2280
Tide Fluor™ 6WS Maleimide [TF6WS Maleimide]	Cy5.5	682	2293
Tide Fluor™ 7WS Maleimide [TF7WS Maleimide]	Cy7®	756	2332
Tide Fluor™ 8WS Maleimide [TF8WS Maleimide]	IRDye® 800	785	2337

in this article. Oligos conjugated with fluorophores can readily be detected in assays involving either gel- or microplate readers, while oligos conjugated with biotin or resin are especially useful for affinity purification procedures. Oligo conjugates are increasingly finding application in molecular diagnostics, and nanotechnology and nucleic acid-based therapeutic applications.

Enzymatic Conjugation Methods

Molecular probes and chemically reactive functional groups are often incorporated into oligos during their synthesis (Wojczewski et al. 1999). While convenient, this strategy can incur additional costs, mainly if an experimental procedure requires both labeled and unlabeled versions of the same oligo sequence (Zearfoss and Ryder, 2012). End-labeling oligos after synthesis provide a simple and easily adapted alternative to probe incorporation during oligo synthesis. By this route, oligo conjugate preparation is achieved by including synthetic steps after the primary oligo synthesis process has been completed.

- *5′ end labeling*

An example of an enzymatic approach to labeling the 5′ end of an oligo employs a two-step reaction scheme (Czworkowski et al. 1991; Zearfoss and Ryder, 2012). The strategy takes advantage of the ability of bacteriophage T4 polynucleotide kinase (T4 PNK) to transfer a phosphate group to the 5′ end of RNA or DNA oligos. For the reaction, ATP is substituted with adenosine 5′-[?-thio] triphosphate (ATP?S), an ATP analog in which the gamma phosphate has been replaced with phosphorothioate. The reaction product is a phosphorylated oligo containing a single reactive sulfur group at the 5′ end of the oligo. The oligo may subsequently be incubated with a haloacetamide or maleimide derivative of a molecular probe (Table 1), which then reacts with the phosphorothioate to generate the labeled oligo product. For example, the fluorophore, 5-(iodoacetamido) fluorescein (5-IAF) (Cat No. 222), can be conjugated to the 5′ end of a DNA or RNA oligo by this basic procedure.

Another enzymatic approach to labeling the 5′end of an oligo is post-synthetic phosphorylation and substitution of the synthesized and purified oligo (Mergny et al. 1994). Much like the previous example, the DNA oligo is enzymatically phosphorylated at the 5′-hydroxyl group using T4 PNK and native ATP. Subsequently, the phosphate residue is activated with N-methyl-imidazole, which is then readily substituted by ethylenediamine to generate a free primary amino group for fluorophore labeling (Table 2).

- *3′ end labeling*

The template-independent DNA polymerase, terminal deoxynucleotidyl transferase (TdT), catalyzes repetitive addition of mononucleotides from dNTPs or NTPs to the terminal 3′-OH of a DNA initiator, accompanied by the release of inorganic phosphate (Guerra, 2006; Sarac and Hollenstein, 2019). 4-thiouridine may be introduced at the 3′-terminus of DNA using the cited enzyme, followed by treatment with ribonuclease and

reaction with thiol-reactive probes (Guerra, 2006; Sarac and Hollenstein, 2019, Table 1).

TdT can also directly incorporate fluorophore-labeled nucleotides to the 3′-hydroxyl terminus of DNA oligos. In the presence of a labeled nucleotide-triphosphate (dNTP or NTP, Table 3), such as Cy3-UTP, the enzyme incorporates ?20 to 100 labeled bases per 3′ end (Guerra, 2006).

This cited approach can easily be customized to the production process and quality control of oligo microarrays since oligos are typically directionally coupled to the solid-phase substrate using a 5′ end linkage (Guerra, 2006).

Recently, a one-step enzymatic method to modify RNA 3′ termini using recombinant human polymerase theta (Pol?) has been reported (Thomas et al. 2019). Pol? efficiently appends 30–50 2′ -deoxyribonucleotides to the 3′ terminus of RNA oligos, extending the termini with any of a variety of modified 2′-deoxy and 2′,3′ -dideoxy ribonucleotide analogs containing fluorophores or affinity tags (Table 3).

Pol displays a strong preference for adding deoxyribonucleotides to RNA, but additionally can add ribonucleotides with relatively high efficiency for certain sequence contexts.

As TdT is not able to act on RNA, Pol? provides a unique capability as an RNA 3′-terminal extension enzyme, capable of efficiently adding canonical nucleotides and a variety of nucleotide analogs to RNA (Thomas et al., 2019).

Chemical Conjugation Methods

Indirect Modification of Oligos with Probes Bearing Reactive Groups

Chemical conjugation methods typically require the synthesis of modified oligos containing susceptible pendant groups that can subsequently be chemically modified with a molecular probe. Oligos are typically synthesized by the phosphoramidite approach on a solid phase support with controlled pore width (Beaucage et al. 1993).

The synthesis is initiated with the 5′-end deprotection of the CPG-bound nucleoside. The free hydroxyl group generated then reacts with the 3′-phosphoramidite of the subsequently introduced nucleoside, activated with 1H-tetrazole to promote the reaction. Any remaining hydroxyl groups are capped to prevent elongation of missense strands. The dinucleoside phosphite generated is then oxidized to the corresponding phosphate using a mixture of iodine in water, and the cycle can then be repeated to add more bases.

Once the synthesis is complete, ammonia is employed to release the product from the solid-phase surface and to deprotect the exocyclic amino groups of the nucleobases. The modular nature of the synthetic procedure allows the covalent introduction of probes for detection at specific positions along the length of a DNA or RNA oligo.

A wide range of substituted fluorophores suitable for labeling oligos is available, including acridine, coumarin, cyanine,

Table 2. Ethylenediamine-reactive succinimidyl esters potentially suitable for labeling of DNA oligos.

Probe	Superior Alternative to	Excitation max (nm)	Emission Max (nm)
Tide Fluor™ 1 Succinimidyl Ester [TF1 SE]	EDANS	341	448
Tide Fluor™ 2 Succinimidyl Ester [TF2 SE]	Fluoresceins (FAM and FITC)	503	525
Tide Fluor™ 2WS Succinimidyl Ester [TF2WS SE]	Fluoresceins (FAM and FITC)	503	525
Tide Fluor™ 3 Succinimidyl Ester [TF3 SE]	Cy3®	554	578
Tide Fluor™ 3WS Succinimidyl Ester [TF3WS SE]	Cy3®	551	563
Tide Fluor™ 4 Succinimidyl Ester [TF4 SE]	ROX/Texas Red®	578	602
Tide Fluor™ 5WS Succinimidyl Ester [TF5WS SE]	Cy5®	649	664
Tide Fluor™ 6WS Succinimidyl Ester [TF6WS SE]	Cy5.5	682	701
Tide Fluor™ 7WS Succinimidyl Ester [TF7WS SE]	Cy7®	756	780
Tide Fluor™ 8WS Succinimidyl Ester [TF8WS SE]	IRDye® 800	785	801

Table 3. Probe-labeled nucleotides potentially suitable for 3′-end labeling of RNA or DNA oligos.

Probe	Unit Size	Catolog Number
2-Aminoethoxypropargyl ddATP	1 µmoles	17084
2-Aminoethoxypropargyl ddCTP	1 µmoles	17080
2-Aminoethoxypropargyl ddGTP	1 µmoles	17086
2-Aminoethoxypropargyl ddTTP	1 µmoles	17082
5-Propargylamino-3'-azidomethyl-dCTP	50 nmoles	17091
5-Propargylamino-3'-azidomethyl-dUTP	50 nmoles	17093
7-Deaza-7-Propargylamino-3'-azidomethyl-dATP	50 nmoles	17090
7-Deaza-7-Propargylamino-3'-azidomethyl-dGTP	50 nmoles	17092
AA-dUTP [Aminoallyl dUTP sodium salt] *4 mM in Tris Buffer (pH 7.5)* *CAS 936327-10-5*	1 µmole	17004
AA-dUTP [Aminoallyl dUTP sodium salt] *4 mM in Tris Buffer (pH 7.5)* *CAS 936327-10-5*	2.5 µmole	17005
AA-UTP [Aminoallyl UTP sodium salt] *4 mM in TE buffer* *CAS 75221-88-4*	250 µL	17021
Aminopropargyl dATP [7-Deaza-7-Propargylamino-2'-deoxyadenosine-5'-triphosphate]	10 µmoles	17056
Aminopropargyl dCTP [5-Propargylamino-2'-deoxycytidine-5'-triphosphate]	10 µmoles	17050
Aminopropargyl ddATP [7-Deaza-7-Propargylamino-2',3'-dideoxyadenosine-5'-triphosphate]	10 µmoles	17074
Aminopropargyl ddCTP [5-Propargylamino-2',3'-dideoxycytidine-5'-triphosphate]	10 µmoles	17070
Aminopropargyl ddGTP [7-Deaza-7-Propargylamino-2',3'-dideoxyguanosine-5'-triphosphate]	10 µmoles	17076
Aminopropargyl ddTTP [5-Propargylamino-2',3'-dideoxyuridine-5'-triphosphate]	10 µmoles	17072
Aminopropargyl dGTP [5-Propargylamino-2'-deoxyguanosine-5'-triphosphate]	10 µmoles	17059
Aminopropargyl dUTP [5-Propargylamino-2'-deoxyuridine-5'-triphosphate]	10 µmoles	17053
Biotin-11-dATP	25 nmoles	17014
Biotin-11-dGTP	25 nmoles	17015
Biotin-11-dUTP *1 mM in Tris Buffer (pH 7.5)* *CAS 86303-25-5*	25 nmoles	17016
Biotin-14-dCTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17019
Biotin-16-dUTP *1 mM in Tris Buffer (pH 7.5)* *CAS 136632-31-0*	25 nmoles	17017
Biotin-20-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17018
Cyanine 5-dATP [Cy5-dATP]	25 nmoles	17038
Cyanine-3- dUTP [Cy3-dUTP] *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17025
Cyanine-5- dUTP [Cy5-dUTP] *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17026

dansyl, fluorescein, oxazine, phenazine, and rhodamine dye derivatives.

- ***Amino-Modified Oligos***

Modified nucleotides, such as 5-aminohexylacrylamido-dUTP and 5-aminohexylacrylamido-dCTP, can be utilized to generate amine-modified DNA using a variety of enzymatic incorporation methods, including nick translation, random primed labeling, reverse transcription, and PCR.

