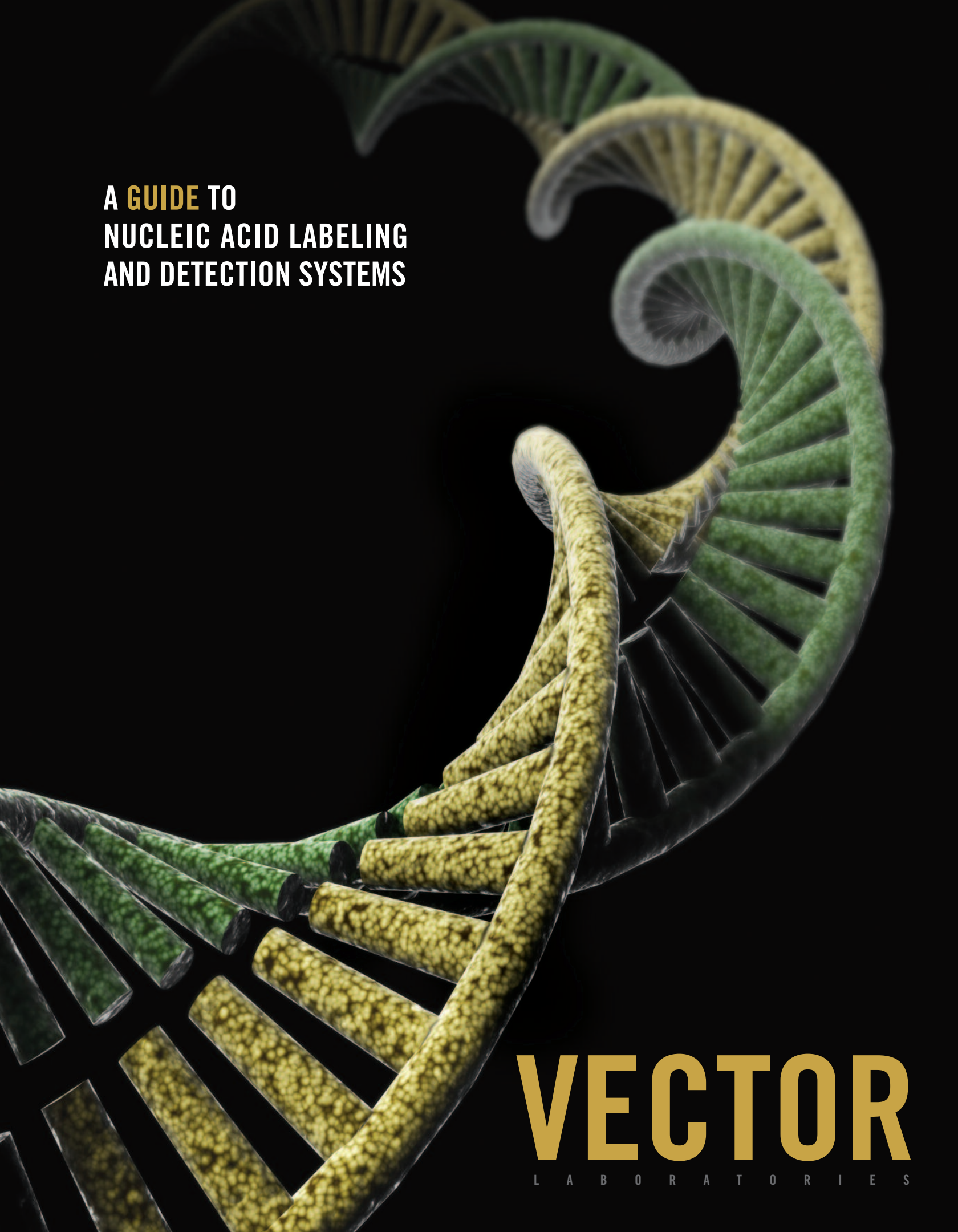
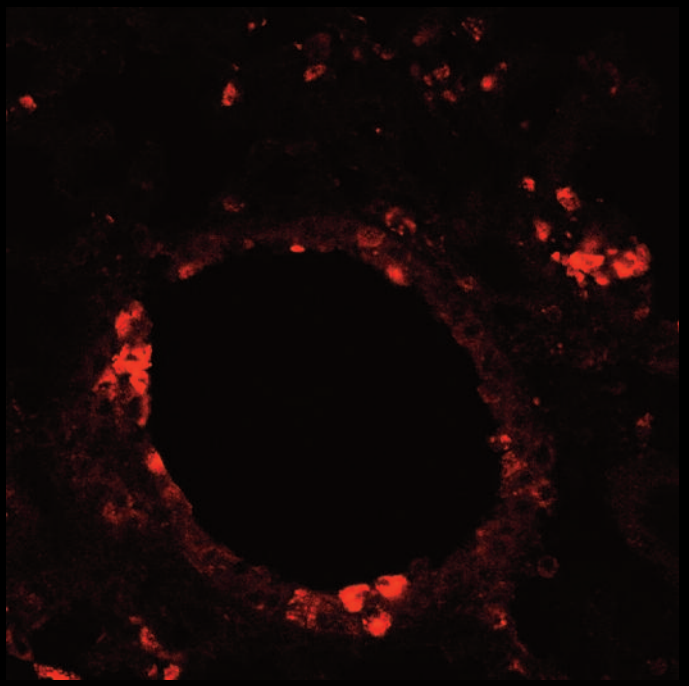
A GUIDE TO NUCLEIC ACID LABELING AND DETECTION SYSTEMS



VECTOR

LABORATORIES



STATE OF THE ART LABELING & DETECTION SYSTEMS

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***Inside front cover**: A plasmid biotinylated with the FastTag® Biotin Labeling System was complexed with a lactosylated polyethylenimine and instilled intranasally to mice. On this tissue slide, the plasmid labeled with rhodamine-conjugated streptavidin (red) is detected in epithelial cells of the bronchus and in the pulmonary cells surrounding it. Image kindly supplied by G.Thévenot and Dr. I. Fajac, Faculté de Médecine Paris 5, Paris, France.*

Introduction

Recognized as a leader in the development of detection systems, Vector Laboratories has been providing the research community with quality reagents yielding high sensitivity and low background since 1976. Continuing in this tradition, our molecular biology tools offer sensitivity, reliability, simplicity, and economy. These systems and reagents facilitate the labeling, detection, and manipulation of nucleic acids. Microarrays, blotting assays, cell trafficking, *in situ* hybridization, comparative genomic hybridization, subtractive hybridization, and affinity purification are just a few applications in which our molecular biology tools can be successfully employed.

Labeling of Nucleic Acids

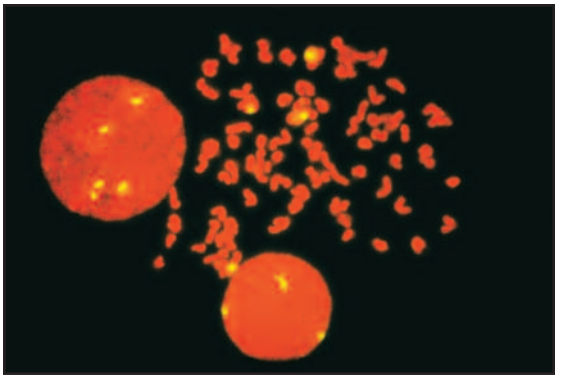
The PHOTOPROBE® reagents, FastTag® Systems, and the 3' and 5' EndTag™ Labeling Systems are efficient and reliable labeling methods that offer choices for optimum labeling for a given application, as well as choices for incorporating fluorochromes, haptens, or affinity tags.

Nucleic acid probes for applications such as *in situ* hybridization, northern and Southern blot hybridization, comparative genomic hybridization, intracellular localization experiments, and DNA microarrays can be easily and optimally labeled and subsequently visualized with our comprehensive range of fluorescent, chemiluminescent, or chromogenic detection systems.

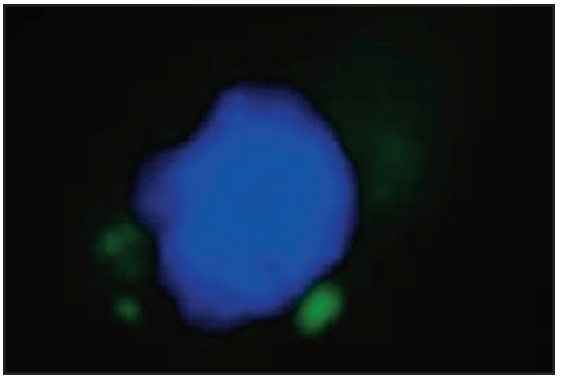
Detection of Nucleic Acid Labels

Each choice of label is complemented by several different fluorescent or enzyme-based detection reagents providing the researcher with flexibility in optimizing experimental systems. Among the extensive range of antibody conjugates and avidin/streptavidin conjugates, amplification reagents such as Biotinylated Anti-Streptavidin and Biotinylated Anti-Avidin are also available. These reagents reliably increase sensitivities of target signals in microarray applications and fluorescence *in situ* hybridization. In addition, the superior anti-fading property of the VECTASHIELD® line of mounting media ensures the preservation of signal intensity in applications such as fluorescence *in situ* hybridization. (Please see pages 10-13 for a listing of selected products.)

For nucleic acid blotting applications, the Vector® UltraSNAP™ Detection System (page 14) containing specially formulated reagents detects biotinylated probes with high sensitivity and low background. This system utilizes the **DuoLuX**™ Chemiluminescent/ Fluorescent Substrate with intense and prolonged light emission characteristics, ideal for Southern and northern hybridization, dot blot, plaque or colony screening.



FastTag® Fluorescein labeled pUC1.77 (yellow-green) detected with Biotinylated Anti-Fluorescein and Fluorescein Avidin DCS. Nuclei were counterstained and mounted with VECTASHIELD® with PI (red-orange).



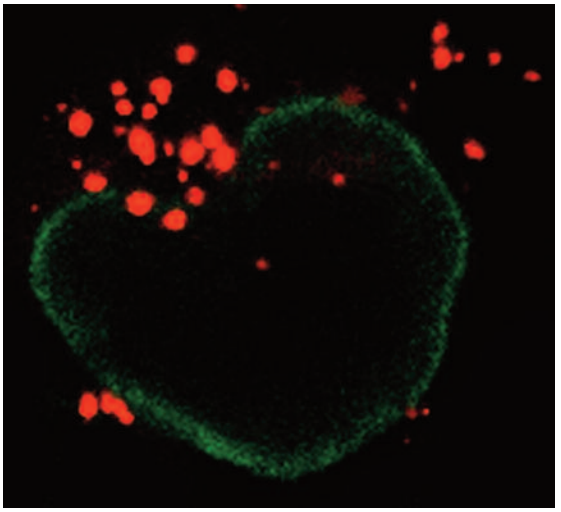
Cellular uptake of labeled plasmid: FastTag® Fluorescein labeled plasmid DNA (green) incubated with COS-7 cells. Nuclei were counterstained and mounted with VECTASHIELD® with DAPI (blue).

Immobilization of Labeled Probes

Vector Laboratories offers both irreversible (VECTREX® Avidin D) as well as reversible (VECTREX® Avidin DLA or VECTREX® AAL) affinity binding matrices. Applications like genomic/ cDNA subtraction or library screening by hybrid capture require labeled nucleic acids to be immobilized onto a solid support. Using our affinity binding matrices in such applications can yield information about differential gene expression or allow the isolation and subsequent amplification of a gene of interest. (Please see page 17 for further information).

Electrophoretic Resolution of Similarly Sized DNA in a Sequence Specific Manner

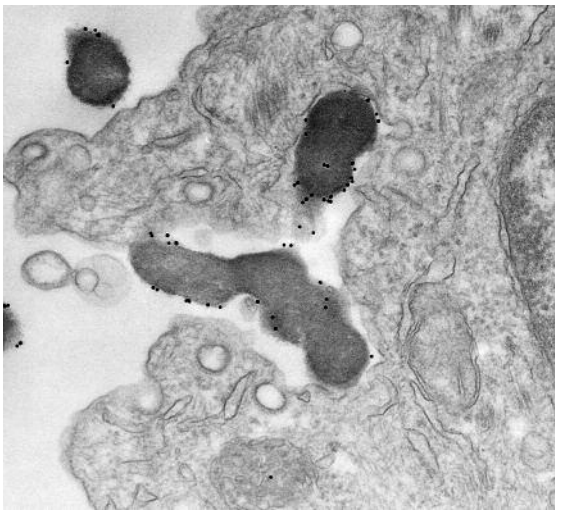
The Resolve-It™ Kit (page 18) contains two sequence-specific DNA ligands which bind to DNA and retard its electrophoretic migration in a sequence specific manner. The separation of the DNA species allows for the gel purification of DNA samples which would otherwise be very difficult to isolate. The ability to resolve DNA in a gel is critical for applications such as differential display in which multiple DNA species of similar size need to be separated before subsequent excision and analysis.



