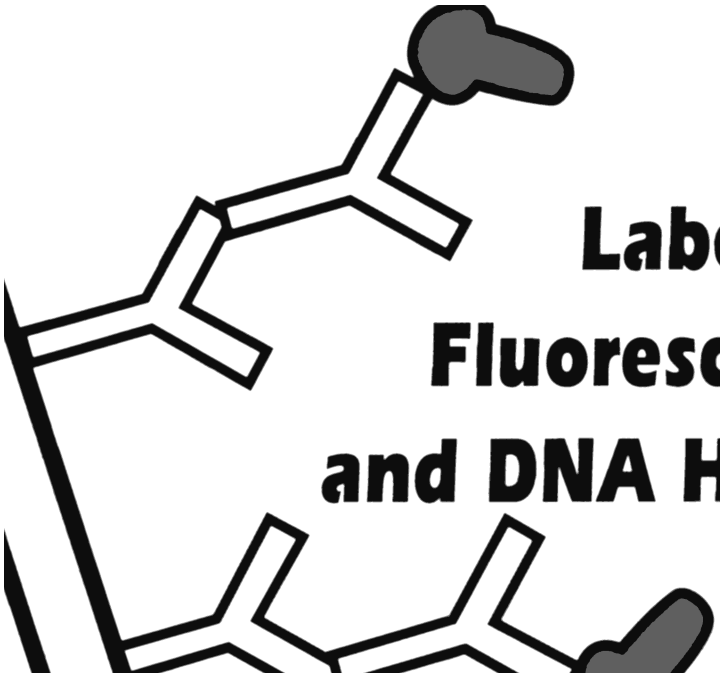

# Europium Chelate Labels in Time-Resolved Fluorescence Immunoassays and DNA Hybridization Assays

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Like many analytical methodologies, immunoassays and nucleic acid hybridization assays rely on the reaction between an analyte of interest and a specific reagent. The analyte concentration is then deduced by measuring either the amount of analyte–reagent complex formed (product) or the amount of residual reagent.

In an immunoassay, the reagent is an analyte-specific antibody, whereas in a nucleic acid hybridization assay, the reagent is an analyte-specific complementary nucleotide sequence (DNA or RNA). The specific reagent is frequently referred to as a "probe" (e.g., an immunological or a DNA probe). Immunological and nucleotide probes are more specific than classical analytical reagents and also have exceptional affinity for the analyte of interest. These two characteristics form the basis of some very sensitive and specific analytical procedures.

To quantify the analyte of interest in an immunological or nucleotide probing assay, a labeled reagent (either the immunological or DNA probe or an analyte analog) is usually introduced into the reaction mixture. Until recently, radionuclides (such as $^{125}I$, $^{32}P$, $^{3}H$, and $^{35}S$) were considered the labels of choice. Recently, however, nonisotopic labeling systems have begun to dominate immunoassay and DNA probing applications (*1*, *2*).

Enzymes, fluorescent and luminescent labels, or combinations (enzymes liberating fluorescent or luminescent products) are the most promising of these nonisotopic labels (Table I). Using such systems, workers have obtained detection limits comparable or superior to those achieved using radionuclides ($<10^6$ molecules per assay). We will describe the application of fluorescent rare-earth chelates to immunoassay and DNA probing.

## Time-resolved fluorescence

Conventional fluorescein and rhodamine derivatives have been used in the past with limited success as immunological labels and have a maximum attainable sensitivity of $\sim10^{-9}$–$10^{-10}$ M (*12*). This limited sensitivity is attributable primarily to high background signals originating from Tyndall, Rayleigh, or Raman scattering from the instrument's optics, the cuvettes, and the sample matrix (e.g., serum). Scattering interferences are aggravated by the small Stokes shifts (usually 24–50 nm) of conventional fluorophores. In addition, background fluorescence signals from biological samples usually arise between 350 and 600 nm and overlap extensively with the emission spectra of many conventional fluorophores.

Another problem in using conventional fluorophores is the so-called inner filter effect, which precludes the use of multiple fluorescence labeling to increase sensitivity. If a macromolecule such as an antibody is multiply labeled with fluorescein, for example, the fluorescence obtained from the labeled macromolecule is much less than expected from the fluorophore load. Significant overlapping of the excitation and emission spectra of many conventional fluorophores results in absorption of fluorescence by fluorophore molecules adjacent to the emitting fluorophore (energy transfer).