The amine-modified DNA can then be labeled with any of a wide variety of amine-reactive dyes or haptens. Certain 5′-amino-modifiers have been specifically designed for use in automated synthesizers to functionalize the 5′-terminus of a target oligo with a primary amine moiety. The resulting amino-modified oligos can be conjugated to various tag molecules such as fluorophores, biotins, alkaline phosphatase, and HRP. Due to the increased possibility of side reactions during the deprotection of modified oligos, it is recommended that the ammonium hydroxide treatment be carried out at a lower temperature than that used for unmodified oligos. Amine-reactive fluorescent probes are widely used to modify amino-modified oligos at the introduced amino residue.

Many fluorescent amino-reactive dyes have been developed to label various oligos, and the resultant conjugates are widely used in biological applications (Table 2). Two major classes of amine-reactive fluorophores are extensively used to label oligos: succinimidyl esters (SE) and sulfonyl chlorides (SC). Succinimidyl esters (SE) are considered more suitable for modifying amine groups since the amide bonds formed are generally quite stable. By contrast, sulfonyl chlorides (SC) are

Table 3. Continued

Probe	Unit Size	Catolog Number
ddATP [2',3'-Dideoxyadenosine-5'-triphosphate]	1 µmole	17209
ddCTP [2',3'-Dideoxycytidine-5'-triphosphate]	1 µmole	17207
ddGTP [2',3'-Dideoxyguanosine-5'-triphosphate]	1 µmole	17210
ddTTP [2',3'-Dideoxythymidine-5'-triphosphate]	1 µmole	17208
DEAC-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17024
Digoxigenin-11-dUTP *1 mM solution in water*	25 nmoles	17012
Fluorescein-12-dUTP (Perkin-Elmer) *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17027
Fluorescein-12-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17028
Fluorescein-12-dUTP *1 mM in Tris Buffer (pH 7.5)* *CAS 214154-36-6*	25 nmoles	17022
iFluor™ 440-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17029
iFluor™488-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17039
MagaDye™ 535-ddGTP	5 nmoles	17063
MagaDye™ 535-ddGTP	50 nmoles	17067
MagaDye™ 561-ddATP	5 nmoles	17062
MagaDye™ 561-ddATP	50 nmoles	17066
MagaDye™ 588-ddTTP	5 nmoles	17061
MagaDye™ 588-ddTTP	50 nmoles	17065
MagaDye™ 613-ddCTP	5 nmoles	17060
MagaDye™ 613-ddCTP	50 nmoles	17064
mFluor™ Violet 450-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17011
Tetramethylrhodamine-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17023
TF1-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17006
TF2-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17007
TF3-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17008
TF4-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17009
TF5-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17010
XFD™488-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17040

highly reactive but unstable in water, especially at the higher pH values required for reactions with aliphatic amines.

Molecular modifications by sulfonyl chlorides need to be carefully performed, preferably at low temperatures. Sulfonyl chlorides may also react with phenols (such as tyrosine), aliphatic alcohols (such as polysaccharides), thiols (such as cysteine), and imidazoles (such as histidine, a consideration when labeling hybrid peptide- or oligosaccharide-oligos.

- ***Thiol-Modified Oligos***

Thiol-modified oligos can be synthesized by incorporating the thiol modification during solid-phase phosphoramidite oligo synthesis at either the 5'- end or the 3'-end of the oligo (Beaucage and Iyer, 1993). Thiol residues must be protected during oligo synthesis since they are strong nucleophiles that can interfere with phosphoramidite chemistry, and unprotected thiol groups spontaneously form disulfide crosslinks under neutral aqueous solution conditions.

Two methods are routinely employed to protect thiol residues during oligo synthesis, disulfide protection, and trityl protection. After oligo synthesis and deprotection, reaction byproducts are removed by gel filtration chromatography. Since free thiol (SH) groups are not present as abundantly as other groups in biopolymers, thiol-reactive reagents often provide a means of selectively modifying a hybrid oligo at a defined site. Therefore thiol-reactive dyes are often used to prepare fluorescent oligos for probing biological structure, function, and interactions (Table 1).

Many types of thiol-reactive dyes are available, including iodoacetamides, disulfides, maleimides, and vinyl sulfones, as

well as various electron-deficient aryl halides and sulfonates. Maleimides are by far the most popular thiol-reactive moiety, since they react readily with thiol moieties of biopolymers to form very stable thioether conjugates, even under neutral conditions. Maleimides require conjugation conditions less stringent than those of iodoacetamides, and do not react with histidine and methionine residues under physiological conditions. Most conjugation reactions can be performed at room temperature and neutral pH conditions.

- ***Click Chemistry Oligos***

Click chemistry is a two-step labeling process involving the quantitative chemical reaction of alkyne and azide moieties to create covalent carbon-heteroatom bonds (Rostovtsev et al. 2002; Moses et al. 2007). The reaction employs the catalyst, copper(I) to form a 1,2,3-triazole bond between an azide and a terminal alkyne moiety. The process has proven itself to be reliably performed, and the resulting bond is relatively stable, making it especially suitable in demanding biological applications. The advantages of the method are that the reaction is a robust catalytic process that can be performed in aqueous solution and at room temperature, generating no side reactions, exhibiting no functional group interference, and producing a thermally and hydrolytically stable triazole linkage. Click chemistry reactions are stereospecific, simple to perform, and can be conducted in easily removable or benign solvents. Click chemistry has proven to be an efficient process for the conjugation of peptides, small molecules, fluorophores, and carbohydrates to oligos.

Direct Incorporation of Modified Bases into Oligonucleotides

5′-Amino-modifying bases may be composed of a cyanoethyl phosphoramidite for automated DNA synthesis and a primary amine serving as a reactive group for post-labeling strategies. Alternatively, 3′-phosphoramidites of 5′-amino-5′-deoxynucleosides can be applied for DNA sequencing with labeled primers (Wojczewski et al. 1999). Since the necessary hydroxyl group for further chain elongation is lacking with 5′-modifying bases, they can only be employed as the final step of oligonucleotide synthesis.

When modification at the 3′-terminus of the oligonucleotide is required, leaving the 5′-end unmodified, a controlled pore glass (CPG) as a solid-phase support is employed, functionalized with a protected amine for post-synthetic probe coupling. Additionally, a dimethoxytrityl-protected hydroxyl group for standard nucleoside coupling can be employed (Wojczewski et al. 1999).

Internal sequence modification permits incorporating a molecular probe at a defined position within the oligo (Wojczewski et al. 1999). This can be necessary when the position of the probe is critical to the application, such as in energy transfer experiments involving donor and acceptor dyes. Internal modifying nucleoside 3′-phosphoramidites typically possess a dimethoxytrityl group that can be removed after the coupling step, unmasking a free hydroxyl group to continue oligo synthesis in the 5′-direction. The modified base employed is typically thymine (T), functionalized with a C-2 or C-6 linker terminating in a primary amino group.

Finally, approaches involving fluorescent probe-modified

Table 4. Dye Azides potentially suitable for labeling of DNA oligos by Click Chemistry.

Dye	Superior Alternative to	Excitation Max (nm)	Emission Max (nm)	Unit Size	Catolog Number
Tide Fluor™ 1 azide [TF1 azide]	EDANS	341	448	5 mg	2236
Tide Fluor™ 2 azide [TF2 azide]	Fluoresceins (FAM and FITC)	503	525	1 mg	2252
Tide Fluor™ 3 azide [TF3 azide]	Cy3®	554	578	1 mg	2254
Tide Fluor™ 4 azide [TF4 azide]	ROX/Texas Red®	578	602	1 mg	2300
Tide Fluor™ 5WS azide [TF5WS azide]	Cy5®	649	664	1 mg	2275
Tide Fluor™ 6WS azide [TF6WS azide]	Cy5.5	682	701	1 mg	2302
Tide Fluor™ 7WS azide [TF7WS azide]	Cy7®	756	780	1 mg	2304
Tide Fluor™ 8WS azide [TF8WS azide] *Near Infrared Emission*	IRDye® 800	785	801	1 mg	2306

Table 5. Fluorophore phosphoramidites potentially suitable for 3′-end labeling of DNA oligos.

Dye	Excitation Max (nm)	Emission Max (nm)	Unit Size	Catolog Number
5'-DABCYL C6 phosphoramidite			1 g	6009
6-FAM phosphoramidite [5'-Fluorescein phosphoramidite] *CAS 204697-37-0*	493	517	100 µmoles	6016
6-FAM phosphoramidite [5'-Fluorescein phosphoramidite] *CAS 204697-37-0*	493	517	10 x 100 µmoles	6017
6-Fluorescein phosphoramidite	498	517	100 µmoles	6018
6-HEX phosphoramidite [5'-Hexachlorofluorescein phosphoramidite]	533	559	100 µmoles	6026
6-TET phosphoramidite [5'-Tetrachlorofluorescein phosphoramidite]	521	542	10 x 100 µmoles	6025
6-TET phosphoramidite [5'-Tetrachlorofluorescein phosphoramidite] *CAS#: 877049-90-6*	521	542	50 µmoles	6021
6-TET phosphoramidite [5'-Tetrachlorofluorescein phosphoramidite] *CAS#: 877049-90-6*	521	542	100 µmoles	6027
FAM-xtra™ Phosphoramidite	493	517	50 µmoles	6037
FAM-xtra™ Phosphoramidite	493	517	100 µmoles	6038
FAM-xtra™ Phosphoramidite	493	517	10 x 100 µmoles	6039
Tide Fluor™ 3 phosphoramidite [TF3 CEP] *Superior replacement to Cy3 phosphoramidite*	560	580	100 µmoles	2274
Tide Quencher™ 1 phosphoramidite [TQ1 phosphoramidite]			100 µmoles	2198
Tide Quencher™ 2 phosphoramidite [TQ2 phosphoramidite]			100 µmoles	2208
Tide Quencher™ 3 phosphoramidite [TQ3 phosphoramidite]			100 µmoles	2228
VIC phosphoramidite	526	543	50 µmoles	6080
VIC phosphoramidite	526	543	100 µmoles	6081
VIC phosphoramidite	526	543	1 g	6082

phosphoramidites may be employed as "pre-synthetic labeling building blocks" during oligo synthesis (Wojczewski et al. 1999). Since conventional automated oligo synthesis proceeds from 3′ to 5′, the 5′-terminus is usually selected for modification.