Detection in an airway epithelial cell of a plasmid biotinylated with the FastTag® Biotin Labeling System and then labeled with rhodamine-conjugated streptavidin (red). The nuclear membrane is labeled with an anti-lamin A/C antibody and appears green. Image kindly supplied by Drs. S. Grosse and I. Fajac, Faculté de Médecine Paris 5, Paris.

Quantitation of Biotin using the Quant*Tag™ System

The Quant*Tag™ Biotin Kit (page 19) is designed to determine the amount of free biotin in solution or the number of biotins attached to proteins, nucleic acids or other macromolecules. Samples do not need to be predigested. The kit reagents chemically react with free or bound biotin, producing a colored product that can be quantified using a spectrophotometer. The absorbance is measured in the visible spectrum, allowing the use of plastic cuvettes or microtitre plates.



Detection by electron microscopy of a plasmid biotinylated with the FastTag® Biotin Labeling System and then gold-labeled. The plasmid, complexed with lactosylated PEI, is taken up by an airway epithelial cell. Image kindly supplied by Drs. S. Grosse and I. Fajac, Faculté de Médecine Paris 5, Paris.

Labeling of Nucleic Acids

Two important considerations in determining what labeling system will work best in a given application are: (1) size and type of the nucleic acid to be labeled; (2) choice of label required for the application.

Size and type. **Longer strands** of DNA (>100 bp) or circular DNA (i.e. plasmid DNA), RNA or PNA (peptide nucleic acid) used in blots, *in situ* hybridization, subtractive hybridization, or other procedures are efficiently and reliably labeled with the PHOTOPROBE® labeling reagents or the FastTag® Nucleic Acid Labeling System. Either labeling method results in the covalent coupling of the label to the sample. These systems ensure multiple site labeling over the entire length of the nucleic acid resulting in greater accessibility of the affinity tag or greater sensitivity in detection.

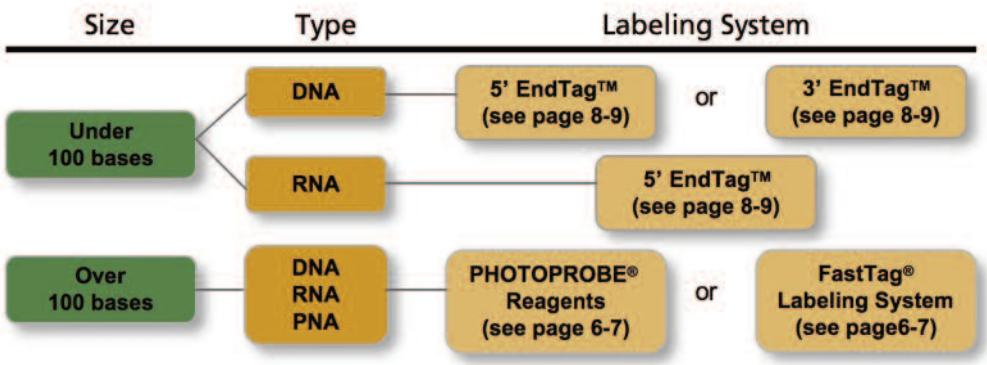
The integrity of the nucleic acid is preserved in this non-destructive reaction making it useful for applications where it is necessary to use the intact, original sample. With the PHOTOPROBE® or FastTag® labeling systems, the entire length of the original nucleic acid sample, rather than copies, is directly, labeled. In contrast, enzymatic labeling methods such as random priming or nick translation are difficult to control and don't label the original nucleic acid sample. Instead, these methods result in a labeled copy produced from the original template.

Thus, the PHOTOPROBE® or FastTag® chemical labeling method is especially convenient for labeling samples that will be used to observe cellular localization of nucleic acid (e.g. plasmid DNA in gene delivery or siRNA) or quantitative comparison.

Shorter strands of nucleic acids such as oligonucleotides, PCR primers, or capture probes used to identify nucleic acid binding proteins are specifically and efficiently labeled at either the 5′ or the 3′ end using the 5′ EndTag™ or the 3′ EndTag™ Nucleic Acid Labeling Systems, respectively. The 5′ EndTag™ or the 3′ EndTag™ Kits can be used to attach a single fluorochrome or affinity tag at the appropriate end of nucleic acids. 5′ EndTag™ Labeling kit uses both DNA and RNA as a substrate whereas the 3′ EndTag™ Kit will selectively label only DNA.

Application. The second important factor in determining the most appropriate labeling system is the choice of label, hapten, or affinity tag required for the given application. The PHOTOPROBE® reagents incorporate a specific tag (e.g. biotin) into nucleic acid in one simple step. The versatility of the FastTag® Labeling kit, 5′ EndTag™ or 3′ EndTag™ Labeling Kits allows a variety of tags to be incorporated. Some commonly used labels that are available from Vector Laboratories are briefly described as follows. Detection reagents for these labels are listed on page 10.

Choose a Labeling System based on Nucleic Acid Type and Size



Biotin - Because of the extraordinary affinity of avidin and streptavidin to biotin and the many biotin-avidin/streptavidin systems available, this label is ideal for a variety of applications including *in situ* hybridization, blotting, and affinity binding.

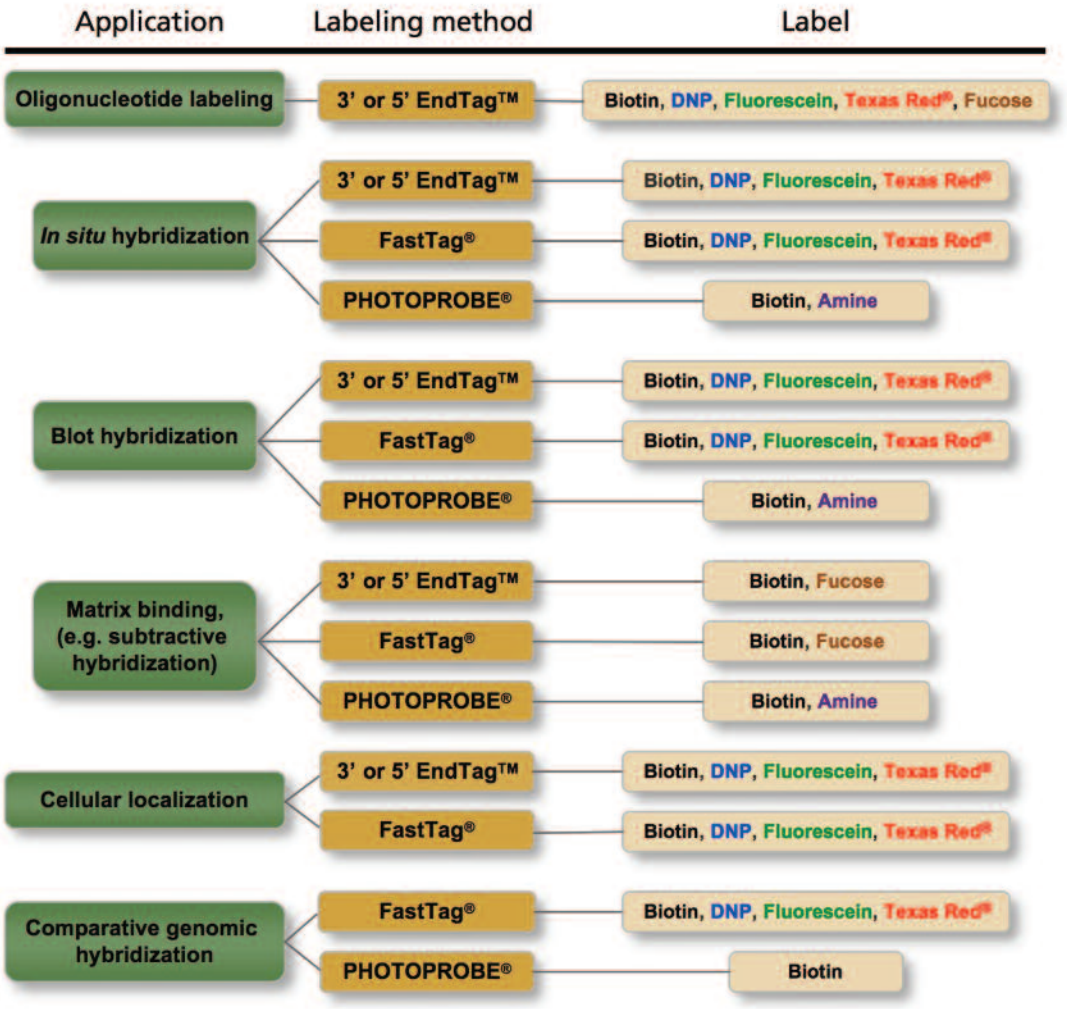
DNP (Dinitrophenyl) - Because DNP is not found endogenously in tissue, it is an excellent alternative to biotin. High affinity, purified antibodies are available for detection or amplification of the signal.

Fluorescein - This fluorescent label can be directly detected or used as a hapten and detected with our high affinity antibody reagents. (Excitation maximum at 495 nm; emission maximum at 515 nm).

Fucose - This unique label is ideal for reversible binding of labeled nucleic acid to the matrix, VECTREX® AAL. Fucose labeled nucleic acids can be bound and eluted under mild conditions. (For more information, please see page 17). Alkaline phosphatase *Aleuria aurantia* lectin can be used in dot blot applications to assess labeling efficiency with the fucose label.

Texas Red® - This fluorescent label is a high quantum yield rhodamine derivative that can be directly visualized. The label can also be detected, and the signal amplified, using our affinity-purified antibody conjugates to rhodamine. (Excitation maximum at 595 nm; emission maximum at 615 nm).

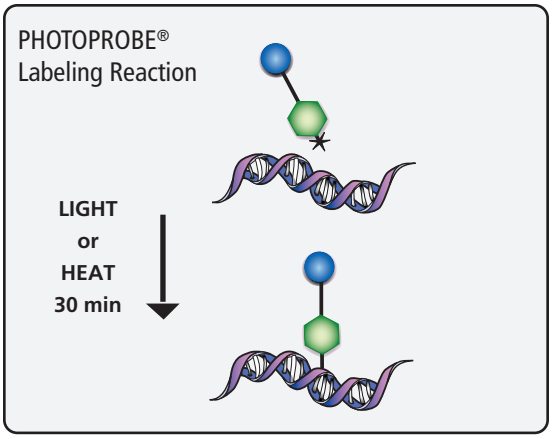
Choose a Labeling System based on Application and Label



PHOTOPROBE® Reagents and the FastTag® System

PHOTOPROBE® Reagents and the FastTag® Nucleic Acid Labeling Systems are the methods of choice for incorporating a label at multiple sites along the entire length of the nucleic acid. The labeling reaction does not destroy the original nucleic acid nor creates its copy. The integrity of the original sample is preserved. Single-[15, 28] or double-stranded[11,20] DNA, circular DNA,[45, 58] RNA,[22] siRNA, [49] or PNA (peptide nucleic acid) can be labeled with the same reagents.

Labeling with either system is based on aryl azide chemistry in which either reagent, when exposed to heat or light, becomes activated and incorporates into the nucleic acid without base specificity. The labeling reaction can be carried out using a mercury vapor bulb (sun lamp), a UV lamp which produces light in the 350 nm to 370 nm range, a halogen lamp, a heating block, or a thermal cycler providing the investigator with great flexibility in experimental design.



PHOTOPROBE® labeling (above) occurs in one short heat- or photo- labeling step.