Fluorescent europium chelates, on the other hand, exhibit large Stokes shifts (~290 nm) with no overlap between the excitation and emission spectra and very narrow (10-nm bandwidth) emission spectra at 615 nm (away from serum native fluorescence). In addition, their long fluorescence lifetimes (600–1000 μs for $Eu^{3+}$ compared with 5–100 ns for conventional fluorophores) allow use of microsecond

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time-resolved fluorescence measurements, which further reduce the observed background signals.

The principle of time-resolved fluorescence is straightforward (Figure 1). When a mixture of fluorescent compounds is excited with a short pulse of light from a laser or flash lamp, the excited molecules emit either short- or long-lived fluorescence. Although both types of fluorescence decay follow an exponential curve (*13*), short-lived fluorescence dissipates to zero in <100 μs. If no measurements are taken during

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**Table I. Nonisotopic labeling systems**

| Type of label | Example | Measurement principle | Sensitivity[a] | Reference no. |
|---|---|---|---|---|
| Enzymatic | Horseradish peroxidase (HRP) | Colorless substrate releases products absorbing in the UV–vis region | $10^{-10}$–$10^{-11}$ M | 3 |
| | Alkaline phosphatase (ALP) | Same as above | $10^{-10}$–$10^{-11}$ M | 3 |
| Fluorometric | Fluorescein, rhodamines | Conventional fluorescence | $10^{-9}$–$10^{-10}$ M | 4 |
| | Europium chelates | Time-resolved fluorescence | $10^{-12}$–$10^{-13}$ M | 1, 5 |
| Chemiluminometric | Luminol, isoluminol | Oxidation by $H_2O_2$ with light emission | $10^{-9}$–$10^{-10}$ M | 6 |
| | Acridinium esters | Same as above | $10^{-11}$–$10^{-12}$ M | 7 |
| Combination | Enhanced luminescence | Oxidation of isoluminol by $H_2O_2$ in the presence of HRP and an enhancer | $10^{-11}$–$10^{-12}$ M | 8 |
| | ALP with fluorogenic substrate | ALP releases fluorescent products from nonfluorescing substrate | $10^{-12}$–$10^{-13}$ M | 9 |
| | ALP with chemiluminogenic substrates | ALP releases chemiluminescent products from nonluminescing substrate | $10^{-12}$–$10^{-13}$ M | 10 |
| | Replicase enzyme with replicable substrate | Replicase exponentially amplifies the substrate population | $10^{-12}$–$10^{-13}$ M | 11 |

[a] Sensitivity may vary significantly depending on the specific assay conditions.

the first 100–200 μs after excitation, all short-lived fluorescence background signals and scattered excitation radiation are completely eliminated, and the long-lived fluorescence signals can be measured with very high sensitivity.

In practice, the only background signal observed when using europium chelate labels is that produced by the nonspecific binding of the labeled reagents to the solid phases used in immunoassay or DNA probing. Precision is improved by counting each sample for a total of 1 s (20–1000 measurements).

### Fluorescence emission of $Eu^{3+}$ chelates

Of the 15 rare-earth metal ions, only four ($Sm^{3+}$, $Eu^{3+}$, $Tb^{3+}$, and $Dy^{3+}$) are fluorescent. When excited by UV radiation of appropriate energy, each ion emits characteristic radiation in the visible region (metal ion fluorescence). Although fluorescence of simple inorganic salts of these ions is weak, fluorescence is dramatically enhanced when the metal ion forms a chelate with appropriate organic ligands.

Radiation is absorbed at a wavelength characteristic of the ligand and emitted as a line spectrum characteristic of the metal ion (*14–17*) because of an intramolecular energy transfer from the ligand to the central metal ion (Figure 2). The organic ligand absorbs energy and is raised from its singlet ground state, $S_0$, to any one of the vibrational multiplets of the first singlet excited state, $S_1$, where it rapidly loses its excess vibrational energy. At this point, there are two possibilities: relaxation by an $S_1 \rightarrow S_0$ transition (ligand fluorescence) or intersystem crossing to one of the triplet states, $T_1$. From the triplet level, the molecule can go to the ground state by either a spin-forbidden $T_1 \rightarrow S_0$ radiative transition (molecular phosphorescence) or a nonradiative process. Alternatively, intramolecular energy transfer can occur from the triplet excited state of the ligand to an appropriate 4f energy level (resonance level) of the central metal ion, which in turn can move up to its