A general approach to modifying the 5′-terminus is to use reagents that couple to the 5′-hydroxyl of an oligo. Fluorophore phosphoramidite reagents are readily adapted for use in automated synthesizers, with little or no modification to existing protocols. Typically, these reagents are highly compatible with automated DNA synthesizers. In general, fluorophore-labeled oligos can be deprotected at room temperature in concentrated ammonium hydroxide. FAM, Dabcyl, and Tide Quencher™ (TQ)-labeled oligos can be heated to 55 °C in ammonium hydroxide for extended periods of time. However, TET, TF-3, and Cy-3 labeled oligos are less stable and survive only for a few hours at 55 °C. HEX, TF-5, and Cy-5 labeled oligonucleotides must be deprotected at room temperature, and the residual ammonia should be removed immediately after deprotection. Numerous fluorophore-containing phosphoramidites are commercially available (Table 5).

In most instances, these fluorophores are incorporated into oligos without substantially impacting hybridization efficiency. Simple fluorophore phosphoramidites can be used as 5′-labeling reagents, while fluorophore phosphoramidites additionally containing a dimethoxytrityl protected hydroxyl group provide an option when the label is to be located at an internal position within the oligo. For 3′-labeling, fluorophores can also be coupled to the solid-phase support.

In this context, Tide Fluor™ dyes (such as TF1, TF2, TF3, TF4, TF5, TF6, TF7, and TF8) are particularly well suited to labeling oligos, offering stronger fluorescence and higher photostability than the classical fluorophores such as fluorescein, rhodamine, and cyanine dyes. For instance, TF2 features similar excitation and emission wavelength maxima as carboxyfluoresceins (FAM), making it readily used for the biological applications that are conducted with fluoresceins.

Compared to FAM probes, TF2 has much stronger fluorescence under physiological conditions, and it is much more photostable. Compared to other fluorescent dye alternatives to fluoresceins and Cy® dyes (such as Alexa Fluor™ and DyLight® dyes), Tide Fluor™ dyes are also much more cost-effective while offering comparable or superior performance for many biological applications. TF3 is much brighter and more photostable on oligonucleotides than Cy3®, Alexa Fluor® 555,

and DyLight™ 555, although TF3 has almost identical spectra to these three dyes.

3′ end labeling of RNA oligos

A chemical approach to 3′ end-labeling of an RNA oligo employs a two-step process (Reines and Cantor, 1974; Zearfoss and Ryder, 2012). Sodium periodate is used to oxidize the 3′ terminal ribose sugar, forming a reactive dialdehyde. Next, the oxidized sugar is conjugated to an aldehyde-reactive molecular probe tag (Table 6), such as fluorescein 5-thiosemicarbazide.

Since periodate oxidation requires vicinal hydroxyl residues, the reaction is highly specific for RNA and only modifies the 3′ terminal ribose. The plethora of aldehyde-reactive molecular probes support conjugation of a wide variety of fluorophores to the 3′ end. In addition, oligos may be conjugated to biotin, using reagents like (+)-biotin amidohexanoic acid hydrazide (BACH).

The cited labeling strategy can readily be performed at the laboratory bench, requiring no specialized equipment.

Dye CPGs for Modifying Oligos

Fluorophore-Chemical Phosphorylation Reagent (CPR) supports can be used to incorporate dye labels at the 3′-terminus of oligos. CPGs are derived from dye carboxylic acids and are attached via an amide linkage, giving an oligo product that is much easier to purify by HPLC. The use of dye CPGs in oligo synthesis proceeds in a manner analogous to using a standard nucleoside support with some necessary modifications. Different fluorophore-CPGs may require different cleavage methods.

The cleavage of oligos from FAM and Tide Quenchers™ (TQs) supports is similar to the standard ammonium hydroxide cleavage, while TAMRA CPG must be deprotected under very mild conditions to safeguard the base-labile TAMRA fluorophore. In the latter instance, it is advised to use UltraMild monomers with potassium carbonate in methanol for deprotection.

An alternative procedure employing t-butylamine/methanol/water (1:1:2) should allow the use of regular monomers. Tide Quencher™ CPGs are especially useful for making FRET probes,

Table 6. Aldehyde-reactive molecular probes potentially suitable for 3′-end labeling of RNA oligos.

Dye	Ex max	Em max	Unit Size	Catalog Number
Biocytin hydrazide *CAS 102743-85-1*			25 mg	3086
Biotin hydrazide *CAS 66640-86-6*			25 mg	3007
Cyanine 3 hydrazide [equivalent to Cy3® hydrazide]	555	569	1 mg	146
Cyanine 5 hydrazide [equivalent to Cy5® hydrazide]	651	670	1 mg	156
Cyanine 5.5 hydrazide [equivalent to Cy5.5® hydrazide]	683	703	1 mg	177
Cyanine 7 hydrazide [equivalent to Cy7® hydrazide]	756	779	1 mg	166
ICG hydrazide	789	814	1 mg	987
iFluor™ 350 hydrazide	345	450	1 mg	1080
iFluor™ 405 hydrazide	403	427	1 mg	1081
iFluor™ 488 hydrazide	491	516	1 mg	1082
iFluor™ 555 hydrazide	557	570	1 mg	1083
iFluor™ 647 hydrazide	656	670	1 mg	1085
iFluor™ 680 hydrazide	684	701	1 mg	1086
iFluor™ 700 hydrazide	690	713	1 mg	1087
iFluor™ 750 hydrazide	757	779	1 mg	1088
iFluor™ 790 hydrazide	787	812	1 mg	1364
ReadiView™ biotin hydrazide			5 mg	3055
Texas Red® hydrazide *Single Isomer*	586	603	5 mg	481

using conventional fluorophores or Tide Fluor™ probes.

Brief Highlight of Applications

Some of the major applications for modified oligos include Dot, Northern, and Southern blotting, RNA and DNA in situ hybridization (ISH), multicolor fluorescence in situ hybridization (mFISH), comparative genome hybridization (CGH), PCR, Real-Time PCR, DNA sequencing, site-directed mutagenesis, single-nucleotide polymorphism (SNP) assays, and microarray analysis. Fluorophore-, quencher- and hapten- labeled oligos are essential tools in both biochemical and cellular studies.

Fluorescent oligos are used extensively in fluorescence fluorimetry, fluorescence microscopy, fluorescence polarization spectroscopy, time-resolved fluorescence (TRF), and fluorescence resonance energy transfer (FRET). FRET oligonucleotides are widely used for diagnosing infectious diseases based upon the molecular beacon and other technologies. FRET oligonucleotides have also been used for cell analysis via fluorescence-associated cell sorting (FACS) either in vivo or in vitro for research and diagnostic purposes. The most important distinguishing features associated with fluorescent oligos correspond to high detection sensitivity without the complications associated with handling radioactivity.

For certain applications, such as DNA sequencing and in situ hybridization (ISH), oligos are usually required to be singly labeled. Subsequent detection and analysis depend upon the fluorescence properties of the dye itself. However, for other biological applications, e.g., probes for real-time PCR quantification of DNA and RNA and allele discrimination (Molecular Beacons™), oligos are required to be doubly labeled.

With doubly labeled oligos, one dye serves as the fluorophore, while the other is a quencher. With dual-labeled probes that are not bound to a target sequence, the light fluorophore emission is undetectable, as the quencher dye absorbs it via a process referred to as Fluorescent Resonance

Table 7. Fluorophore-CPG probes potentially suitable for 3′-end labeling of oligos.

Dye	Abs/Ex (nm)	Em (nm)	Unit Size	Catalog Number
3'-(6-Fluorescein) CPG *1000 Å*	498	517	1 g	6014
3'-DABCYL CPG *1000 Å*	454		1 g	6008
6-TAMRA CPG *1000 Å*	552	578	1 g	6051
BXQ-1 CPG (1000 A)	522		100 mg	2410
BXQ-1 CPG (500 A)	522		100 mg	2408
BXQ-2 CPG (1000 A)	554		100 mg	2430
BXQ-2 CPG (500 A)	554		100 mg	2428
CDPI3-CPG [Minor Groove Binder CPG] *1000A*			100 mg	6902
CDPI3-CPG [Minor Groove Binder CPG] *500A*			100 mg	6900
Tide Fluor™ 1 CPG [TF1 CPG] *1000 Å* *Superior replacement for EDANS*	341	448	100 mg	2241
Tide Fluor™ 1 CPG [TF1 CPG] *500 Å* *Superior replacement for EDANS*	341	448	100 mg	2240
Tide Quencher™ 1 CPG [TQ1 CPG] *1000 Å*	492		100 mg	2194
Tide Quencher™ 1 CPG [TQ1 CPG] *500 Å*	492		100 mg	2193
Tide Quencher™ 2 CPG [TQ2 CPG] *1000 Å*	516		100 mg	2204
Tide Quencher™ 2 CPG [TQ2 CPG] *500 Å*	516		100 mg	2203
Tide Quencher™ 3 CPG [TQ3 CPG] *1000 Å*	573		100 mg	2224
Tide Quencher™ 3 CPG [TQ3 CPG] *500 Å*	573		100 mg	2223
Tide Quencher™ 4 CPG [TQ4 CPG] *1000 Å*	603		100 mg	2063
Tide Quencher™ 4 CPG [TQ4 CPG] *500 Å*	603		100 mg	2062
Tide Quencher™ 5 CPG [TQ5 CPG] *1000 Å*	661		100 mg	2078
Tide Quencher™ 5 CPG [TQ5 CPG] *500 Å*	661		100 mg	2077

Energy Transfer (FRET). Because FRET is a distance-dependent interaction between the excited state of the donor and acceptor dye molecules, their eventual separation through hybridization to target in the detection event allows the fluorescence to be detected.

To maximize the FRET efficiency, the FRET pairs need to be carefully selected based upon the consideration of fluorescence lifetime and the spectral overlap of donor emission with acceptor excitation. A variety of FRET building blocks for labeling oligos are commercially available, including classic dyes and optimized Tide Fluor™ (donors) and Tide Quenchers™ (acceptors).

Summary

Radioactively labeled oligos provide high detection sensitivity, and the probes can readily be added to an oligo post-synthesis. However, their use can be problematic due to the hazardous nature of radioisotopes, the regulatory burden associated with their laboratory use and disposal, and the limited half-lives of the probes. Molecular probes, such as fluorescent dyes and small molecule haptens, are suitable alternatives to radioactive labeling, providing abundant sensitivity for most applications.