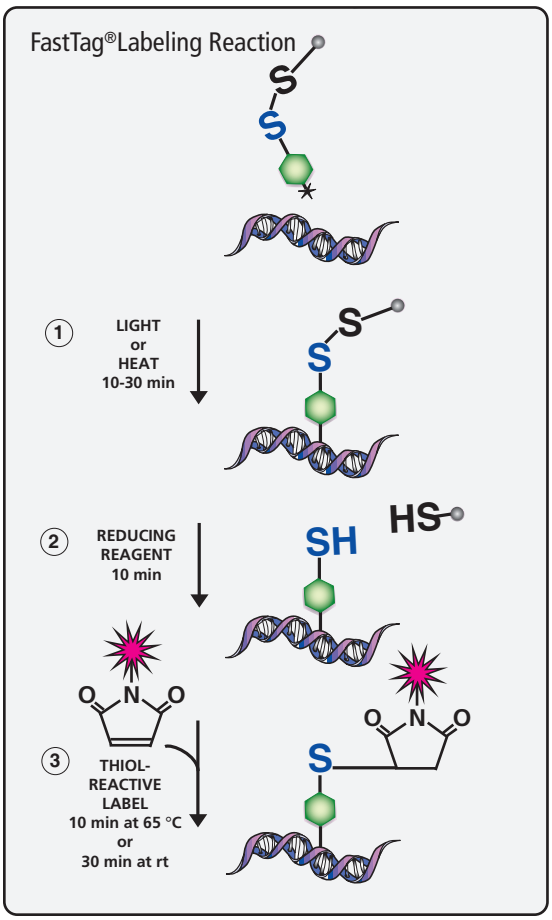
FastTag® labeling (right) occurs in three easy steps:

***Step 1**. The FastTag® Universal Linker is incorporated into the nucleic acid upon exposure to heat or light.*
***Step 2.** The disulfide bond is reduced, yielding a free thiol group.*
***Step 3**. A covalent bond is formed between the FastTag® reagent thiol and a thiol-reactive hapten, fluorochrome, affinity ligand, or other marker.*

A distinct advantage of the simple setup in each of these labeling options is the ease and economy of scaling up.

PHOTOPROBE® Biotin incorporates biotin in one reaction step. PHOTOPROBE® (Long Arm) Biotin has an extra long linker arm and should be used if increased distance between the sample and the tag is needed. In a similar manner, PHOTOPROBE® Amine incorporates primary amines into nucleic acids. These amino groups can subsequently be used to attach haptens, affinity tags, or fluorochromes, or to immobilize nucleic acids to a solid matrix.

The FastTag® Nucleic Acid Labeling System utilizes a disulfide-containing universal linker that is incorporated into nucleic acid simply by using heat or light. Reducing the disulfide bond yields a free thiol group. A variety of thiol-reactive haptens, fluorochromes, affinity ligands or other markers can then be covalently bound to the nucleic acid via the FastTag® reagent thiol group. See page 7 for a list of thiol-reactive reagents available from Vector Laboratories.



Each **PHOTOPROBE® Biotin Kit** contains the following components to label up to 250 µg of nucleic acid (or to carry out up to 50 labeling reactions):

- PHOTOPROBE® Biotin Reagent
- Tris Buffer
- sec-Butanol
- Biotinylated DNA Standard
- Precipitant

The **PHOTOPROBE® Amine Kit** includes the following components to label up to 360 µg of nucleic acid (or to carry out up to 72 labeling reactions):

- PHOTOPROBE® Amine
- Borate Buffer
- sec-Butanol
- Precipitant

The **FastTag® Labeling Kit**† contains the following reagents to label up to 250 µg of nucleic acid (or to carry out up to 50 labeling reactions):

- FastTag® Reagent
- Tris Buffer
- Citrate Buffer
- Reducing Reagent
- sec-Butanol
- Precipitant

†Use this kit with any thiol-reactive label.

Labeling Systems

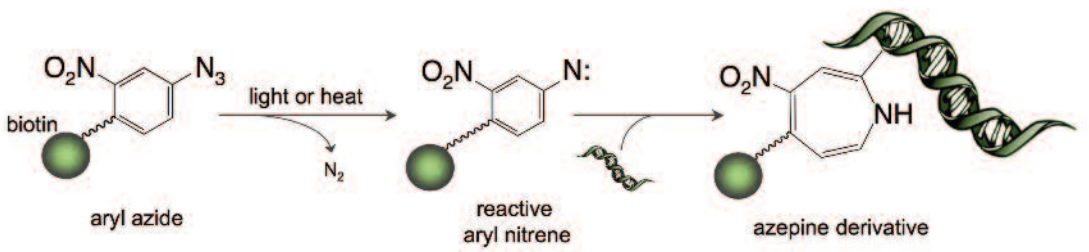
FastTag® Labeling Kit	MB-8000	• 1 Kit
PHOTOPROBE® Biotin	SP-1000	• 0.5mg
PHOTOPROBE® (Long Arm) Biotin	SP-1020	• 0.5mg
PHOTOPROBE® Biotin Labeling and Detection System	SPK-1906	• 1 Kit
PHOTOPROBE® Amine	SP-1070	• 0.5mg

Thiol-reactive Labeling Reagents

Biotin Maleimide	SP-1501	• 12mg
DNP Maleimide	SP-1503	• 1mg
Fluorescein Maleimide	SP-1502	• 12mg
Fucose Maleimide	SP-1504	• 500µg
Texas Red® Maleimide	SP-1505	• 3.6mg

These thiol-specific labels can be used with the FastTag® Labeling Kit, 5′ EndTag™ or 3′ EndTag™ Labeling Kits.

Aryl Azide Chemistry



5′ EndTag™ and 3′ EndTag™ Labeling Systems

End labeling is a favored method for applications where an internal label might interfere with hybridization or sequence-specific protein binding. Short oligonucleotides are labeled more efficiently with these systems than with other methods. In addition, end labeling of oligonucleotides is an economical alternative to having labels inserted during synthesis.

Both the 5′ EndTag™ and the 3′ EndTag™ Nucleic Acid Labeling Systems enable the covalent attachment of a variety of fluorescent dyes, haptens or affinity tags to the respective ends of the nucleic acids using thiol-specific chemistry.

5′ EndTag™ is ideal for labeling PCR primers because a label is attached only at the 5′ end, leaving the 3′ end available for polymerization. The end position of the label generally does not interfere with hybridization or nucleic acid binding and is, therefore, appropriate for binding of capture probes to affinity matrices[30, 52] and for electrophoretic mobility shift assays (EMSA).[19, 37]

The 5′ EndTag™ system labels 5′ ends of DNA, RNA,[30, 60] or unmodified oligonucleotides.[47, 57]

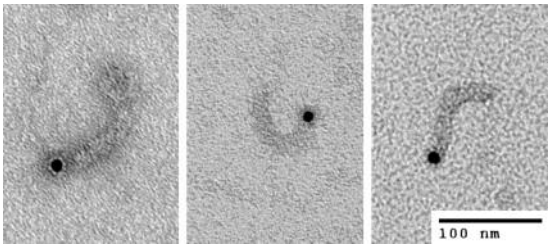
3′ end labeling is preferred over 5′ end labeling if the terminal phosphate at the 5′ end must be preserved. 3′ EndTag™ labeled nucleic acids can be used for applications such as DNA hybridization, PCR, *in situ* hybridization, or EMSA. The 3′ EndTag™ system enables simple and uniform labeling of 3′ ends of DNA.

5′ EndTag™ Kit	MB-9001	•	1 Kit
3′ EndTag™ Kit	MB-9002	•	1 Kit

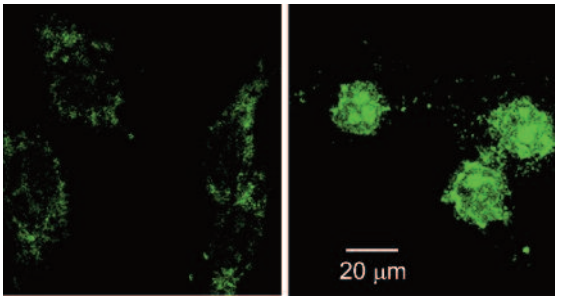
A thiol-reactive label is not included in the kit, but one can be selected from the list below.

Thiol-reactive Labeling Reagents

Biotin Maleimide	SP-1501	•	12mg
DNP Maleimide	SP-1503	•	1mg
Fluorescein Maleimide	SP-1502	•	12mg
Fucose Maleimide	SP-1504	•	500µg
Texas Red® Maleimide	SP-1505	•	3.6mg



Transmission electron micrographs of molecules of influenza A viral ribonucleoprotein particles (vRNPs) labeled at the 5′ end of the vRNA with biotin using the 5′ EndTag™ Kit, and further labeled with streptavidin gold. vRNPs from influenza A were purified according to Kemler et al., (1994 Virology, 202:1028-1033) on a glycerol gradient with modifications as described in Wu et al., (2007, Virol J. Jun 4;4:49). Courtesy of Drs. Winco WH Wu and Nelly Panté, University of British Columbia, Vancouver BC, Canada.



- energy
- cytosol

+ energy
+ cytosol

Nuclear import assay in digitonin-permeabilized HeLa cells of biotinylated vRNPs. vRNPs were labeled first at the 5′ end of the vRNA with biotin using the 5′ EndTag™ Kit, and then with Vector® Fluorescein Streptavidin. This allowed for direct fluorescence visualization of the vRNPs on a confocal fluorescence microscope. Nuclear import assays were carried out as described in Wu et al., (2007, Virol J. Jun 4;4:49). The negative control consists of vRNPs added to the cells in the absence of energy and exogenous cytosol. In the presence of energy and cytosol, the fluorescein-labeled vRNPs successfully enter the nucleus, with a high degree of nucleolar staining. Courtesy of Drs. Winco WH Wu and Nelly Panté, University of British Columbia, Vancouver BC, Canada.

5′ End Labeling Kit

Labeling with the 5′ EndTag™ Kit is achieved by a simple two step process. The first step is to incorporate a thiol group onto the 5′ end of the oligonucleotide. This is accomplished by T4 polynucleotide kinase which transfers a thiol-phosphate from ATPγS to the 5′-OH group of the oligonucleotide. In the second step the thiol-containing oligonucleotide is incubated with a thiol-reactive label (e.g. biotin-maleimide). Labeling requires about 1 hour with very few minutes of hands-on time.

The 5′ EndTag™ Kit is designed to perform 10 labeling reactions of up to 0.6 nmols of 5′ ends (e.g. about 5 µg of a 25 base oligo) per reaction and includes:

- T4 polynucleotide kinase
- 10x reaction buffer
- ATPγS
- Precipitant
- Alkaline Phosphatase

(A thiol-reactive label is not included.)

Figure A schematically shows the 3′ EndTag™ labeling reaction. **Figure B** shows the labeling reaction for the 5′ EndTag™ Labeling System.

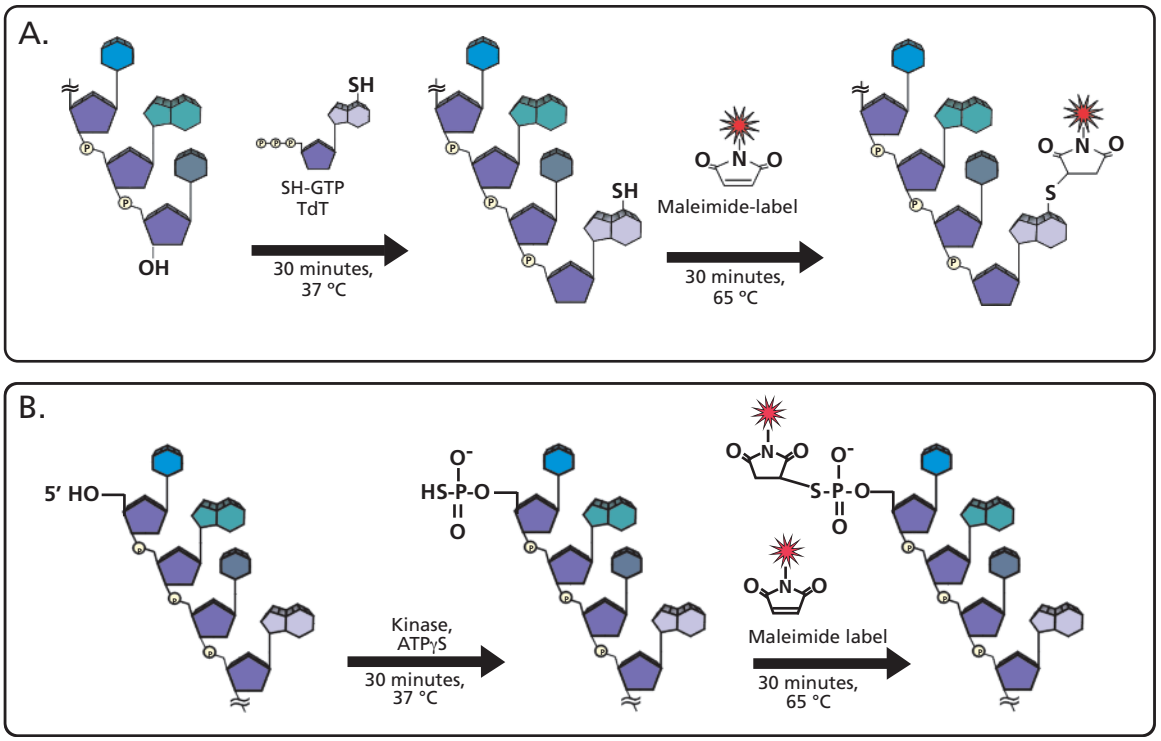
3′ End Labeling Kit

Labeling using the 3′ EndTag™ Labeling Kit is achieved in two steps. The first step is to incorporate a thiol group onto the 3′ end of the DNA. This is accomplished by using a terminal transferase enzyme (TdT) to attach SH-GTP, a modified guanosine triphosphate containing a thiol, to the double or single stranded DNA end. Blunt, overhanging, or recessed 3′-OH ends may also be used. In the second step the thiol-containing oligonucleotide is incubated with a thiol-reactive label (e.g. biotin maleimide). Labeling time is about 1 hour with very little hands-on time. Labeling efficiency is equivalent to that achieved with traditional methods but the 3′ EndTag™ labeling system offers greater versatility in the choice of label at a fraction of the cost.

