Nonisotopic DNA Probe Techniques

Edited by Larry J. Kricka

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Front cover photograph: Color enchanced digitized image of a DNA sequence obtained using the chemiluminescent substrate CSPD to visualize bound alkaline phosphatase conjugate. This illustration was kindly provided by Irena Bronstein and Chris Martin of Tropix, Inc.

This book is printed on acid-free paper. ♾

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PREFACE

Numerous nonisotopic methods have now been developed as replacements for radioactive labels such as ^{32}phosphorous and ^{125}iodine in DNA probe hybridization assays. Most have been developed within the last five years; the range of nonisotopic methods is now so extensive that it is difficult to determine the relative merits and demerits for particular applications.

The objective of this book is to bring together descriptions of the principal nonisotopic methods for DNA hybridization assays, together with experimental details of the methods, including labeling and detection of the label. This book contains descriptions of bioluminescent, chemiluminescent, fluorescent, and time-resolved fluorescent detection methods. It covers the following combinations of label and detection reaction: acridinium esters/chemiluminescence; alkaline phosphatase/bioluminescence, colorimetry, chemiluminescence, time-resolved fluorescence; lanthanide chelates/time-resolved fluorescence; glucose 6-phosphate dehydrogenase/bioluminescence; fluorescence/fluorescence; and horseradish peroxidase/enhanced chemiluminescence/colorimetry. Non-separation DNA probe assay strategies based on selective hydrolysis of acridinium esters and energy transfer involving pairs of probes, one labeled with a chemiluminescent molecule and the other labeled with a fluorophore, are also presented.

Each chapter has been prepared by the inventor or developer of a particular nonisotopic method and thus provides an expert account of the method. Practical details for a range of applications are presented in step-by-step experimental procedures that provide a valuable source of authoritative information.

This book is intended to give research workers and assay developers a single source of information on nonisotopic procedures for DNA hybridization based assays.

Larry J. Kricka

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PART TWO
Detection Methods

PART ONE
Introduction

1 Nucleic Acid Hybridization Test Formats: Strategies and Applications

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I. INTRODUCTION

Nucleic acid hybridization tests for the detection of specific DNA and RNA sequences are now extensively used in research and routine laboratories (Diamandis, 1990; Leary and Ruth, 1989; Matthews and Kricka, 1988; Pollard-Knight, 1991). Hybridization assays have diverse applications in medicine and forensics, and some representative examples of these applications are listed in Table I. Labeled nucleic acid probes are utilized in a variety of assay formats including dot blots, Southern blots (DNA target), Northern blot (RNA target), *in situ* hybridization, plaque hybridization, and colony hybridization. An important aspect of nucleic hybridization assays is the choice of the substance used to label a nucleic acid probe and the label detection method. As yet there is no consensus on which substance is the ideal label for nucleic acid probes for use in the various assay formats. The first assays used a radioactive ^{32}phosphorus label. However, this label has the major disadvantage of a relatively short

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Table I
Applications of Nucleic Acid Hybridization Assays

Application	Reference
Arteriosclerosis	Williams (1985)
Cell line authentication	Thacker *et al.* (1988)
Forensics	Budowle *et al.* (1990); Cawood (1989); Thornton (1989)
Blood stains	Gill *et al.* (1985)
Inherited disorders	Dawson (1990); Ropers (1987)
Cystic fibrosis	Kerem *et al.* (1989); Riordan *et al.* (1989)
Duchenne muscular dystrophy	Kunkel *et al.* (1989)
Phenylketonuria	DiLella *et al.* (1986); Woo *et al.* (1983)
Sickle cell anemia	Saiki *et al.* (1985)
Microbiology	Buck (1989); McGowan (1989); Wolfe (1988)
E coli	Miller *et al.* (1988)
Neisseria gonorrhoeae	Sanchez-Pescador *et al.* (1988)
Legionella	Wilkinson *et al.* (1986)
Mycoplasma pneumoniae	Dular *et al.* (1988)
Oncology	Knudson (1986)
Leukemia	Lovell (1989)
Neu oncogene	Slamon *et al.* (1987)
Paternity testing	Odelberg *et al.* (1988)
Virology	Landry (1990)
Cytomegalovirus	Spector and Spector (1985)
Hepatitis B	Kam *et al.* (1982)
Rotavirus	Flores *et al.* (1983)

half-life (14.2 d) (cf. ^{125}iodine used in immunoassay has a half-life of 60 d). Thus nucleic acid hybridization probes have a very short shelf-life. This has placed severe limitations on the routine use and commercialization of probe tests; hence, there are extensive efforts to develop and implement alternatives to the radioactive ^{32}phosphorus label. Many different substances have been tested as nonisotopic replacements for ^{32}phosphorus, and subsequent chapters of this book provide background and practical details of the application of various nonisotopic labels.

II. NUCLEIC ACID LABELS

The majority of the substances used as labels for nucleic acid hybridization probes have been tested previously in immunoassay. Nonisotopic labels have been the focus of development because of the limitations of radioactive labels such as ^{32}phosphorus (Kricka, 1985). These limitations are principally (1) a short half-life that restricts the shelf life of labeled probes

and hence hybridization assay kits, (2) possible health hazards during preparation and use of the labeled nucleic acid, and (3) disposal of radioactive waste from the assay. The ideal label for a nucleic acid hybridization probe would have the following properties.