Additionally, the modification of oligos with fluorophores broadens the range of experimental procedures that can be performed with oligos. For instance, assays based upon fluorescence resonance energy transfer (FRET) and fluorescence polarization (FP) depend upon the physicochemical properties of fluorescent dyes and cannot be undertaken using radioisotope labels.

Similarly, biotin-conjugated oligos can be noncovalently attached to streptavidin-conjugated resins, facilitating retrieval and purification of specific target molecules. The majority of molecular biology techniques employed in modern biomedical research laboratories require chemically synthesized DNA or RNA oligos.

Oligo conjugates are widely employed in various biotechnology applications (ISH, microarray analysis, CGH), in research, as diagnostics tools, and for nucleic acid-based therapeutics.

References

1. Beaucage, S.L.; Iyer, R.P. "The functionalization of oligonucleotides via phosphoramidite derivatives." Tetrahedron 1993, 49, 1925–1963
2. Czworkowski J, Odom OW, Hardesty B. "Fluorescence study of the topology of messenger RNA bound to the 30S ribosomal subunit of Escherichia coli". Biochemistry. 1991; 30:4821–4830
3. Guerra CE. "Analysis of oligonucleotide microarrays by 3' end labeling using fluorescent nucleotides and terminal transferase." Biotechniques. 2006 Jul;41(1):53-6
4. Mergny, J.-L.; Boutorine, A. S.; Garestier, T.; Belloc, F.; Rougée, M.; Bulychev, N. V.; Koshkin, A. A.; Bourson, J.; Lebedev, A. V.; Valeur, B.; Thuong, N. T.; Hélène, C. "Fluorescence energy transfer as a probe for nucleic acid structures and sequences." Nucleic Acids Res. 1994, 22, 920-928
5. Reines SA, Cantor CR. "New fluorescent hydrazide reagents for the oxidized 3'-terminus of RNA". Nucleic Acids Res. 1974; 1:767–786
6. Rostovtsev, V.V., Green, L.G., Fokin, V. V.,Sharpless, K. B. "A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes." Angew Chem Int Ed Engl, 2002. 41(14): 2596-9
7. Sarac I, Hollenstein M. "Terminal deoxynucleotidyl transferase in the synthesis and modification of nucleic acids." Chembiochem. 2019 Apr 1;20(7):860-871
8. Thomas C, Rusanov T, Hoang T, Augustin T, Kent T, Gaspar I, Pomerantz RT. "One-step enzymatic modification of RNA 3' termini using polymerase ?." Nucleic Acids Res. 2019 Apr 23;47(7):3272-3283
9. Wojczewski, C., Stolze, K. Engels, J. W. "Fluorescent oligonucleotides – Versatile tools as probes and primers for DNA and RNA analysis." Synlett 1999, 10, 1667–1678
10. Zearfoss NR, Ryder SP. "End-labeling oligonucleotides with chemical tags after synthesis." Methods Mol Biol. 2012; 941: 181–193

Product	Unit Size	Cat No.
Tide Fluor™ 1 Maleimide [TF1 Maleimide]	5 mg	2242
Tide Fluor™ 2 Maleimide [TF2 Maleimide]	1 mg	2247
Tide Fluor™ 2WS Maleimide [TF2WS Maleimide]	1 mg	2350
Tide Fluor™ 3 Maleimide [TF3 Maleimide]	1 mg	2270
Tide Fluor™ 3WS Maleimide [TF3WS Maleimide]	1 mg	2344
Tide Fluor™ 4 Maleimide [TF4 Maleimide]	1 mg	2287
Tide Fluor™ 5WS Maleimide [TF5WS Maleimide]	1 mg	2280
Tide Fluor™ 6WS Maleimide [TF6WS Maleimide]	1 mg	2293
Tide Fluor™ 7WS Maleimide [TF7WS Maleimide]	1 mg	2332
Tide Fluor™ 8WS Maleimide [TF8WS Maleimide]	1 mg	2337
Tide Fluor™ 1 Succinimidyl Ester [TF1 SE]	5 mg	2244
Tide Fluor™ 2 Succinimidyl Ester [TF2 SE]	5 mg	2248
Tide Fluor™ 2WS Succinimidyl Ester [TF2WS SE]	5 mg	2349
Tide Fluor™ 3 Succinimidyl Ester [TF3 SE]	5 mg	2271
Tide Fluor™ 3WS Succinimidyl Ester [TF3WS SE]	5 mg	2346
Tide Fluor™ 4 Succinimidyl Ester [TF4 SE]	5 mg	2289
Tide Fluor™ 5WS Succinimidyl Ester [TF5WS SE]	5 mg	2281
Tide Fluor™ 6WS Succinimidyl Ester [TF6WS SE]	1 mg	2294
Tide Fluor™ 7WS Succinimidyl Ester [TF7WS SE]	1 mg	2333
Tide Fluor™ 8WS Succinimidyl Ester [TF8WS SE]	1 mg	2338
2-Aminoethoxypropargyl ddATP	1 µmoles	17084
2-Aminoethoxypropargyl ddCTP	1 µmoles	17080
2-Aminoethoxypropargyl ddGTP	1 µmoles	17086
2-Aminoethoxypropargyl ddTTP	1 µmoles	17082
5-Propargylamino-3'-azidomethyl-dCTP	50 nmoles	17091
5-Propargylamino-3'-azidomethyl-dUTP	50 nmoles	17093
7-Deaza-7-Propargylamino-3'-azidomethyl-dATP	50 nmoles	17090
7-Deaza-7-Propargylamino-3'-azidomethyl-dGTP	50 nmoles	17092
AA-dUTP [Aminoallyl dUTP sodium salt] *4 mM in Tris Buffer (pH 7.5)* *CAS 936327-10-5*	1 µmole	17004
AA-dUTP [Aminoallyl dUTP sodium salt] *4 mM in Tris Buffer (pH 7.5)* *CAS 936327-10-5*	2.5 µmole	17005
AA-UTP [Aminoallyl UTP sodium salt] *4 mM in TE buffer* *CAS 75221-88-4*	250 µL	17021
Aminopropargyl dATP [7-Deaza-7-Propargylamino-2'-deoxyadenosine-5'-triphosphate]	10 µmoles	17056
Aminopropargyl dCTP [5-Propargylamino-2'-deoxycytidine-5'-triphosphate]	10 µmoles	17050
Aminopropargyl ddATP [7-Deaza-7-Propargylamino-2',3'-dideoxyadenosine-5'-triphosphate]	10 µmoles	17074

Product	Unit Size	Cat No.
Aminopropargyl ddCTP [5-Propargylamino-2',3'-dideoxycytidine-5'-triphosphate]	10 µmoles	17070
Aminopropargyl ddGTP [7-Deaza-7-Propargylamino-2',3'-dideoxyguanosine-5'-triphosphate]	10 µmoles	17076
Aminopropargyl ddTTP [5-Propargylamino-2',3'-dideoxyuridine-5'-triphosphate]	10 µmoles	17072
Aminopropargyl dGTP [5-Propargylamino-2'-deoxyguanosine-5'-triphosphate]	10 µmoles	17059
Aminopropargyl dUTP [5-Propargylamino-2'-deoxyuridine-5'-triphosphate]	10 µmoles	17053
Biotin-11-dATP	25 nmoles	17014
Biotin-11-dGTP	25 nmoles	17015
Biotin-11-dUTP *1 mM in Tris Buffer (pH 7.5)* *CAS 86303-25-5*	25 nmoles	17016
Biotin-14-dCTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17019
Biotin-16-dUTP *1 mM in Tris Buffer (pH 7.5)* *CAS 136632-31-0*	25 nmoles	17017
Biotin-20-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17018
Cyanine 5-dATP [Cy5-dATP]	25 nmoles	17038
Cyanine-3- dUTP [Cy3-dUTP] *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17025
Cyanine-5- dUTP [Cy5-dUTP] *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17026
ddATP [2',3'-Dideoxyadenosine-5'-triphosphate]	1 µmole	17209
ddCTP [2',3'-Dideoxycytidine-5'-triphosphate]	1 µmole	17207
ddGTP [2',3'-Dideoxyguanosine-5'-triphosphate]	1 µmole	17210
ddTTP [2',3'-Dideoxythymidine-5'-triphosphate]	1 µmole	17208
DEAC-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17024
Digoxigenin-11-dUTP *1 mM solution in water*	25 nmoles	17012
Fluorescein-12-dUTP (Perkin-Elmer) *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17027
Fluorescein-12-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17028
Fluorescein-12-dUTP *1 mM in Tris Buffer (pH 7.5)* *CAS 214154-36-6*	25 nmoles	17022
iFluor™ 440-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17029
iFluor™488-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17039
MagaDye™ 535-ddGTP	5 nmoles	17063
MagaDye™ 535-ddGTP	50 nmoles	17067
MagaDye™ 561-ddATP	5 nmoles	17062
MagaDye™ 561-ddATP	50 nmoles	17066
MagaDye™ 588-ddTTP	5 nmoles	17061
MagaDye™ 588-ddTTP	50 nmoles	17065
MagaDye™ 613-ddCTP	5 nmoles	17060
MagaDye™ 613-ddCTP	50 nmoles	17064
mFluor™ Violet 450-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17011