The 3′ EndTag™ Labeling Kit is designed to perform 20 labeling reactions of up to 0.5 nmols of 3′ ends (e.g. about 3.3 µg of a 20 base oligonucleotide) per reaction and includes:

- Terminal transferase (TdT)
- SH-GTP
- 10x TdT buffer
- Precipitant

(A thiol-reactive label is not included.)



Detection of the Nucleic Acid Label

Our range of products offers various options for detection of a given label. To select the desired visualization method, choose the reagent according to the label being detected. Then choose the complementary visualization reagents, either fluorescence or enzyme-based, from the lists on the following pages.

All antibodies to the labels are affinity-purified and are ideal for detecting labeled probes in such applications as *in situ* hybridization (please see pages 22-25 for protocols) or northern and Southern blotting (please see pages 20-21 for protocol).

Biotin - Because of the many biotin-avidin/streptavidin systems available, this label is ideal for a variety of applications including *in situ* hybridization, blotting, and affinity binding.

Alkaline Phosphatase Anti-Biotin *made in goat*	SP-3020	•	1ml
Alkaline Phosphatase Streptavidin	SA-5100	•	1ml
Peroxidase Anti-Biotin *made in goat*	SP-3010	•	1mg
Peroxidase Streptavidin	SA-5004	•	1mg
VECTASTAIN® Elite ABC Kit (Standard)	PK-6100	•	1 kit
Fluorescein Anti-Biotin *made in goat*	SP-3040	•	0.5mg
Fluorescein Avidin DCS	A-2011	•	1mg
Fluorescein Streptavidin	SA-5001	•	1mg
Texas Red® Avidin DCS	A-2016	•	1mg
Texas Red® Streptavidin	SA-5006	•	1mg
AMCA Avidin D	A-2008	•	5mg
AMCA Streptavidin	SA-5008	•	1mg
Phycoerythrin Avidin D	A-2007	•	1mg
Phycoerythrin Streptavidin	SA-5007	•	1mg
Anti-Biotin-M (monoclonal) *made in mouse*	MB-9100	•	1ml
Anti-Biotin (polyclonal) *made in goat*	SP-3000	•	1mg
Biotinylated Anti-Avidin *made in goat*	BA-0300	•	0.5mg
Biotinylated Anti-Streptavidin *made in goat*	BA-0500	•	0.5mg
UltraSNAP™ Detection Kit for blots	MB-6500	•	1 Kit

DNP (Dinitrophenyl) - Because DNP is not found endogenously in tissue, it is an excellent alternative label to biotin. High affinity, purified antibodies are available for detection or amplification of the signal.

Alkaline Phosphatase Anti-DNP *made in rabbit*	MB-3100	•	150µg
Biotinylated Anti-DNP *made in rabbit*	BA-0603	•	0.5mg

Fluorescein - This fluorescent label can be directly detected or used as a hapten and detected with any of our high affinity antibody reagents. (Excitation maximum at 495 nm; emission maximum at 515 nm).

Anti-Fluorescein *made in goat*	SP-0601	•	1mg
Alkaline Phosphatase Anti-Fluorescein *made in goat*	MB-2100	•	150µg
Peroxidase Anti-Fluorescein *made in goat*	SP-1910	•	0.5mg
Biotinylated Anti-Fluorescein *made in goat*	BA-0601	•	0.5mg

Fucose - This unique label is ideal for reversible binding of labeled nucleic acid to the matrix, VECTREX® AAL. Fucose labeled nucleic acids can be bound and eluted under mild conditions. (For more information, please see page 17). Alkaline phosphatase *Aleuria aurantia* lectin can be used in dot blot applications to assess labeling efficiency with the fucose label.

Alkaline Phosphatase *Aleuria aurantia* lectin (AAL)	MB-4100	•	150µg
Biotinylated *Aleuria aurantia* lectin (AAL) *made in goat*	B-1395	•	1mg

Texas Red® - This fluorescent label is a rhodamine derivative that can be directly visualized. The label can also be detected, and the signal amplified using our affinity-purified antibody conjugates to rhodamine. (Excitation maximum at 595 nm; emission maximum at 615 nm).

Alkaline Phosphatase Anti-Rhodamine* *made in goat*	MB-1920	•	150µg
Biotinylated Anti-Rhodamine* *made in goat*	BA-0605	•	0.5mg

**Binds most rhodamines including Texas Red®*

Complementary Detection Reagents

Fluorescent Systems

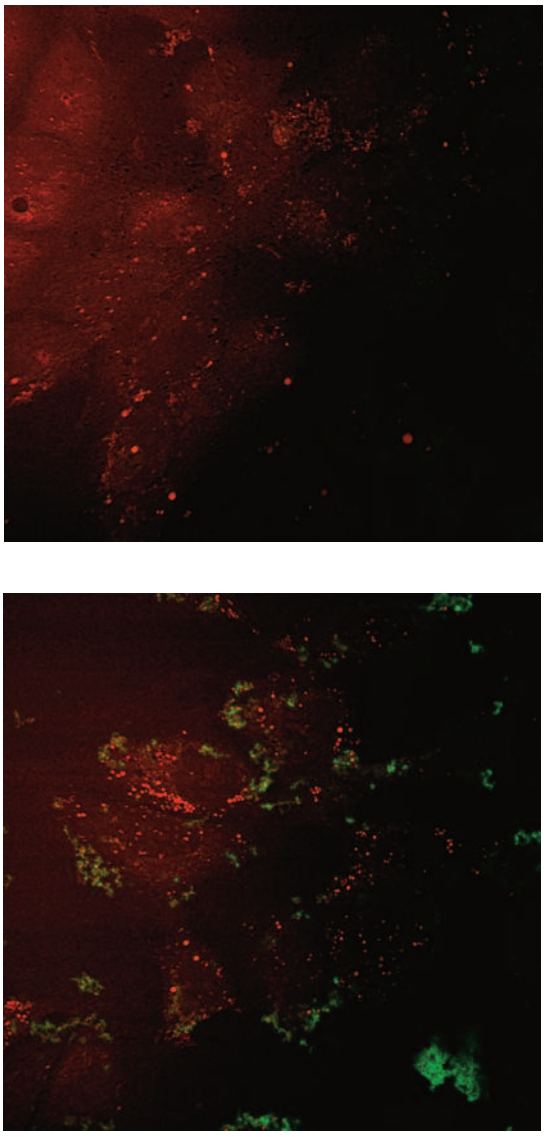
The following fluorescent detection reagents are ideal for applications such as fluorescence *in situ* hybridization. Biotinylated Anti-Avidin D[5, 18, 29] and Biotinylated Anti-Streptavidin[56] are effectively used for amplifying fluorescent signals in biotin-avidin/streptavidin systems (please see page 22 for protocol).

After choosing a fluorescent detection method, choose one of the VECTASHIELD® mounting media on page 12 for mounting and coverslipping. VECTASHIELD® mounting media are unsurpassed in preserving and preventing the fading of fluorescently labeled sections, cells or spreads.

Fluorescein Avidin DCS	A-2011	• 1mg
Fluorescein Streptavidin	SA-5001	• 1mg
Texas Red® Avidin DCS	A-2016	• 1mg
Texas Red® Streptavidin	SA-5006	• 1mg
AMCA Avidin D	A-2008	• 5mg
AMCA Streptavidin	SA-5008	• 1mg
Phycoerythrin Avidin D	A-2007	• 1mg
Phycoerythrin Streptavidin	SA-5007	• 1mg
Biotinylated Anti-Avidin* *made in goat*	BA-0300	• 0.5mg
Biotinylated Anti-Streptavidin* *made in goat*	BA-0500	• 0.5mg
Fluorescein Anti-Goat IgG *made in rabbit*	FI-5000	• 1.5mg
Fluorescein Anti-Mouse IgG *made in horse*	FI-2000	• 1.5mg
Texas Red® Anti-Goat IgG *made in rabbit*	TI-5000	• 1.5mg
Texas Red® Anti-Mouse IgG *made in horse*	TI-2000	• 1.5mg
AMCA Anti-Goat IgG *made in rabbit*	CI-5000	• 1.5mg
AMCA Anti-Mouse IgG *made in horse*	CI-2000	• 1.5mg

* Amplifying Reagent

FLUOROPHOR	EXCITATION	EMISSION
AMCA	350 nm	450 nm
Fluorescein	495 nm	515 nm
Texas Red®	595 nm	615 nm
Phycoerythrin	450-570 nm	574 nm



Coherent anti-Stokes Raman scattering (CARS) and two-photon fluorescence (TPF) imaging of fluorescein-labeled HCV RNA inside Huh-7 cells. HCV RNA in-vitro transcripts were labeled with a fluorescein-maleimide label (SP-1502) using the 5′ EndTag™ Nucleic Acid Labeling System. Huh-7 cells were transfected with 3 µg of 5′-fluorescein-labeled HCV RNA and imaged at 4 h post-transfection. Top: CARS and TPF of Mock transfected Huh-7 cells. Bottom: CARS and TPF of Huh-7 cells transfected with fluorescein-labeled HCV RNA. Figure courtesy of Jennifer Haley, Sylvie Belanger, Adrian Pegoraro, Albert Stolow, and John P. Pezacki, The Steacie Institute for Molecular Sciences, The National Research Council of Canada. Unpublished data.

VECTASHIELD® Mounting Media

VECTASHIELD® Mounting Media are unsurpassed in preventing the rapid loss of fluorescence during microscopic examination. The different formulations of VECTASHIELD® Mounting Media all offer the same outstanding anti-fade and anti-photobleaching properties. They are all compatible with fluorescein, Texas Red®, AMCA and a wide variety of other fluorochromes. Different formulations are available to best suit the investigators' priorities and experimental design.

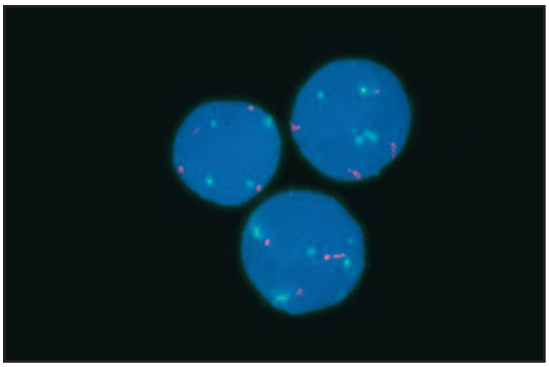
The original VECTASHIELD® Mounting Medium is a glycerol-based, aqueous mountant that does not solidify but remains a viscous liquid on the slide. After mounting, coverslipped slides will not readily dry out and can be reviewed for weeks afterwards without sealing. For prolonged storage, coverslips can be permanently sealed around the perimeter with nail polish.

VECTASHIELD® Hard✦Set™ Mounting Medium is an aqueous mountant that hardens when allowed to set at room temperature for at least 20 minutes. This facilitates handling of the slide, eliminates the need to secure the coverslip, and is more convenient for use with oil immersion microscopy. Although stained and mounted slides may be stored for several weeks, VECTASHIELD® Hard✦Set™ is not a permanent mounting medium. To remove coverslip and mounting medium, slides can simply be incubated in buffer until the mounting medium is removed. Slides can be remounted in fresh mounting medium.