1. Easy to attach to a nucleic acid using a simple and reproducible labeling procedure;
2. Stable under nucleic acid hybridization conditions, typically temperatures up to 80° C, and exposure to solutions containing detergents and solvents such as formamide;
3. Detectable at very low concentrations using a simple analytical procedure and noncomplex instrumentation;
4. Nonobstructive on the nucleic acid hybridization reaction;
5. Applicable to solution or solid-phase hybridizations. In a solid-phase application, e.g., membrane-based assay, the label must produce a long-lived signal (e.g., enzyme label detected chemiluminescently or by time-resolved fluorescence);
6. Nondestructive. The label must be easy to remove for successive reprobing of membranes. Generally, reprobing is not problematic for ^{32}phosphorus labels, but it is less straightforward for some nonisotopic labels (e.g., insoluble diformazan product of 5-bromo-4-chloro-3-indolyl-phosphate (BICP)-nitroblue tetrazolium (NBT)-alkaline phosphatase reaction has to be removed from a membrane with hot formamide);
7. Adaptable to nonseparation (homogenous) formats. Hybridization of labeled DNA probe to its complementary DNA sequence should modulate a property of the label so that it is detectable and distinguishable from unhybridized probe;
8. Stable during storage, providing longer shelf-life for commercial hybridization assay kits; and
9. Compatible with automated analysis. Widespread and large-scale applications of hybridization assays will lead to the need for automated analyzers. The label and the assay for the label must be compatible with a high throughput analyzer (rapid detection using the minimum number of reagents and analytical steps).

None of the labels listed in Table II fulfills all of these criteria and, just as in the case of immunoassays, there is still no agreement on the most appropriate nonisotopic label. Enzymes, such as horseradish peroxidase and alkaline phosphatase, have become particularly popular in recent years as a range of sensitive detection methods has evolved. Alkaline phosphatase, for example, can be detected using chemiluminescent, bioluminescent, and time-resolved fluorescent methods.

Table II
Direct Labels for Nucleic Acid Hybridization Assays

Chemiluminescent compounds
Acridinium ester
Isoluminol
Luminol
Enzymes
Alkaline phosphatase
Bacterial luciferase
Firefly luciferase
Glucose oxidase
Glucose 6-phosphate dehydrogenase
Hexokinase
Horseradish peroxidase
Microperoxidase
Papain
Fluorescent compounds
Fluorescein
Bimane
Ethidium
Methylcoumarin
Nitrobenzofuran
Pyrenebutyrate
Rhodamine
Terbium chelate
Tetramethylrhodamine
Texas Red
Miscellaneous
Latex particle
PolyAMP
Pyrene
Radioluminescent
^{125}Iodine
^{32}Phosphorus
^{35}Sulfur
Tritium

III. NUCLEIC ACID LABELING PROCEDURES

Detection of probe : nucleic acid target hybrids can be accomplished by direct or indirect labeling methods. In the former case, a label is attached directly to the nucleic acid by a covalent bond, or the label intercalates noncovalently between the double strand of the probe : nucleic acid target complex. The latter method, indirect labeling, employs a hapten (e.g., biotin) attached to the nucleic acid probe. The hapten is detected using a

labeled specific binding protein (e.g., antibiotin, avidin, or streptavidin) (Table III). A slightly more complex format uses an intermediate binding protein to bridge between the hapten and the labeled binding protein (Table IV). Alternatively, a binding protein specific for double-stranded DNA can be used (e.g., monoclonal anti-dsDNA), and complexes are then detected using a labeled antispecies antibody (Mantero *et al.*, 1991). More complex indirect procedures have been developed to improve assay sensitivity (Wilchek and Bayer, 1990). In one design, a biotin-labeled probe is hybridized to the target DNA, followed by reaction of the biotinylated probe with streptavidin. The remaining binding sites on tetravalent streptavidin are then reacted with a biotinylated poly(alkaline phosphatase) to obtain a cluster of alkaline phosphatase labels around the bound biotinylated probe (Leary *et al.*, 1983).

Procedures for the direct labeling of a nucleic acid probe with a hapten or a direct label can be categorized into chemical, enzymatic, and synthetic procedures (Keller and Manak, 1989; Leary and Ruth, 1989; Matthews and Kricka, 1988). One of the goals of a labeling method is to

Table III
Indirect Labels for Nucleic Acid Hybridization Assays

Hapten	Binding protein	Label
Biotin	Antibiotin	Gold colloid
	Avidin	Alkaline phosphatase
		β-Galactosidase
		Ferritin
		Fluorescein
		Horseradish peroxidase
	Streptavidin	β-Galactosidase
		b-Phycoerythrin
Digoxigenin	Antidigoxigenin	Alkaline phosphatase
Ethidium	Antiethidium-DNA	β-Galactosidase
Glucosyl	Concanavilin A	Acid phosphatase
		Glucose oxidase
IgG	Antispecies IgG	Horseradish peroxidase
IgG, Fab fragment	Antispecies IgG	Horseradish peroxidase
Lacoperon DNA	*Lac* repressor protein	Fluorescein
Poly(dA)	Poly(dT)-DNA	Horseradish peroxidase
Poly(dT)	Poly(dA)-DNA	Horseradish peroxidase
Protein A	IgG	Horseradish peroxidase
Protein G	IgG	Horseradish peroxidase
Sulfone	Antisulfone	Europium chelate
	Anti-RNA : DNA hybrid	Fluorescein
	Histone	^{125}Iodine