Product	Unit Size	Cat No.
Tetramethylrhodamine-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17023
TF1-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17006
TF2-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17007
TF3-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17008
TF4-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17009
TF5-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17010
XFD™488-dUTP *1 mM in Tris Buffer (pH 7.5)*	25 nmoles	17040
Tide Fluor™ 1 azide [TF1 azide]	5 mg	2236
Tide Fluor™ 2 azide [TF2 azide]	1 mg	2252
Tide Fluor™ 3 azide [TF3 azide]	1 mg	2254
Tide Fluor™ 4 azide [TF4 azide]	1 mg	2300
Tide Fluor™ 5WS azide [TF5WS azide]	1 mg	2275
Tide Fluor™ 6WS azide [TF6WS azide]	1 mg	2302
Tide Fluor™ 7WS azide [TF7WS azide]	1 mg	2304
Tide Fluor™ 8WS azide [TF8WS azide] *Near Infrared Emission*	1 mg	2306
5′-DABCYL C6 phosphoramidite	1 g	6009
6-FAM phosphoramidite [5′-Fluorescein phosphoramidite] *CAS 204697-37-0*	100 µmoles	6016
6-FAM phosphoramidite [5′-Fluorescein phosphoramidite] *CAS 204697-37-0*	10 x 100 µmoles	6017
6-Fluorescein phosphoramidite	100 µmoles	6018
6-Fluorescein phosphoramidite	10 x 100 µmoles	6019
6-HEX phosphoramidite [5′-Hexachlorofluorescein phosphoramidite]	100 µmoles	6026
6-HEX phosphoramidite [5′-Hexachlorofluorescein phosphoramidite]	10 x 100 µmoles	6024
6-TET phosphoramidite [5′-Tetrachlorofluorescein phosphoramidite]	10 x 100 µmoles	6025
6-TET phosphoramidite [5′-Tetrachlorofluorescein phosphoramidite] *CAS#: 877049-90-6*	50 µmoles	6021
6-TET phosphoramidite [5′-Tetrachlorofluorescein phosphoramidite] *CAS#: 877049-90-6*	100 µmoles	6027
FAM-xtra™ Phosphoramidite	50 µmoles	6037
FAM-xtra™ Phosphoramidite	100 µmoles	6038
FAM-xtra™ Phosphoramidite	10 x 100 µmoles	6039
Tide Fluor™ 3 phosphoramidite [TF3 CEP] *Superior replacement to Cy3 phosphoramidite*	100 µmoles	2274
Tide Quencher™ 1 phosphoramidite [TQ1 phosphoramidite]	100 µmoles	2198
Tide Quencher™ 2 phosphoramidite [TQ2 phosphoramidite]	100 µmoles	2208
Tide Quencher™ 3 phosphoramidite [TQ3 phosphoramidite]	100 µmoles	2228
VIC phosphoramidite	50 µmoles	6080
VIC phosphoramidite	100 µmoles	6081

Product	Unit Size	Cat No.
VIC phosphoramidite	1 g	6082
Biocytin hydrazide *CAS 102743-85-1*	25 mg	3086
Biotin hydrazide *CAS 66640-86-6*	25 mg	3007
Cyanine 3 hydrazide [equivalent to Cy3® hydrazide]	1 mg	146
Cyanine 5 hydrazide [equivalent to Cy5® hydrazide]	1 mg	156
Cyanine 5.5 hydrazide [equivalent to Cy5.5® hydrazide]	1 mg	177
Cyanine 7 hydrazide [equivalent to Cy7® hydrazide]	1 mg	166
ICG hydrazide	1 mg	987
iFluor™ 350 hydrazide	1 mg	1080
iFluor™ 405 hydrazide	1 mg	1081
iFluor™ 488 hydrazide	1 mg	1082
iFluor™ 555 hydrazide	1 mg	1083
iFluor™ 647 hydrazide	1 mg	1085
iFluor™ 680 hydrazide	1 mg	1086
iFluor™ 700 hydrazide	1 mg	1087
iFluor™ 750 hydrazide	1 mg	1088
iFluor™ 790 hydrazide	1 mg	1364
ReadiView™ biotin hydrazide	5 mg	3055
Texas Red® hydrazide *Single Isomer*	5 mg	481
3'-(6-Fluorescein) CPG *1000 Å*	1 g	6014
3'-DABCYL CPG *1000 Å*	1 g	6008
6-TAMRA CPG *1000 Å*	1 g	6051
BXQ-1 CPG (1000 A)	100 mg	2410
BXQ-1 CPG (500 A)	100 mg	2408
BXQ-2 CPG (1000 A)	100 mg	2430
CDPI3-CPG [Minor Groove Binder CPG] *1000A*	100 mg	6902
CDPI3-CPG [Minor Groove Binder CPG] *500A*	100 mg	6900
Tide Fluor™ 1 CPG [TF1 CPG] *1000 Å* *Superior replacement for EDANS*	100 mg	2241
Tide Fluor™ 1 CPG [TF1 CPG] *500 Å* *Superior replacement for EDANS*	100 mg	2240
Tide Quencher™ 1 CPG [TQ1 CPG] *1000 Å*	100 mg	2194
Tide Quencher™ 1 CPG [TQ1 CPG] *500 Å*	100 mg	2193
Tide Quencher™ 2 CPG [TQ2 CPG] *1000 Å*	100 mg	2204
Tide Quencher™ 2 CPG [TQ2 CPG] *500 Å*	100 mg	2203
Tide Quencher™ 3 CPG [TQ3 CPG] *1000 Å*	100 mg	2224

Product	Unit Size	Cat No.
Tide Quencher™ 3 CPG [TQ3 CPG] *500 Å*	100 mg	2223
Tide Quencher™ 4 CPG [TQ4 CPG] *1000 Å*	100 mg	2063
Tide Quencher™ 4 CPG [TQ4 CPG] *500 Å*	100 mg	2062
Tide Quencher™ 5 CPG [TQ5 CPG] *1000 Å*	100 mg	2078
Tide Quencher™ 5 CPG [TQ5 CPG] *500 Å*	100 mg	2077

Sensitive Identification of Newly Synthesized

DNA In situ Using Click Chemistry-based Bucculite

Abstract

The measurement of cell proliferation in situ is fundamental to the assessment of cell health, genotoxicity, and drug efficacy evaluation. Precisely labeling and quantifying cells in the synthesis phase (S phase) of cell cycle progression is not only adequate for characterizing basic biology but can provide insight into cell cycle kinetics, mechanisms of cell growth, and cytotoxicity. In drug discovery, cell proliferation assays can facilitate the determination of novel therapeutics, define cellular responses to drug treatments, and evaluate the antiproliferative potency and toxicity of drug candidates. Traditionally, cell proliferation measurements rely on incorporating radioactive nucleosides, such as 3H-thymidine, into DNA or incorporating the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) during synthesis, with subsequent detection by an anti-BrdU antibody conjugate. While BrdU proliferation assays provided a safer alternative to radioactive 3H-thymidine, the harsh treatments needed to denature the DNA and facilitate the detection of the incorporated BrdU molecules by antibodies can adversely affect cell morphology and antigen recognition sites resulting in poor image quality. The Bucculite™ XdU Proliferation assays represent a significant breakthrough in the evolution of cell proliferation measurements. Unlike traditional cell proliferation assays, the Bucculite™ XdU Cell Proliferation assays are not dependent upon radioactivity or antibodies for detection. Instead, a proprietary mixture of alkyne-containing thymidine analogs, XdU, is incorporated into DNA during active DNA synthesis. The incorporated XdU is then detected by a copper-catalyzed azide-alkyne cycloaddition or click reaction using a fluorescent dye containing a picolyl azide moiety.

Introduction

Quantitative analysis of newly synthesized DNA in situ in the presence of a label is considered one of the most reliable and accurate assay types for measuring cell proliferation. Initially, proliferation measurements relied on the incubation of 3H-thymidine for several hours to overnight. Proliferating cells would incorporate the radioactive 3H-thymidine into their newly synthesized DNA. After subsequent washing and adherence to filters, a scintillation beta-counter is used to measure the radioactivity in DNA recovered from the cells to determine the degree of cell division. Besides a lengthy and tedious protocol, the health hazards of working with radioactive materials are well recognized, as are the accompanying economic considerations required for appropriate handling and disposal of radioactive waste.

Years later, a nonradioactive approach was introduced based on the detection of incorporated 5-bromo-2'-deoxyuridine (BrdU) by anti-BrdU antibody conjugates. Although BrdU proliferation assays eliminated the risks and complications associated with handling radioactive materials, it too is burdened by a tedious and time-consuming protocol. Assay

workflow requires harsh treatments either by acid, heat, or enzymes to denature the DNA and facilitate the detection of the incorporated BrdU molecules by anti-BrdU antibody conjugates. Without DNA denaturation, BrdU is accessible to antibodies for detection. Consequently, these harsh treatments damage antigen recognition sites and adversely affect sample integrity and cell morphology. Furthermore, the denaturants used to enable antibody accessibility of the incorporated BrdU also limits its compatibility to be multiplexed with other organelle stains, fluorescent proteins, such as GFP or RFP, or phycobiliproteins, such as phycoerythrin (PE) or allophycocyanin (APC).

Click- Chemistry Driven Bucculite™ XdU Cell Proliferation Assays

The click chemistry-based Bucculite™ XdU Cell Proliferation Assays represents the newest addition of AAT Bioquest's growing family of proliferation reagents. Unlike traditional cell proliferation assays, Bucculite™ XdU Cell Proliferation Assays do not rely on radioactivity or antibodies to quantify nascent DNA. Rather, Bucculite™ XdU Cell Proliferation Assays use a proprietary mixture of alkyne-containing thymidine analogs, XdU, which are incorporated into newly synthesized DNA during the S phase. The incorporated XdU is subsequently detected via a copper-catalyzed azide-alkyne cycloaddition (i.e., click reaction) using various fluorophore containing a picolyl azide moiety, such as iFluor™ 488 azide.

Since the click reaction utilizes biorthogonal moieties to detect proliferating cells, background interference is minimal. More importantly, because of the mild reaction conditions, Bucculite™ XdU Cell Proliferation Assays preserve cell morphology, antigen-binding sites, and sample integrity, affording the user an opportunity for multiplexing analysis. After thymidine incorporation, the click reaction and subsequent wash steps can be completed in less than 60 minutes, and nascent DNA can be quantified using standard imaging systems (Figure 2) or a flow-cytometer (Figure 3).

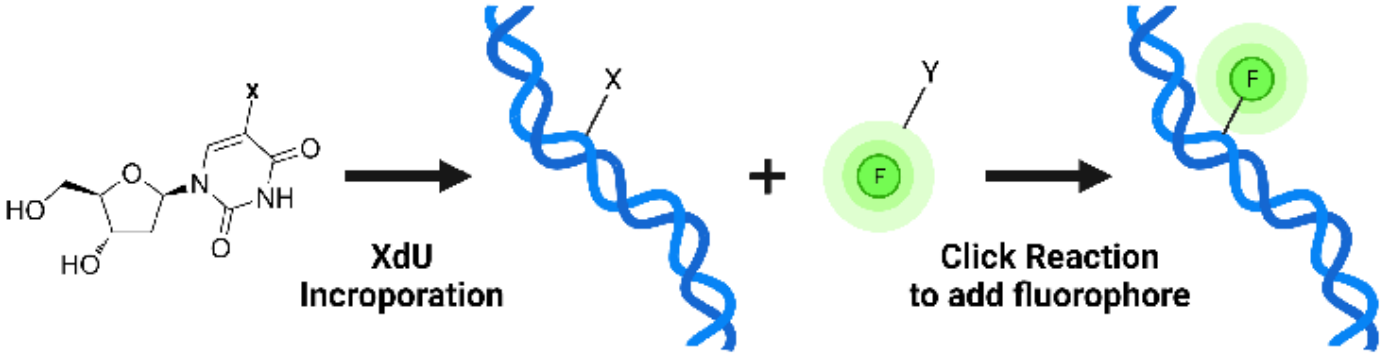

Figure 1. Bucculite™ XdU Cell Proliferation Assay Principle. Proliferating cells in the presence of XdU incorporate the compound at thymidine bases during the S phase. Fluorophore-labeled azide reacts with the incorporated XdU to allow detection by imaging or flow cytometry (figure made in BioRender).