Both the VECTASHIELD® Hard✦Set™ and the original VECTASHIELD® Mounting Media are available with or without the counterstain DAPI (4,6'diamidino-2-phenylindole; blue). The original VECTASHIELD® Mounting Medium is also available with the counterstain propidium iodide (PI; red). These counterstains are provided at an optimal concentration of 1.5 µg/ml. Lower concentrations can be achieved by dilutions with the appropriate VECTASHIELD® Mounting Medium.

DAPI produces a blue fluorescence when bound to DNA with excitation at about 360 nm and emission at 460 nm. PI has a broad excitation range with a maximum at about 535 nm and emission at about 615 nm when bound to DNA.

VECTASHIELD® Mounting Medium	H-1000	•	10ml
VECTASHIELD® Mounting Medium with DAPI	H-1200	•	10ml
VECTASHIELD® Mounting Medium with PI	H-1300	•	10ml
VECTASHIELD® Hard✦Set™ Mounting Medium	H-1400	•	10ml
VECTASHIELD® Hard✦Set™ Mounting Medium with DAPI	H-1500	•	10ml



Fluorescence in situ *hybridization of human chromosomes using FastTag® Fluorescein-labeled pHuR 98 detected with Biotinylated Anti-Fluorescein and Fluorescein Avidin DCS, and FastTag® Texas Red® - labeled pUC1.77 directly detected. Counterstained and mounted with VECTASHIELD® with DAPI.*

Chromogenic Substrates for Enzyme-based Detection

The reagents in these enzyme substrate kits are supplied as concentrated stock solutions in convenient dropper bottles. All substrates can be used in ISH and blotting applications.

Alkaline Phosphatase

BCIP/NBT Substrate Kit	SK-5400	•	1 Kit
Vector® Red Substrate Kit	SK-5100	•	1 Kit

BCIP/NBT substrate (blue/violet) is the most sensitive of all the chromogenic substrates because it continues to develop over many hours, darkening with time.

Vector® Red is unique. It is chromogenic and fluorescent, and dehydratable. It can be viewed with a fluorescence microscope using a rhodamine or Texas Red® filter. Vector® Red can be used in fluorescent multiple label protocols in combination with fluorochromes like fluorescein and is compatible with fluorescent counterstains like DAPI. Vector® Red can also be used for sequential, multiple label ISH or IHC/ISH because of its stability through ISH procedures.

The alkaline phosphatase substrate kits provide sufficient reagents to make approximately 200 ml of working solution.

Peroxidase

ImmPACT™ DAB	SK-4105	•	120ml
DAB Substrate Kit	SK-4100	•	1 Kit
TMB Substrate Kit	SK-4400	•	1 Kit
Vector® *NovaRED*™ Substrate Kit	SK-4800	•	1 Kit
Vector® VIP Substrate Kit	SK-4600	•	1 Kit

Vector® *NovaRED*™ (red), Vector® VIP (purple), DAB and DAB/Ni (brown or black), are similar in sensitivity and can be permanently mounted. All produce dense precipitates with crisp localization. ImmPACT™ DAB has these same attributes but is up to four times more sensitive. DAB or ImmPACT™ DAB can be used for multiple label ISH or IHC/ISH because of their stability. TMB is the most sensitive peroxidase substrate, albeit with a more diffuse, less localized reaction product. Each peroxidase substrate kit provides all reagents necessary to prepare approximately 300 ml of working solution. ImmPACT™ DAB provides 120 ml of working solution.

DuoLuX™ Chemiluminescent/Fluorescent Substrates

For Peroxidase	SK-6604	•	200 ml
For Alkaline Phosphatase	SK-6605	•	100 ml

DuoLuX™ Chemiluminescent/ Fluorescent Substrate is a unique formula based on acridan chemistry with very high sensitivity, prolonged chemiluminescent light emission, as well as permanent fluorescence. The **DuoLuX**™ Substrate is available for alkaline phosphatase[39, 53, 55] or peroxidase[10, 17, 43] and is recommended for blotting applications.[16, 25, 48] Sensitivities ranging from 100 fg to 10 pg of nucleic acid can be achieved.

The **UltraSNAP™ Detection Kit**, which includes the **DuoLuX**™ Substrate, is designed for detection of biotinylated probes in nucleic acid blotting applications (Please see page 14 for details).

With either substrate formula, reacted **DuoLuX**™ substrate luminesces in the blue range with a peak emission at 453 nm. The half-life of luminescent emission exceeds that of other luminescent substrates. The fluorescent signal can be seen visually or captured digitally.[7, 34] The excitation maximum is at 405 nm, but other wavelengths (254 nm and 365 nm) also excite. Maximum fluorescent emission occurs at 453 nm.

Permanent Mounting Medium for Chromogenic Substrates

VectaMount® Permanent Mounting Medium	H-5000	•	60ml

VectaMount® Mounting Medium is a non-flammable, non-hazardous, optically clear, odorless formula for permanently preserving enzyme substrates in tissue sections or cell preparations. VectaMount® Mounting Medium retains the color and intensity of preparations stained with enzyme substrates such as BCIP/NBT, Vector® Red, DAB, ImmPACT™ DAB, TMB, Vector® *NovaRED*™, and Vector® VIP.

Applications

Southern and Northern Blot Hybridization

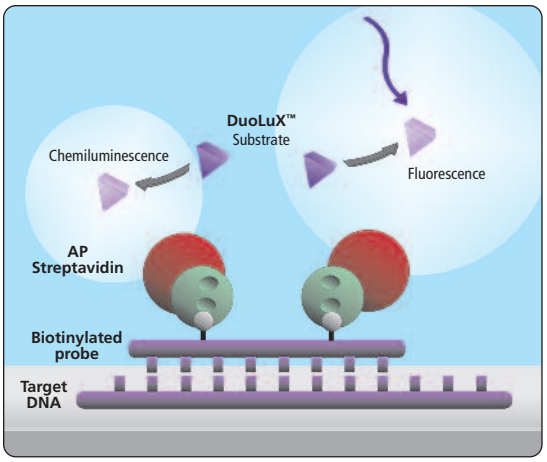
FastTag®-, EndTag™-, and PHOTOPROBE® Biotin-labeled probes can be used to detect targets on Southern and northern blots as well as colony or plaque lifts. The same well established protocols for radioactive hybridization are employed but without the hazards and disposal issues associated with radioactivity.

Hybridization of the nucleic acid probe to the target on the blot is simplified using the ready-to-use **HYBEX™ Hybridization Solution**. HYBEX™ Hybridization Solution contains no formamide and can be used with both nylon and nitrocellulose membranes.

DNA Molecular Weight Markers can be used as easy size references. These ladders range from 0.2 to 10 kbps, are available biotinylated or unlabeled, are ready-to-use, and contain loading dye for monitoring electrophoretic mobility.

The UltraSNAP™ Detection System for Nucleic Acid Blots allows for the convenient detection of biotinylated nucleic acids on nylon or nitrocellulose blots.[32, 40, 54] (Please see protocol on page 20). This kit features the **DuoLuX™** Chemiluminescent/Fluorescent Substrate (see page 13), alkaline phosphatase streptavidin, and a specially optimized PolyBlock™ Blocking Reagent and washing solutions developed for high sensitivity and low background. One kit contains sufficient reagents to develop approximately twenty 100 cm^2 blots.

Detection is accomplished using biotinylated probes that are recognized by streptavidin coupled to alkaline phosphatase. The probe is visualized by the conversion of the **DuoLuX™** Substrate to a luminescent and fluorescent product by a dephosphorylation reaction catalyzed by alkaline phosphatase. The sensitivity is enhanced with the use of the PolyBlock™ Blocking Reagent and wash buffers included in the kit.



Chemiluminescence can be documented and recorded by exposure to film or by image capture with digital imaging systems. Blots can be exposed to film several times over an extended period of time, with typical film exposure times of 30 seconds to 10 minutes. Sensitivities as low as 100 fg of target nucleic acid can be achieved. Optimal exposure times using digital imaging systems depend on the sensitivity of the instrument. Chemiluminescence can be developed on either nylon or nitrocellulose membranes, although nylon is preferred because of faster signal production.

Alternatively, the fluorescence of the developed substrate can be recorded with a digital imaging system or conventional camera even months after the chemiluminescence has faded. Acquiring an image from the fluorescent signal requires a much shorter exposure time than from the chemiluminescent signal, often just a fraction of a second.

Selected Reagents:

HYBEX™ Hybridization Solution	MB-1230	• 200ml
DNA Molecular Weight Markers		
Unlabeled	MB-1301	• 25µg
Biotinylated	MB-1302	• 25µg
10x Casein Solution	SP-5020	• 250ml
Animal-Free Blocker™ (5x)	SP-5030	• 250ml
UltraSNAP™ Detection Kit	MB-6500	• 1 Kit
UltraSNAP™ Accessory Kit (buffers and blocking agent only)	MB-6501	• 1 Kit

In Situ Hybridization

The availability of many different labels and the wide range of detection systems allows the flexibility and sensitivity necessary for *in situ* hybridization (ISH). For example, for fluorescence *in situ* hybridization, biotin-labeled probes can be detected with fluorescently labeled avidin or streptavidin. For brightfield microscopy, alkaline phosphatase-conjugated streptavidin followed by the enzyme substrate, BCIP/NBT, provides a sensitive detection method. Additional color choices are available with the use of the peroxidase-based VECTASTAIN® Elite ABC Kit followed by the appropriate substrate (e.g. TMB, DAB, ImmPACT™ DAB, Vector® VIP, or Vector® *NovaRED*™).

Haptens such as dinitrophenyl (DNP), fluorescein, and Texas Red®, for example, provide nearly equally sensitive alternatives to biotin. Several detection options are also available for these labels. Antibodies to these haptens, together with many fluorescent and enzymatic detection methods, provide a great array of possibilities for detecting probes.

With the options available, the researcher has substantial flexibility in optimizing experimental systems, especially in cases where endogenous biotin or enzyme activity, or antibody cross-reactivity present potential problems. The available options also allow the simultaneous detection of multiple, differentially labeled probes in a single experiment. (See pages 22-25).

Optimization of Probe Length for *In Situ* Hybridization

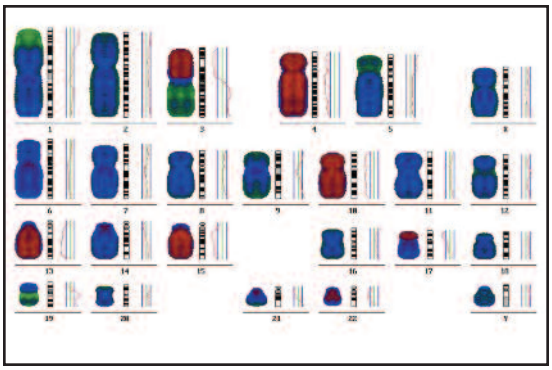
NICKIT™ p.s.o. has been developed to reduce the size of large DNA probes using a photo/enzymatic procedure without the problem of over- or under-digestion experienced with other methods. A smaller probe size generally results in better signal and lower background for ISH applications, probably due to better tissue penetration of the probe.

NICKIT™ p.s.o. Probe Size Optimization Kit
MB-1905 • 1 Kit

Comparative Genomic Hybridization

Comparative genomic hybridization (CGH), based on fluorescence *in situ* hybridization, is a technique for screening chromosomal imbalances in tumor tissue. Labeled DNA samples from two sources are competitively hybridized onto normal chromosome metaphase spreads: one labeled DNA sample is isolated from tumor tissue, the other, differently labeled DNA from normal tissue. Based on the differential hybridization of the two different sources of DNA, the over- or under-expression of chromosomal regions can be mapped on the normal chromosomes.

PHOTOPROBE® Biotin has been shown to be an effective labeling technique of highly degraded DNA from archival material for this analysis.[26, 27, 59] Often, DNA isolated from formalin-fixed, paraffin-embedded archival tissue is reduced in quality, tending to be smaller than 1000 bp. Using the classical method of nick translation, the resulting labeled DNA fragments are usually too small to give a detectable signal. Because labeling with PHOTOPROBE® Biotin is non-destructive, the original, larger fragments of DNA are labeled allowing for the analysis of gains and losses of DNA from the tumor tissue sample. Over-represented chromosomal regions may correspond to tumor promoting genes, and chromosomal deletions potentially contain tumor suppressor genes.



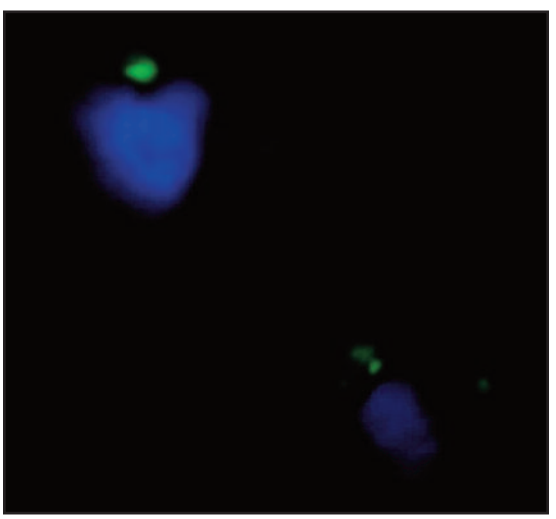
CGH sum karyogram was created after analyzing PHOTOPROBE® labeled tumor DNA from a small cell lung cancer. Biotin was detected with Vector® Fluorescein Avidin D (green). Chromosomes from 15 metaphase spreads were analyzed for each hybridization. Figure provided courtesy of Dirk Korinth, Konrad Donhuijsen, Ulrike Bockmühl, and Iver Petersen, Institute of Pathology, University Hospital Charité, Berlin, Germany.

Cellular Localization

FastTag®-, EndTag™ -, and PHOTOPROBE® Biotin-labeled nucleic acids have been successfully employed in the study of intracellular trafficking of gene delivery complexes and nuclear import mechanisms of viral nucleoprotein molecules. Because these labeling systems do not destroy the nucleic acid nor create nascent nucleic acid, the original DNA or RNA can be tagged and traced throughout the experiment.

For example, in the study of gene delivery, plasmid DNA can be tagged with a FastTag® label. Intracellular trafficking of gold-labeled plasmid DNA can be followed under electron microscopy.[13, 14] In addition, fluorescent staining patterns of fluorescently labeled plasmid DNA can be followed under confocal microscopy.[12, 42, 61] In this manner, cellular interactions of the plasmid DNA with different gene delivery vehicles and with cytoskeletal components can be studied. Such investigations can yield insights into gene delivery mechanisms. siRNA samples labeled with FastTag® can be used as internal controls to evaluate transfection efficiency or cell viability.[49]

In a similar manner, 5′ EndTag™ labeling of nucleic acid is also used successfully in the study of cellular trafficking and localization of nucleic acids and complexes including viral RNA,[35, 36, 44] ribonucleoprotein particles,[60] and oligonucleotide/peptide complexes.[57]



Cellular uptake of labeled plasmid: FastTag® Fluorescein labeled plasmid DNA (green) incubated with COS-7 cells. Nuclei were counterstained and mounted with VECTASHIELD® with DAPI (blue).

EndTag™-Labeled Primers Used in PCR Amplification

Several applications involve the incorporation of labeled primers into a PCR amplification product:

• Immobilization of DNA target to a solid support for isolating sequence-specific DNA-binding molecules. Synthesizing a DNA fragment by PCR using a labeled primer ensures that the label is located only near the end of the DNA. The target DNA is thus bound to the matrix only at one end, minimizing interference in the binding domains.

• Cloning or library screening by hybrid capture. Immobilized single-stranded DNA can be prepared by PCR amplification in which one of a pair of primers is EndTag™-Biotin labeled. Only a single strand of the PCR product is biotinylated. After binding the labeled DNA to a matrix such as VECTREX® Avidin D, the unlabeled strand can be removed, yielding a matrix ready for hybrid capture.

Covalent Attachment of 5′ EndTag™-Labeled Nucleic Acids to Gold

Nucleic acids containing sulfur atoms can form covalent bonds with gold. The phosphoro-thioate group of 5′ EndTag™-labeled RNA has been employed for immobilization to a gold substrate for atomic force microscopy.[33]

Affinity Binding

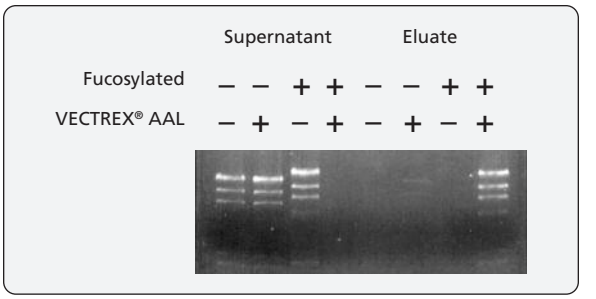
Genomic/cDNA subtraction,[1, 31, 51] microsatellite isolation,[2, 21, 50, 62] and library screening by hybrid capture[24] require the removal or immobilization of nucleic acid hybrids. Affinity binding matrices are available for use with PHOTOPROBE® -, FastTag®-, or EndTag™- labeled probes for such applications.

Irreversible binding of biotinylated nucleic acids to a solid support can be accomplished using VECTREX® Avidin D. Following incubation of VECTREX® Avidin D/biotinylated probe with a cellular extract, hybridized nucleic acids can be recovered by melting the doubled stranded nucleic acid and eluting the unbiotinylated single strands.[4, 23, 41] (Guidelines for this procedure are described on page 26).

Reversible binding of nucleic acids can be achieved with either of two systems:

1. VECTREX® Avidin DLA matrix has a significantly reduced binding affinity for biotin. Biotinylated nucleic acids that are bound to this matrix can be eluted with biotin.[8]

2. Nucleic acids that have been labeled with FastTag® Fucose or EndTag™ Fucose bind specifically to VECTREX® AAL, a matrix containing the fucose-specific lectin from *Aleuria aurantia*. These nucleic acids are then easily eluted under mild conditions using the sugar L-fucose at physiological pH and salt concentrations.



Reversible binding of FastTag® Fucose-labeled λ Hind III DNA to VECTREX® AAL. Labeled (+) or unlabeled (-) DNA was incubated with VECTREX® AAL (+) or binding buffer (-). Following centrifugation of binding reactions, VECTREX® AAL was washed and the bound DNA eluted with L-fucose. Supernatants and eluates from the binding reactions were fractionated by agarose gel electrophoresis and DNA visualized by ethidium bromide staining. Lane 4 shows that the fucosylated DNA bound to the VECTREX® AAL and lane 8 shows that fucosylated DNA can be easily eluted.

Irreversible Binding Matrix

VECTREX® Avidin D	A-2020	•	1 ml

Reversible Binding Matrices

For Biotin Labeled Nucleic Acids

VECTREX® Avidin DLA	MB-2021	•	1 ml
VECTREX® Avidin DLA Binding and Elution Kit	MB-2022	•	1 Kit

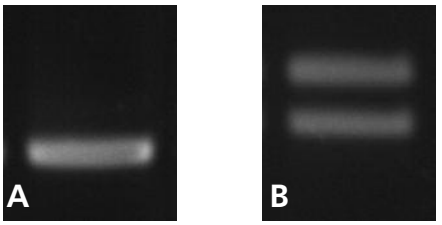
For Fucose Labeled Nucleic Acids

VECTREX® AAL	MB-1396	•	1 ml
VECTREX® AAL Binding and Elution Kit	MB-1397	•	1 Kit

Resolve Same-Sized DNA by Sequence Variation

The ability to resolve DNAs of similar size in a gel is critical for applications such as differential display and Rapid Amplification of Polymorphic DNAs (RAPD) in which multiple DNA species need to be separated before subsequent excision and analysis. Two sequence specific DNA ligands in the Resolve-It™ Kit bind to GC- or AT- rich sites on DNA and retard the electrophoretic migration of DNA in a sequence specific manner. AT-Yellow™, a minor groove binding bisbenzimide-PEG conjugate, binds to AT-rich regions; GC-Red™, an intercalating phenyl neutral red-PEG conjugate, binds GC-regions. A ligand is added to the agarose during gel preparation, and, during electrophoresis, the increased friction caused by ligand association with the DNA retards the mobility of DNA depending on the amount of ligand bound. The resolved bands can be excised and electrophoresed on a gel containing the second ligand, if desired, for confirmation of homogeneity or for additional resolution. The resolved DNA sequences can then be extracted for cloning, sequencing, or other analysis. The quantity of each ligand supplied is sufficient to prepare about 200 ml of agarose gel.

Resolve-It™ Kit - Sequence Specific DNA Ligands
MB-1401 • 1 Kit



Two 600 bp DNAs of different sequence form a single band using typical electrophoretic conditions (A). The same DNAs are separated into two distinct bands in a gel containing Resolve-It™ AT-Yellow™ (B).

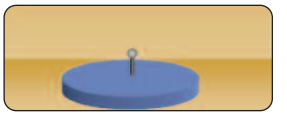
Amplification of Fluorescent Signal in Nucleic Acid Microarrays

Fluorescent amplification of biotinylated probes is common in microarray-based analysis. The multiple binding capability of Biotinylated Anti-Streptavidin provides a method for significant signal amplification. This antibody binds to streptavidin through the antigen binding sites and through the covalently attached biotin residues. Following the first application of a fluorescent streptavidin such as phycoerythrin- or fluorescein-labeled streptavidin, the signal is amplified by incubation with Biotinylated Anti-Streptavidin followed by a second incubation with the fluorescent streptavidin. The same procedure can be performed with labeled avidin and Biotinylated Anti-Avidin. This procedure results in the introduction of more fluorochromes at the target site.[3, 6, 9, 38]

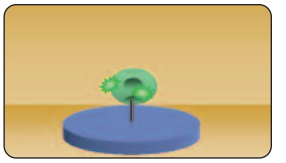
Biotinylated Anti-Streptavidin
made in goat BA-0500 • 0.5mg
Phycoerythrin Streptavidin
SA-5007 • 1mg
Fluorescein Streptavidin SA-5001 • 1mg

Amplification of Signal Using Biotinylated Anti-Streptavidin

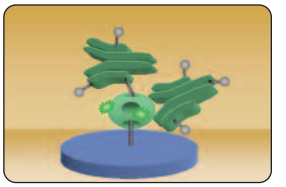
Biotinylated Target



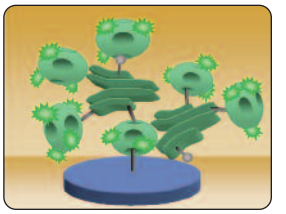
Step 1:
Add Fluorescent Streptavidin



Step 2:
Add Biotinylated Anti-Streptavidin



Step 3:
Add Fluorescent Streptavidin



Protocols

Assessing Labeling Efficiency

In applications involving several steps - from producing and labeling a probe to detecting the labeled probe - assessing labeling efficiency can be an essential part of assay design.

Estimation of Labeling by Comparative Dot Blot

The efficiency of labeling can be estimated by comparing the relative detection sensitivity of the labeled nucleic acid to a standardized sample in a side by side dot blot dilution series.

1. Dilute both the labeled nucleic acid sample and the labeled DNA standard to 1 µg/ml, 100 ng/ml, 10 ng/ml, and 1 ng/ml in TE.

2. Dot 1 µl of each dilution on nitrocellulose or nylon membrane. Crosslink the membrane according to the manufacturers' protocol.

3. Block the membrane in blocking solution

4. Detect by incubating with the appropriate alkaline phosphatase conjugate and an AP substrate such as BCIP/NBT or **DuoLuX**™ Chemiluminescent/Fluorescent Substrate according to the instructions provided with the substrate. Efficiency of labeling can be estimated by the comparison of the signals of the labeled sample and the labeled DNA standard.

Selected Reagents:

10x Casein Solution	SP-5020	• 250ml
Animal-Free Blocker™	SP-5030	• 250ml
BCIP/NBT Substrate Kit	SK-5400	• 1 kit
DuoLuX™ Chemiluminescent/Fluorescent Substrate for Alkaline Phosphatase	SK-6605	• 100 ml

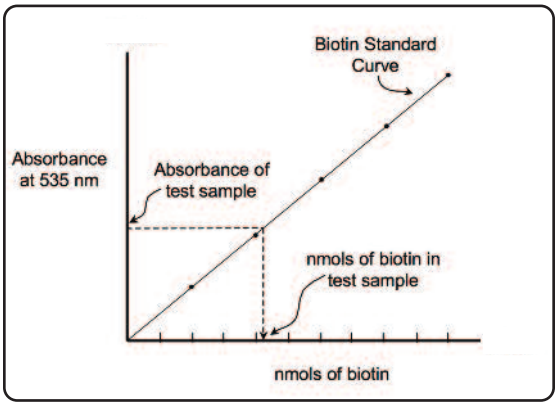
Quantitation of Biotin using the Quant*Tag™ Biotin Kit

For biotin-labeled probes, labeling efficiency can be determined using either the dot blot technique or an alternative method - Quant*Tag™ Biotin quantitation. The Quant*Tag™ Biotin Kit is designed to determine the amount of free biotin in solution or the number of biotins attached to nucleic acids, proteins, or other macromolecules. The kit reagents chemically react with free or bound biotin, producing a colored product that can be quantified using a spectrophotometer. The absorbance is measured in the visible spectrum, allowing the use of plastic cuvettes or microtitre plates.