Table 1. Click-chemistry based cell proliferation kits suitable for imaging or flow cytometry.

Name	Size	Catalog Number
Bucculite™ Flow Cytometric XdU Cell Proliferation Assay Kit *Violet Laser-Comptatible*	100 Tests	22321
Bucculite™ Flow Cytometric XdU Cell Proliferation Assay Kit *Blue Laser-Comptatible*	100 Tests	22323
Bucculite™ Flow Cytometric XdU Cell Proliferation Assay Kit *Red Laser-Comptatible*	100 Tests	22325
Bucculite™ XdU Cell Proliferation Fluorescence Imaging Kit *Green Fluorescence*	200 Tests	22326
Bucculite™ XdU Cell Proliferation Fluorescence Imaging Kit *Red Fluorescence*	200 Tests	22327
Bucculite™ XdU Cell Proliferation Fluorescence Imaging Kit *Deep Red Fluorescence*	200 Tests	22328

Microscopic Imaging with Bucculite™ XdU

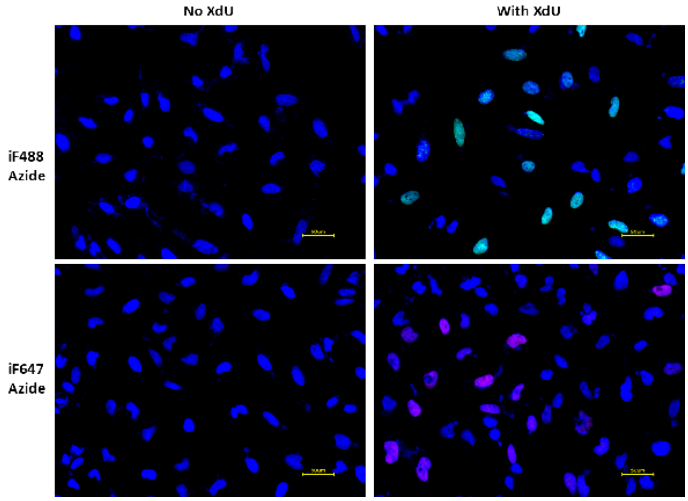

Figure 2. Cell proliferation detected using the Bucculite™ XdU Cell Proliferation Assay. HeLa cells were processed using the reagents and fixation/detection protocol provided in the Bucculite™ XdU Cell Proliferation iFluor™ 444 Imaging kit (Cat No. 22326) and the Bucculite™ XdU Cell Proliferation iFluor™ 647 Imaging kit (Cat No. 22328). Both samples were counter-stained with Hoechst® 33342 nucleic acid stain (blue, Cat No. 17530) and imaged using a fluorescence microscope.

Flow Cytometric Analysis with Bucculite™ XdU

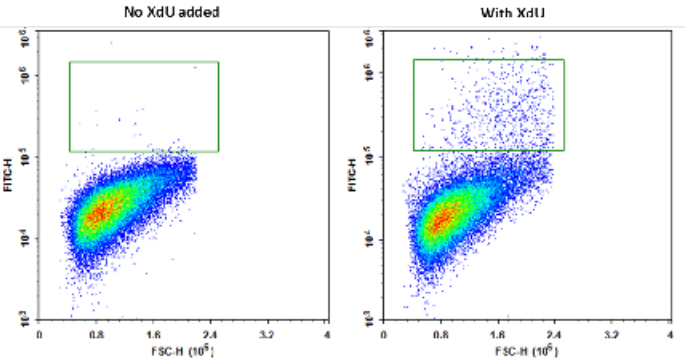

Figure 3. Cell proliferation detected using the Bucculite™ XdU Cell Proliferation Assay. Jurkat cells were processed using the reagents and fixation/detection protocol provided in the Bucculite™ XdU Cell Proliferation iFluor™ 444 Imaging kit (Cat No. 22326). Data were collected and analyzed using an ACEA Novocyte flow cytometer with 488 nm excitation and a 530/30 nm bandpass emission filter for detection of the iFluor™ 488–labeled XdU.

References

1. Buck, S. B., Bradford, J., Gee, K. R., Agnew, B. J., Clarke, S. T., & Salic, A. (2008). Detection of S-phase cell cycle progression using 5-ethynyl-2′-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2′-deoxyuridine antibodies. BioTechniques, 44(7), 927–929. https://doi.org/10.2144/000112812
2. Clarke, S. T., Calderon, V., & Bradford, J. A. (2017). Click Chemistry for Analysis of Cell Proliferation in Flow Cytometry. Current protocols in cytometry, 82, 7.49.1–7.49.30. https://doi.org/10.1002/cpcy.24
3. Diermeier-Daucher, S., Clarke, S. T., Hill, D., Vollmann-Zwerenz, A., Bradford, J. A., & Brockhoff, G. (2009). Cell type specific applicability of 5-ethynyl-2′-deoxyuridine (EdU) for dynamic proliferation assessment in flow cytometry. Cytometry. Part A : the journal of the International Society for Analytical Cytology, 75(6), 535–546. https://doi.org/10.1002/cyto.a.20712
4. Li, X., & Darzynkiewicz, Z. (1995). Labelling DNA strand breaks with BrdUTP. Detection of apoptosis and cell proliferation. Cell proliferation, 28(11), 571–579. https://doi.org/10.1111/j.1365-2184.1995.tb00045.x
5. Salic, A., & Mitchison, T. J. (2008). A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proceedings of the National Academy of Sciences of the United States of America, 105(7), 2415–2420. https://doi.org/10.1073/pnas.0712168105

Product	Unit Size	Cat No.
Bucculite™ Flow Cytometric XdU Cell Proliferation Assay Kit *Violet Laser-Compatible*	100 Tests	22321
Bucculite™ Flow Cytometric XdU Cell Proliferation Assay Kit *Blue Laser-Compatible*	100 Tests	22323
Bucculite™ Flow Cytometric XdU Cell Proliferation Assay Kit *Red Laser-Compatible*	100 Tests	22325
Bucculite™ XdU Cell Proliferation Fluorescence Imaging Kit *Green Fluorescence*	200 Tests	22326
Bucculite™ XdU Cell Proliferation Fluorescence Imaging Kit *Red Fluorescence*	200 Tests	22327
Bucculite™ XdU Cell Proliferation Fluorescence Imaging Kit *Deep Red Fluorescence*	200 Tests	22328

A Practical Guide for the Detection

and Analysis of PCR Products

Abstract

PCR, or polymerase chain reaction, is a core technology used routinely to amplify a specific DNA sequence rapidly. Offering exceptional speed, specificity, and sensitivity as a tool, PCR, and its many adaptations, such as real-time PCR and reverse transcription-PCR, have revolutionized how DNA and RNA species are identified and measured. These techniques have become ubiquitous across all fields of life science. They have been adapted for various detection strategies, including end-point analysis via gel electrophoresis and subsequent DNA staining and quantitative analysis using DNA-binding fluorescent dyes, such as Helixye™ Green and Q4ever™ Green, or hybridization probes, such as TaqMan® probes and Molecular Beacons.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an analytical technique widely used in molecular biology research to amplify a specific DNA template in vitro rapidly. It enables researchers to generate significant quantities of sample DNA for a wide range of downstream laboratory and clinical applications, including cloning, genotyping, sequencing, mutagenesis, forensics, and the detection of pathogens to diagnose infectious diseases. Since being introduced in 1985, several iterations of the PCR process have been developed, including quantitative PCR (qPCR) for monitoring DNA amplification in real-time and reverse-transcription PCR (RT-PCR) for the detection of RNA, a tool that has become instrumental in viral diagnostics.

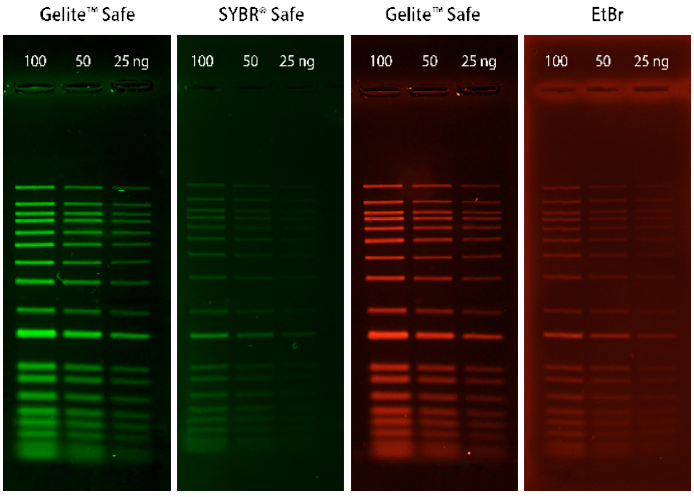

Figure 1. Comparison of DNA detection in 1% agarose gel in TBE buffer using Gelite™ Safe, EtBr, and SYBR® Safe. Two-fold serial dilutions of 1 kb DNA ladder were loaded in amounts of 100 ng, 50 ng, and 25 ng from left to right. Gels were stained for 60 minutes with Gelite™ Safe, EtBr, and SYBR® Safe according to the manufacturer's recommended concentrations and imaged using the ChemiDoc™ Imaging System (Bio-Rad®). Gels were illuminated using a 300 nm transilluminator fitted with GelGreen and GelRed filters.

Visualizing PCR Amplification Products

The analysis of PCR amplification products (i.e., amplicons) is an essential step in both the quantitative and qualitative assessment of the DNA target that has been amplified. Techniques used to analyze PCR products are generally divided

into two distinct groups:

1. End-point techniques - where analysis is performed outside of the PCR reaction vessel, such as analysis via DNA gel electrophoresis and DNA intercalating dyes.

2. Real-time techniques - where analysis is performed inside the PCR reaction vessel and during PCR thermocycling using DNA binding fluorescent dyes, DNA hybridization, or dNTP nucleotides with fluorescent dyes.

End-Point PCR Analysis Methods

Intercalating Chemical Dyes and DNA Gel Stains

Intercalating chemical dyes insert between the organic bases within duplex strands of DNA, facilitating a significant fluorescence increase, and as such are valuable for determining DNA concentrations. Many intercalating dyes useful in PCR are currently commercially available, such as ethidium bromide (EtBr) or SYBR® Gold. For quantitative DNA calculations, EtBr can be used to quantify DNA within the concentration range of 20 ng/mL-20 µg/mL, with the relationship between staining intensity and the quantity of DNA being linear. However, the health hazards associated with handling toxic chemicals such as EtBr (e.g., mutagenicity, UV-illumination, etc.) are well recognized, creating a need for better alternatives that minimize health risks and environmental impact. DNA-binding dyes, such as Gelite™ Safe (Cat No. 17700), Helixyte™ Gold (Cat No. 17595), and SYBR® Safe, offer safer alternatives readily detected using the 488 nm argon-ion laser or Blue LED. With Gelite™ Safe, the limit of DNA detection is as little as 0.67 ng DNA.

Real-Time PCR Analysis Methods

DNA-Binding Dyes

DNA-binding dyes used for measuring PCR amplification, such as Helixyte™ Green (Cat No. 17592), Q4ever™ Green (Cat No. 17609), and SYBR® Green, bind nonspecifically to double-stranded DNA (dsDNA) (Figure 2). Helixyte™ Green exhibits minimal fluorescence when it is free in solution. Upon binding to DNA, its fluorescence increases up to 1,000-fold. More importantly, the fluorescence output is proportional to the amount of dsDNA present, and increases as more PCR product is produced. Along with target amplification, the fluorescence signal increases exponentially until the DNA primers are depleted.

The benefits of using dsDNA-binding dyes include a simple PCR primer design. The assay requires only two sequence-specific DNA primers. The ability to test multiple genes rapidly,

Table 1. Nucleic acid stains for agarose and polyacrylamide gel electrophoresis

Product	Ex (nm)	Filter	Unit Size	Catalog Number
Helixyte™ Green Nucleic Acid Gel Stain *10,000X DMSO Solution*	254 mn	Long path green filter	1 mL	17590
Helixyte™ Green Nucleic Acid Gel Stain *10,000X DMSO Solution*	254 mn	Long path green filter	100 µL	17604
Helixyte™ Gold Nucleic Acid Gel Stain *10,000X DMSO Solution*	254 mn	Long path green filter	1 mL	17595
Gelite™ Green Nucleic Acid Gel Staining Kit	254 nm or 300 nm	Long path green filter	1 Kit	17589
Gelite™ Orange Nucleic Acid Gel Staining Kit	254 nm or 300 nm	Long path green filter	1 Kit	17594
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	254 nm, 300 nm or 520 nm	Ethidium Bromide, Gel Star, Gel Green, Gel Red and SYBR filters	100 µL	17700
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	254 nm, 300 nm or 520 nm	Ethidium Bromide, Gel Star, Gel Green, Gel Red and SYBR filters	500 µL	17701
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	254 nm, 300 nm or 520 nm	Ethidium Bromide, Gel Star, Gel Green, Gel Red and SYBR filters	1 mL	17702
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	254 nm, 300 nm or 520 nm	Ethidium Bromide, Gel Star, Gel Green, Gel Red and SYBR filters	10 mL	17703
Gelite™ Safe DNA Gel Stain *10,000X DMSO Solution*	254 nm, 300 nm or 520 nm	Ethidium Bromide, Gel Star, Gel Green, Gel Red and SYBR filters	100 µL	17704
Gelite™ Safe DNA Gel Stain *10,000X DMSO Solution*	254 nm, 300 nm or 520 nm	Ethidium Bromide, Gel Star, Gel Green, Gel Red and SYBR filters	500 µL	17705

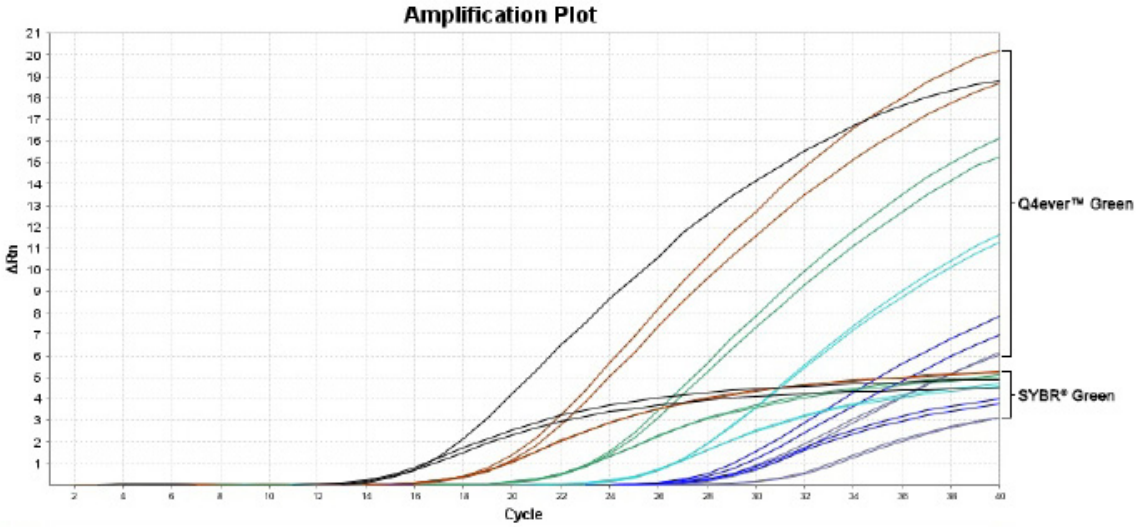

Figure 2. A comparison of the relative fluorescence signal from qPCR reactions performed with Q4ever™ Green and SYBR® Green. Q4ever™ Green has much brighter signal than SYBR® Green.

such as in the validation of gene expression data. Lower initial cost and the ability to perform a melt curve analysis to verify amplification reaction specificity. Compared to microarrays, dye-based qPCR is more sensitive at detecting modest changes in expression levels, making it well-suited for investigating small subsets of genes.

The major drawback of DNA-binding dyes is that they are non-specific. Any dsDNA produced from off-target and non-template amplification (NTC) will be observed, including PCR primer-dimers, reducing the accuracy of quantification. Moreover, DNA-binding dyes don't afford multiplexing since the fluorescence signals from different products cannot be distinguished.

Probe-Based Chemistries

Probe-based qPCR is a favored method for measuring transcript abundance. It is one of the most sensitive detection methods that provide an accurate and reproducible analysis. In probe-based qPCR, fluorescently-labeled target-specific probes are used to measure DNA amplification in real-time, offering the least background fluorescence compared to other (dye-based) chemistries. This method benefits from extreme specificity and affords the end-user the opportunity for multiplexing multiple targets in a single reaction. Several platforms are available that use probe-based chemistry to quantitate transcript abundance. Of them, hydrolysis probes, such as TaqMan® probes, and Molecular Beacons are the most widely used.

- ***Hydrolysis Probes***

Hydrolysis assays exploit the 5′ exonuclease activity of specific thermostable polymerases such as Taq DNA polymerase. Hydrolysis probes for 5′ nuclease assays are synthesized with a fluorescent reporter, such as FAM, HEX, NED, TET, VIC, Cy3, or Tide Fluor™ dyes, covalently attached to the 5′ end and a quencher dye, such as DABCYL, TAMRA, BXQ-1, BXQ-2 or Tide Quencher™ dyes, to the 3′ end of a sequence-specific oligonucleotide probe, which is complementary to the target DNA sequence. While the probe is intact, the reporter and quencher remain in close proximity to each other, FRET occurs, and consequently, the reporter dye signal is quenched. During PCR cycling, both the primers and probe anneal to the target. As Taq DNA polymerase binds to and extends the primer upstream of the probe, any probe bound to the correct target sequence is hydrolyzed. The reporter dye fragment is released, resulting in a fluorescence signal proportional to the amount of amplicon produced.

Not only does this method benefit from high sensitivity and specificity, but it also allows multiplexing qPCR using probes

with different combinations of reporter dyes. This allows for an increase in throughput, meaning multiple samples can be assayed per plate, and consequently, there is a reduction in both sample and reagent usage.

- ***Molecular Beacons***

Molecular Beacons are single-stranded nucleic probes held in a hairpin-loop conformation (typically 20 to 40 nucleotides) by a complementary stem sequence of 4 to 6 nucleotides at each terminus. The hairpin-loop comprises of a nucleotide sequence complementary to the template, and the hydrogen-bonded stem sequences allow the 3′ quencher to suppress the fluorescence of the 5′ reporter when the probe is free in solution. Molecular Beacons hybridization to their specific target sequence causes the hairpin-loop structure to open, displacing the 5′ reporter from the 3′ quencher. As the quencher and reporter are no longer in proximity, fluorescence emission takes place. Unlike hydrolysis probes, the detection mechanism of Molecular Beacons does not depend on degradation during the reaction. Instead, the measured fluorescence signal is directly proportional to the amount of amplicon produced. Molecular Beacons are highly specific and can be used for multiplexing. More importantly, if the target sequence does not match the beacon sequence exactly, hybridization and fluorescence will not occur—an advantageous characteristic for allelic discrimination experiments.

The main disadvantage of using molecular beacons is that they are challenging to design. The hairpin stem must be strong enough that the molecule will not spontaneously fold into non-hairpin conformations that result in unintended fluorescence. Conversely, the hairpin stem must not be too strong, or the beacon may not properly hybridize to the target.

- ***Scorpion® Primers***

Scorpion® primers are highly sensitive, sequence-specific probes for qPCR, genotyping, allelic discrimination, and SNP detection. They feature a bi-functional design that combines a target specific PCR primer together with a bi-labeled target specific DNA probing sequence. Like Molecular Beacons, the

Table 2. Double-stranded DNA-binding dyes for qPCR

Product	Ex (nm)	Em (nm)	Unit Size	Catalog Number
Helixyte™ Green *20X Aqueous PCR Solution*	498 nm	522 nm	5x1 mL	17591
Helixyte™ Green *10,000X Aqueous PCR Solution*	498 nm	522 nm	1 mL	17592
Helixyte™ Green dsDNA Quantifying Reagent *200X DMSO Solution*	490 nm	525 nm	1 mL	17597
Helixyte™ Green dsDNA Quantifying Reagent *200X DMSO Solution*	490 nm	525 nm	1 mL	17598
Q4ever™ Green *2000X DMSO Solution*	503 nm	527 nm	50 µL	17608
Q4ever™ Green *2000X DMSO Solution*	503 nm	527 nm	1 mL	17609

Table 3. Relative effectiveness of PCR detection strategies for common applications.