Unlike other methods of measuring biotin incorporation, no pre-digestion of the nucleic acid is required, saving time and increasing consistency. The assay can be completed in 30 minutes, and is able to detect less than 1 nmol of biotin.

Quant*Tag™ Biotin Kit	BDK-2000	• 1 Kit

Biotin Quantitation using Quant*Tag™ Biotin Kit



The sample to be tested is reacted with the kit reagents along with standards containing known amounts of biotin. The absorbance readings of the known samples are plotted producing a standard curve. The absorbance of the test sample is located on the standard curve indicating the amount of biotin present.

Protocol:
Southern Blot Hybridization and Detection

Perform Southern transfer and hybridization of the biotinylated probe followed by stringency washes using standard procedures.[46]

Blotting can be done onto either nylon or nitrocellulose (nylon membranes usually require a shorter exposure time than nitrocellulose and are therefore recommended, especially when using CCD camera-based digital imagers).

Hybridization: Prehybridization and hybridization incubations can be carried out in appropriate hybridization tubes or in heat-sealable polyethylene bags. To ensure constant agitation, incubations can be performed in a commercial hybridization oven or water bath equipped with a shaking platform.

Prehybridization: Preincubate the membrane in sufficient HYBEX™ Hybridization Solution to ensure that the membrane is completely covered (usually 0.2 ml of hybridization solution is enough for each square centimeter of membrane). Nitrocellulose membranes are prehybridized for 1-2 hours and nylon membranes for 30 minutes. Prehybridization temperature should be the same as the temperature used for hybridization.

Hybridization: Double stranded DNA probes need to be denatured before hybridization. Dilute denatured probe in HYBEX™ solution. Use sufficient hybridization solution to ensure that the membrane will be completely covered.

Decant prehybridization solution and immediately add diluted probe to the membrane. Optimal hybridization temperature will depend on the probe type and size and may have to be empirically determined. As a guideline, temperatures can range from 68 °C for large DNA fragments to room temperature for short oligonucleotide probes. (For oligonucleotide probes it is necessary to determine the melting temperature of the oligo).

Incubate the membrane with the HYBEX™ solution containing the probe for two hours to overnight. Following hybridization, wash the membrane with stringency solutions: 2 x 5 min in 2x SSC, 0.1% SDS, followed by 2 x 15 min in 0.1x SSC, 0.1%SDS. (1x SSC is 0.15 M NaCl, 15 mM Trisodium Citrate, pH 7.0).

Detection: This protocol is adapted from the Ultra-SNAP™ Detection Kit which has been optimized for the chemiluminescent or fluorescent detection of biotinylated nucleic acid. Solution volumes are for a 100 cm^2 blot and can be scaled accordingly. A general protocol using the chromogenic alkaline phosphatase substrate, BCIP/NBT, as the substrate is described on the following page.

1. Block blot in 20 ml of 1x PolyBlock™ Reagent for 30 minutes at room temperature with gentle shaking.

2. Incubate the blot in diluted Alkaline Phosphatase-Streptavidin (1 µg/ml in 1x PolyBlock™ Reagent) for 30 minutes at room temperature with gentle shaking.

3. Wash blot 3 times for 10 minutes each in 30 ml of 1x Wash A at room temperature with gentle shaking.

4. Rinse blot in 30 ml of 1x Wash B.

5. Remove excess Wash B by touching edge of blot to absorbent paper.

6. Place blot target-side up on plastic wrap on a level surface.

7. Cover the blot surface with **DuoLuX**™ Substrate (about 5 ml). Incubate for 5 minutes under subdued light or in the dark.

8. Wash the blot in 30 ml of 1x Wash B for 1 minute at room temperature with gentle shaking. Remove excess liquid from the blot by touching edge to absorbent paper. *See Note A.*

9. Place the blot in a sheet protector or between layers of plastic wrap and smooth away any bubbles trapped between layers. The signal can be recorded with either film or a digital imager.

10a. Chemiluminescence:

X-ray film: Expose to film for 5 seconds to several hours. *See Note B.*

Digital imager: For optimal exposure time, please consult the manufacturer of the digital imaging system. A digital imager also enables recording of fluorescent signal. *See Notes C and D.*

10b. Fluorescence:

DuoLuX™ fluorescence signal can be achieved with a UV transilluminator. The signal can be acquired with a digital imager or conventional camera, equipped with an ethidium bromide filter. Pre-exposure to UV light for at least 2 minutes frequently enhances the fluorescent signal. **DuoLuX**™ fluorescent signal can usually be captured in just a few seconds. However, for the best picture quality some optimization may be needed. The fluorescence of the developed substrate can be recorded months after the chemiluminescence has faded. *See Note D.*

Notes:

A. Extensive washing will reduce signal strength; do not extend the wash time unless high background is experienced. If background is excessive, repeat steps 6-9 with a wash time of 5-10 minutes in step 8. Ideal wash time is dependent on the degree of background previously detected and, therefore, may require optimization.

B. The long emission lifetime of **DuoLuX**™ allows the user to re-expose the same blot until optimal signal to noise ratio is achieved. Typical exposure times are from 5 to 60 minutes.

C. Exposure time for chemiluminescent signal development will depend on the sensitivity of the instrument. Often, imagers require longer exposure times than x-ray film.

D. Prolonged exposure to UV will weaken chemiluminescent emission. ***If fluorescence imaging is to be performed, chemiluminescence should be recorded first.***

General Protocol for Southern Blot Detection Using BCIP/NBT Substrate for Visualization

1. Block blot in 20 ml of 1x Casein Solution for 30 minutes at room temperature with gentle shaking.

2. Incubate the blot in diluted Alkaline Phosphatase-Streptavidin (1 µg/ml in 1x Casein Solution) for 30 minutes at room temperature with gentle shaking.

3. Wash blot 3 times for 5 minutes each in 30 ml of TBST (Tris buffered saline, pH 7.5, with 0.1% Tween) at room temperature with gentle shaking.

4. Rinse blot for 5 minutes in 30 ml 0.1 M Tris, pH 9.5.

5. Prepare BCIP/NBT substrate solution according to kit instructions in a separate staining dish, and submerge the blot in the solution until signal develops.

Probes other than biotin can be detected by substituting Alkaline Phosphatase-Streptavidin in step 2 with the appropriate AP-conjugated antibody.

Selected Reagents:

HYBEX™ Hybridization Solution	MB-1230	• 200ml
DNA Molecular Weight Markers		
Unlabeled	MB-1301	• 25µg
Biotinylated	MB-1302	• 25µg
10x Casein Solution	SP-5020	• 250ml
Animal-Free Blocker™ (5x)	SP-5030	• 250ml
BCIP/NBT Substrate Kit	SK-5400	• 1 Kit
UltraSNAP™ Detection Kit	MB-6500	• 1 Kit
UltraSNAP™ Accessory Kit*	MB-6501	• 1 Kit

*Contains PolyBlock™ Reagent and wash buffers only

Protocol:
Fluorescent Detection of Biotin-Labeled ISH Probes

This procedure uses successive rounds of Fluorescein Avidin DCS and Biotinylated Anti-Avidin to detect and amplify *in situ* hybridization signals. The multiple binding capability of Biotinylated Anti-Avidin D can provide significant amplification. This antibody binds to Avidin D through the antigen binding sites and through the biotin residues that are covalently attached to the molecule. Following the first application of Fluorescein Avidin DCS, the signal is amplified by incubation with Biotinylated Anti-Avidin, followed by a second incubation with Fluorescein Avidin DCS. This results in the introduction of several more fluorochromes at the target site. The same procedure can be performed with labeled streptavidin and Biotinylated Anti-Streptavidin (see diagram on page 18).

1. After hybridization of biotinylated DNA/ RNA probes, block tissue sections or chromosome spreads for at least 30 minutes in 1x ISH Blocking Solution*. The effectiveness of the blocking solution may be enhanced by pre-warming the solution to 37 °C and incubating tissue sections or chromosome spreads for 30 minutes or longer at 37 °C.

2. Dilute each of the detection reagents (Fluorescein Avidin DCS and Biotinylated Anti-Avidin; or fluorescent streptavidin and Biotinylated Anti-Streptavidin) to 5 µg/ml in 1x ISH Blocking Solution approximately 30 minutes before use to minimize any non-specific binding.

3. Drain off the blocking solution from the specimen and add the Fluorescein Avidin DCS solution (5 µg/ml). Incubate for 30 minutes at room temperature.

4. Wash slides for 2 x 3 minutes in blocking solution.

[If satisfactory sensitivity has been achieved, skip to step 8. To amplify the fluorescent signal, continue with steps 5 through 7].

5. Incubate sections or spreads with the Biotinylated Anti-Avidin solution (5µg/ml) for 30 minutes at room temperature.

6. Wash slides for 2 x 3 minutes in blocking solution.

7. Perform a second incubation of the sections or spreads with the same Fluorescein Avidin DCS solution (5 µg/ml) for 30 minutes at room temperature.

8. Wash slides 2 x 5 minutes in 4x SSC + 0.1% Tween 20 before coverslipping with a VECTASHIELD® Mounting Medium.

* Note: 5% nonfat dry milk plus 0.1% Tween 20 in 4x SSC can be used as an alternative blocking solution. (4x SSC is 0.6 M NaCl, 60 mM sodium citrate, pH 7.0.) However, non-fat dry milk can contain variable amounts of biotin which could reduce staining if used as a diluent for (strept)avidin conjugates.

Detection Reagents

ISH Blocking Solution (5x)	MB-1220	•	100ml
Fluorescein Avidin DCS	A-2011	•	1mg
Biotinylated Anti-Avidin *made in goat*	BA-0300	•	0.5mg
Biotinylated Anti-Streptavidin *made in goat*	BA-0500	•	0.5mg

Mounting Media

VECTASHIELD® Mounting Medium	H-1000	•	10ml
VECTASHIELD® Mounting Medium with DAPI	H-1200	•	10ml
VECTASHIELD® Mounting Medium with PI	H-1300	•	10ml
VECTASHIELD® Hard✦Set™ Mounting Medium	H-1400	•	10ml
VECTASHIELD® Hard✦Set™ Mounting Medium with DAPI	H-1500	•	10ml

Protocol:
Fluorescence Detection of Fluorescein-Labeled ISH Probes

1. After hybridization of fluorescein-labeled DNA/RNA probes, block tissue sections or chromosome spreads for at least 30 minutes in 1x ISH Blocking Solution*. The effectiveness of the blocking solution may be enhanced by pre-warming the solution to 37 ºC and incubating tissue sections or chromosome spreads for 30 minutes or longer at 37 ºC.

2. Dilute each of the detection reagents (Biotinylated Anti-Fluorescein and Fluorescein Avidin DCS) to 10 µg/ml in 1x ISH Blocking Solution for approximately 30 minutes before use to minimize any non-specific binding.

3. Drain off the blocking solution from the specimen and incubate with Biotinylated Anti-Fluorescein solution (10 µg/ml) for 30 minutes at room temperature.

4. Wash slides for 2 x 3 minutes in blocking solution.

5. Incubate with the Fluorescein Avidin DCS solution (10 µg/ml) for 30 minutes at room temperature.

6. Wash slides 2 x 5 minutes in 4x SSC + 0.1% Tween 20 before coverslipping with a VECTASHIELD® Mounting Medium.

* Note: 5% nonfat dry milk plus 0.1% Tween 20 in 4x SSC can be used as an alternative blocking solution. (4x SSC is 0.6 M NaCl, 60 mM sodium citrate, pH 7.0.) However, non-fat dry milk can contain variable amounts of biotin which could reduce staining if used as a diluent for (strept)avidin conjugates.