Application	Helixyte™ Green/Q4ever™ Green	Hydrolysis Probes (Taqman®)	Molecular Beacons	Scorpions® Probes
Allelic Discrimination			√	√√
Gene Expression Analysis	√	√√	√√	√√
Gene Copy Determination		√	√	√√
End-Point Genotyping			√√	√√
In vitro Quantification			√√	
Mass Screening	√√			
Microarray Validation	√√	√		
Multiple Target Genes	√	√		
Multiplexing		√√	√√	√√
Pathogen Detection	√	√	√	√√
SNP Detection			√	√√
Viral Load Quantification		√	√	√√

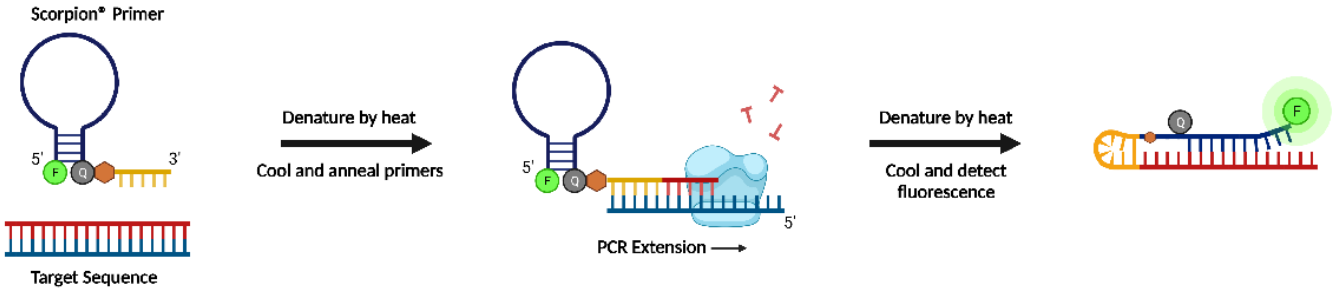

Figure 3. An illustration of the mechanism of action of Scorpion® primers. Scorpion® primers combine a target-specific primer and probe in a single sequence for rapid qPCR quantification (figure made in BioRender).

DNA probing component is a self-complementary sequence labeled with a reporter at the 5′ end. However, the internal quencher is directly linked to the 5′ end of the PCR primer via a hexathylene glycol (HEG) blocker. This HEG blocker is an essential element of Scorpion® primers as it prevents the polymerase from extending the PCR primer.

Following the completion of one PCR cycle, the probe and newly synthesized target region will be attached to the same strand.

During subsequent denaturation and annealing, the hair-pin loop unfolds and the specific probe sequence hybridzes to its complement within the extended amplicon, and a signal is observed (Figure 3).

This uni-moleculer mechanism increases reaction kinetics, which enables Scorpion® primers to generate fluorescent signals instantaneously without incurring any competing side reactions.

Summary

There are many considerations when selecting a detection strategy for a particular PCR application. While Helixyte™ Green and TaqMan® hydrolysis assays generally work well, there are situations in which the other detection systems might be preferable.

References

1. Holland, P. M., Abramson, R. D., Watson, R., & Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5′----3′ exonuclease activity of Thermus aquaticus DNA polymerase. Proceedings of the National Academy of Sciences of the United States of America, 88(16), 7276–7280. https://doi.org/10.1073/pnas.88.16.7276
2. Garibyan, L., & Avashia, N. (2013). Polymerase chain reaction. The Journal of investigative dermatology, 133(3), 1–4. https://doi.org/10.1038/jid.2013.1
3. Ng, C. T., Gilchrist, C. A., Lane, A., Roy, S., Haque, R., & Houpt, E. R. (2005). Multiplex real-time PCR assay using Scorpion probes and DNA capture for genotype-specific detection of Giardia lamblia on fecal samples. Journal of clinical microbiology, 43(3), 1256–1260. https://doi.org/10.1128/JCM.43.3.1256-1260.2005
4. Rockett, R. J., Tozer, S. J., Peatey, C., Bialasiewicz, S., Whiley, D. M., Nissen, M. D., Trenholme, K., Mc Carthy, J. S., & Sloots, T. P. (2011). A real-time, quantitative PCR method using hydrolysis probes for the monitoring of Plasmodium falciparum load in experimentally infected human volunteers. Malaria journal, 10, 48. https://doi.org/10.1186/1475-2875-10-48
5. Tyagi, S., & Kramer, F. R. (2012). Molecular beacons in diagnostics. F1000 medicine reports, 4, 10. https://doi.org/10.3410/M4-10√

Product	Unit Size	Cat No.
Helixyte™ Green Nucleic Acid Gel Stain *10,000X DMSO Solution*	1 mL	17590
Helixyte™ Green Nucleic Acid Gel Stain *10,000X DMSO Solution*	100 µL	17604
Helixyte™ Gold Nucleic Acid Gel Stain *10,000X DMSO Solution*	1 mL	17595
Gelite™ Green Nucleic Acid Gel Staining Kit	1 Kit	17589
Gelite™ Orange Nucleic Acid Gel Staining Kit	1 Kit	17594
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	100 µL	17700
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	500 µL	17701
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	1 mL	17702
Gelite™ Safe DNA Gel Stain *10,000X Water Solution*	10 mL	17703
Gelite™ Safe DNA Gel Stain *10,000X DMSO Solution*	100 µL	17704
Gelite™ Safe DNA Gel Stain *10,000X DMSO Solution*	500 µL	17705
Gelite™ Safe DNA Gel Stain *10,000X DMSO Solution*	1 mL	17706
Gelite™ Safe DNA Gel Stain *10,000X DMSO Solution*	10 mL	17707

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Featured Articles

Development and Applications of Coronavirus Protease-Specific Covidyte™ and Covipyte™ Peptide Substrates

Prolonged Retention of Calbryte™ 520 AM for In Vitro Analysis

T Lymphocyte Immunophenotyping Using Multicolor Flow Cytometry

A Practical Guide for Labeling Antibodies

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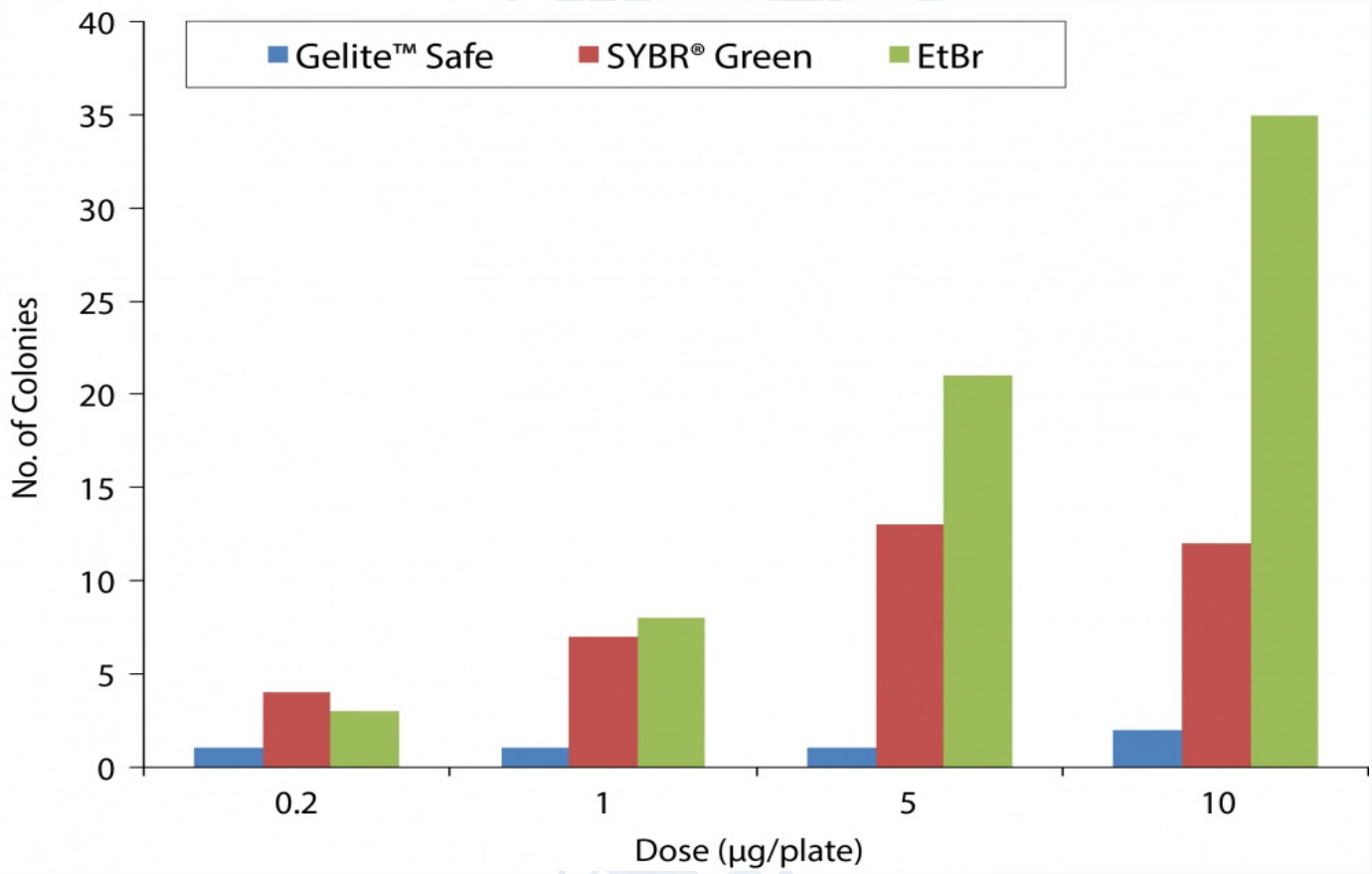
Safety Comparison



Summary of Ames Test Results

Ames mutagenicity test was performed in a dose-dependent manner for Gelite™ Safe, SYBR® Green, and EtBr. Samples were pretreated with an S9 fraction liver extract and then tested. With S. Typhimurium strain TA1538, an increase in revertants of more than two-fold over the background indicates a positive result for mutagenicity.

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