Detection Reagents

ISH Blocking Solution (5x)	MB-1220	•	100ml
Biotinylated Anti-Fluorescein	BA-0601	•	0.5mg
Fluorescein Avidin DCS	A-2011	•	1mg

Mounting Media

VECTASHIELD® Mounting Medium	H-1000	•	10ml
VECTASHIELD® Mounting Medium with DAPI	H-1200	•	10ml
VECTASHIELD® Mounting Medium with PI	H-1300	•	10ml
VECTASHIELD® Hard✦Set™ Mounting Medium	H-1400	•	10ml
VECTASHIELD® Hard✦Set™ Mounting Medium with DAPI	H-1500	•	10ml

Protocol:
Chromogenic Detection of Biotin-Labeled ISH Probes

1. After hybridization of biotin-labeled DNA/RNA probes, block tissue sections or chromosome spreads for at least 30 minutes in 1x ISH Blocking Solution. The effectiveness of the blocking solution may be enhanced by pre-warming the solution to 37 °C and incubating tissue sections or chromosome spreads for 30 minutes or longer at 37 °C. *Please see Note A.*

2. Dilute Alkaline Phosphatase Streptavidin to 1–5 µg/ml in 1x ISH Blocking Solution approximately 30 minutes before use to minimize any non-specific binding. *Please see Note B.*

3. Drain off the blocking solution from the specimen and incubate with Alkaline Phosphatase Streptavidin solution for 30 minutes at room temperature.

4. Wash slides for 2 x 3 minutes in 100 mM Tris, pH 9.5 buffer.

5. Visualize the probe by incubating the tissue section or chromosome spread in BCIP/NBT substrate working solution prepared according to kit instructions (BCIP/NBT Substrate Kit). Incubate until desired sensitivity is achieved.

6. Wash in 100 mM Tris, pH 9.5 buffer for 5 minutes.

7. Rinse in tap water and counterstain if desired (BCIP/NBT substrate is compatible with Vector® Nuclear Fast Red counterstain and Vector® Methyl Green counterstain).

8. For permanent mounting, dehydrate, clear, and mount sections in VectaMount™ Mounting Medium which minimizes crystal formation in mounted sections. For aqueous mounting, use VectaMount™ AQ Mounting Medium.

Notes:

A. 5% nonfat dry milk plus 0.1% Tween 20 in 4x SSC can be used as an alternative blocking solution. (4x SSC is 0.6 M NaCl, 60 mM sodium citrate, pH 7.0.) However, non-fat dry milk can contain variable amounts of biotin which could reduce staining if used as a diluent for (strept)avidin conjugates.

B. For an overnight incubation in the BCIP/NBT substrate solution, use the Alkaline Phosphatase Streptavidin reagent at a concentration of 0.3 – 0.5 µg/ml.

Detection Reagents

ISH Blocking Solution (5x)	MB-1220	•	100ml
Alkaline Phosphatase Streptavidin	SA-5100	•	1ml
BCIP/NBT Substrate Kit	SK-5400	•	1 kit
Levamisole Solution	SP-5000	•	18ml
Vector® Nuclear Fast Red Counterstain	H-3403	•	500ml
Vector® Methyl Green Counterstain	H-3402	•	500ml

Mounting Media

VectaMount™ Permanent Mounting Medium	H-5000	•	60ml
VectaMount™ AQ Aqueous Mounting Medium	H-5501	•	60ml

Protocol:
Chromogenic Detection of Fluorescein-Labeled ISH Probes

1. After hybridization of fluorescein-labeled DNA/RNA probes, block tissue sections or chromosome spreads for at least 30 minutes in 1x ISH Blocking Solution. The effectiveness of the blocking solution may be enhanced by pre-warming the solution to 37 °C and incubating tissue sections or chromosome spreads for 30 minutes or longer at 37 °C. *Please see Note A*.

2. Dilute Alkaline Phosphatase Anti-Fluorescein to 5 µg/ml in 1x blocking solution approximately 30 minutes before use to minimize any non-specific binding. *Please see Note B*.

3. Drain the blocking solution from the specimen and incubate with Alkaline Phosphatase Anti-Fluorescein solution for 30 minutes at room temperature.

4. Wash slides for 2 x 3 minutes in 100 mM Tris, pH 9.5 buffer.

5. Visualize the probe by incubating the tissue section or chromosome spread in BCIP/NBT substrate working solution prepared according to kit instructions. Incubate until desired sensitivity is achieved.

6. Wash in 100 mM Tris, pH 9.5 buffer for 5 minutes.

7. Rinse in tap water and counterstain if desired (BCIP/NBT substrate is compatible with Vector® Nuclear Fast Red counterstain and Vector® Methyl Green counterstain).

8. For permanent mounting, dehydrate, clear, and mount sections in VectaMount™ Mounting Medium which minimizes crystal formation in mounted sections. For aqueous mounting, use VectaMount™ AQ Mounting Medium.

Notes:

A. 5% nonfat dry milk plus 0.1% Tween 20 in 4x SSC can be used as an alternative blocking solution. (4x SSC is 0.6 M NaCl, 60 mM sodium citrate, pH 7.0.) However, non-fat dry milk can contain variable amounts of biotin which could reduce staining if used as a diluent for (strept)avidin conjugates.

B. For an overnight incubation in the BCIP/NBT substrate solution, use the Alkaline Phosphatase Anti-Fluorescein reagent at a concentration of 0.2 – 2.0 µg/ml.

Detection Reagents

ISH Blocking Solution (5x)	MB-1220	•	100ml
Alkaline Phosphatase Anti-Fluorescein	MB-2100	•	150µg
BCIP/NBT Substrate Kit	SK-5400	•	1 kit
Levamisole Solution	SP-5000	•	18ml
Vector® Nuclear Fast Red Counterstain	H-3403	•	500ml
Vector® Methyl Green Counterstain	H-3402	•	500ml

Mounting Media

VectaMount™ Permanent Mounting Medium	H-5000	•	60ml
VectaMount™ AQ Aqueous Mounting Medium	H-5501	•	60ml

Protocol:
Methods used to elute single strands of DNA from VECTREX® - bound biotinylated DNA

The procedures outlined below use heat to denature the DNA without disrupting the biotin-avidin interaction. The temperature of washes and elution will depend on the length and base composition of the DNA.

According to Carriero (2002), PCR amplified genomic DNA was denatured and mixed with biotinylated oligonucleotide $(GA)_{20}$. This mixture was allowed to anneal and was mixed with VECTREX® Avidin D. After two washes to remove unbound DNA, a more stringent wash at 55 °C was employed. Single-stranded DNA was collected by incubating in solution at 65 °C.

Kandpal (1994) heat denatured a genomic digest or PCR product and hybridized it to a biotinylated 29-base oligomer. The hybridization mixture was added to VECTREX® Avidin D that had been blocked with salmon DNA in 0.1 M Tris, pH 7.5, 150 mM NaCl. The VECTREX® Avidin D was washed in Tris/HCl to remove unbound salmon DNA. After binding at room temperature, the supernatant was removed. The matrix was washed three times with Tris/NaCl at room temperature, followed by one wash with the same buffer at 50°C. Single-stranded DNA was recovered by incubation at various stringencies. $(AGAT)_{11}$ repeats were dissociated in 150 mM NaCl at 65 °C. $(CAG)_{15}$ repeats were recovered in 15 mM NaCl at 65 °C, while $(CA)_n$ repeats were dissociated in water at 65 °C.

Rehner (2003) annealed PCR products to biotinylated oligonucleotides specifying CA, GA, AAG, or GTT motifs. These hybrids were incubated with VECTREX® Avidin D. After washing in 0.2x SSC/0.1% SDS (1x SSC is 15 mM Citrate, 150 mM NaCl, pH 7.2) to remove unbound DNA, all were suspended in TLE (10 mM Tris, 0.1 mM EDTA, pH 8.0) and heated to 95 °C for 5 minutes to elute single strands (regardless of the biotinylated oligonucleotide used).

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Protocol:
Nucleic Acid Purification

There are many methods used to purify nucleic acid samples at different steps in sample preparation (isolation, labeling, etc.). Which is the best suited will depend on the nature of the sample (DNA, RNA, oligos), the nature of the contaminant (proteins, nucleotides, reactive chemicals) or the desired level of purity. The simple protocols below can be used for preparation of the samples used in protocols on pages 19-26.

Ethanol precipitation

Ethanol precipitation is a commonly used method to precipitate nucleic acids out of solution due to their low solubility in alcohols. DNA and RNA will precipitate out, leaving soluble contaminants in the solution.

1. Adjust sample volume to 50 µl with water or TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA).

2. Add, in this order, the following components:
   a) 10 µl of 10 M ammonium acetate
   b) 2 µl of 1 M $MgCl_2$ (for nucleic acids ≤500 bases)
   c) 1 µl of 20 mg/ml glycogen (only necessary with oligonucleotides and dilute nucleic acid solutions, e.g. ≤10 ng/µl)
   d) 125 µl of 95% ethanol (-20 °C)

3. Pellet the precipitated nucleic acid by centrifugation at 10,000 x g (ca. 13,000 rpm) for 15 minutes.

4. Remove supernatant and wash the pellet with 70% ethanol. Centrifuge at 10,000 x g for 5 minutes.

5. Remove supernatant and resuspend the pelleted nucleic acid in TE. Store at -20 °C to -80 °C for up to 1 year.

Gel filtration

Due to the difference in molecular weight, a nucleic acid sample can be separated from small contaminants such as nucleotides, unincorporated labels, small primers, etc., using gel filtration on spin columns. For this method, use a commercially available spin column or prepare a spin column with Sephadex® G-25 or other gel filtration matrix with a similar separation range (1 - 3 ml column volume). While the small molecular weight contaminants enter pores of the gel filtration media, the large molecular weight sample remains in the void volume and is easily recovered by subsequent centrifugation.

1. Wash the column three times with TEN buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl) by centrifuging the buffer through the column for 1-2 minutes at 1,000 x g (ca. 4,000 rpm). Do not exceed the manufacturer's recommendations.

2. Load the sample (in TEN buffer) onto the column and allow to equilibrate for 5 minutes.

3. Centrifuge the sample (1-2 minutes at 1,000 x g) through the column into a clean tube.

Fast Facts

Formula for Calculation of Primer Melting Temperature (T_m)

T_m (°C) = 81.5 + 16.6(logM) + 0.41(%GC) - (500/n)

n = length of primer
M = molarity of the salt in the buffer

Formula for Calculation of nmoles of 5′ or 3′ ends per µl of nucleic acid sample

$$\text{nmoles ends} = \frac{A}{BxC} \times 1000 \text{ nmoles/µmol}$$

A = the concentration of nucleic acid (µg/µl)
B = average molecular weight of nucleotide (333 µg/ µmol for DNA; 317 µg/ µmol for RNA)
C = total number of bases (for ssDNA or RNA) or base pairs (for dsDNA)

Characteristics of Common Proteins

Protein	Molecular Weight	Subunits	pI	Extinction coefficient at 280 nm (at 1 mg/ml)	Optimal pH (for enzymes)
Alkaline Phosphatase, (calf intestinal)	140,000	2	5.7	1.0	9.5
Avidin	68,000	4	10	1.54	-
BSA	67,000	1	4.9	0.67	-
GFP	25,000	1	5.0	1.05	-
Glucose Oxidase	160,000	2	4.2	1.38	5.5
Horseradish Peroxidase	40,000	1	7.2	0.63	7.0
Immunoglobulin G (IgG)	150,000	4	5-12	1.34	-
Streptavidin	60,000	4	5.5	3.4	-

Spectrophotometric Conversions of Nucleic Acids

1 A_{260} of dsDNA = 50 µg/ml = 0.15 mM (in nucleotides)
1 A_{260} of ssDNA = 33 µg/ml = 0.1 mM (in nucleotides)
1 A_{260} of ssRNA = 40 µg/ml = 0.11 mM (in nucleotides)

$MW_{average}$ of a deoxyribonucleotide = 333 g/mol
$MW_{average}$ of a ribonucleotide = 350 g/mol

Weight Conversions

1 mg = 10^{-3} gm
1 µg = 10^{-6} gm
1 ng = 10^{-9} gm
1 pg = 10^{-12} gm
1 fg = 10^{-15} gm
1 ag = 10^{-18} gm

Amino Acid Symbols:

A	Alanine	**G**	Glycine	**P**	Proline
R	Arginine	**H**	Histidine	**S**	Serine
N	Asparagine	**I**	Isoleucine	**T**	Threonine
D	Aspartic Acid	**L**	Leucine	**W**	Tryptophan
C	Cysteine	**K**	Lysine	**Y**	Tyrosine
Q	Glutamine	**M**	Methionine	**V**	Valine
E	Glutamic Acid	**F**	Phenylalanine		

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