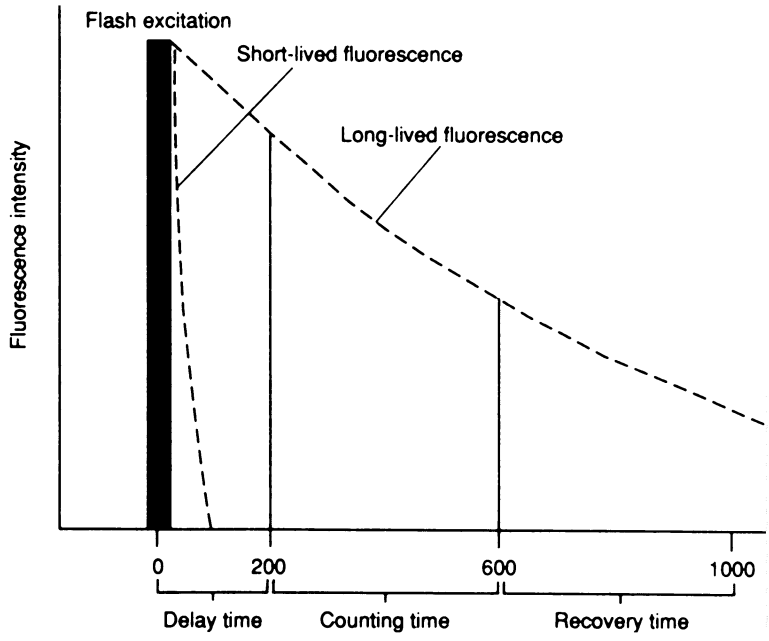


**Figure 1.** Principle of time-resolved fluorescence.
All times are in μs.

own excited singlet state and subsequently emit characteristic radiation (ion fluorescence).

For this series of events to take place, the following requirements must be fulfilled: deactivating ligand transitions $S_1 \rightarrow S_0$ or $T_1 \rightarrow S_0$ (either radiative or nonradiative) should be minimal; the energy of the ion resonance level should be just below that of the triplet-state level of the ligand, so that the probability of the triplet to resonance-level transition ($T_1 \rightarrow d$) is high; and the radiationless transitions of the excited metal ion should be low. The predominant radiative transition of excited $Eu^{3+}$ after an energy transfer is $^5D_0 \rightarrow {}^7F_2$ with an emission wavelength of ~613 nm.

### Fluorescence lifetime measurement

After instantaneous pulse excitation of a fluorescent molecule, the fluorescence decay curve follows first-order kinetics (*13*) and can be quantitatively described by Equation 1

$$I_t = I_0 e^{-kt} \quad (1)$$

where $I_0$ and $I_t$ are the fluorescence intensities at times zero and $t$, respectively, and $k$ is a rate constant. The fluorescence lifetime, $\tau$, is defined as the time required for the fluorescence emission to decay to $1/e$ of its initial intensity following excitation. Substitution of $I_t$ in Equation 1 by $I_0 e^{-1}$ (fluorescence intensity after time $\tau$) yields

$$\tau = k^{-1} \quad (2)$$

indicating that the lifetime is equal to the reciprocal of the rate constant. Combining Equations 1 and 2 yields

$$I_t = I_0 e^{-t/\tau} \quad (3)$$

or

$$\ln I_t = \ln I_0 - t/\tau \quad (4)$$

The plot of $\ln I_t$ versus $t$ is a straight line with a slope of $-\tau^{-1}$. If there is only one emitting species, the fluorescence lifetime can then be calculated from such linear plots assuming that the duration of the exciting pulse is substantially less than the fluorescence lifetime.

For nanosecond and subnanosecond lifetime measurements, phase-resolved fluorescence, in which the sample is excited by a continuous but sinusoidally modulated radiation source, is the method of choice. The emitted fluorescence as a function of time is then phase-shifted and partially demodulated to an extent dependent on the lifetime of the fluorescing species (*18*).

### Biotin–streptavidin system

The noncovalent, specific, and very strong binding of the water-soluble vitamin biotin to the tetrameric protein avidin (or streptavidin) has become one of the most useful tools in the fields of immunoassay and DNA hybridization (*19*, *20*). The affinity (formation) constant for the biotin–avidin interaction is among the highest reported at ~$10^{15}$ $M^{-1}$. (This can be compared with the affinity constant for the antigen–antibody interaction, which is at best ~$10^{11}$–$10^{12}$ $M^{-1}$.)

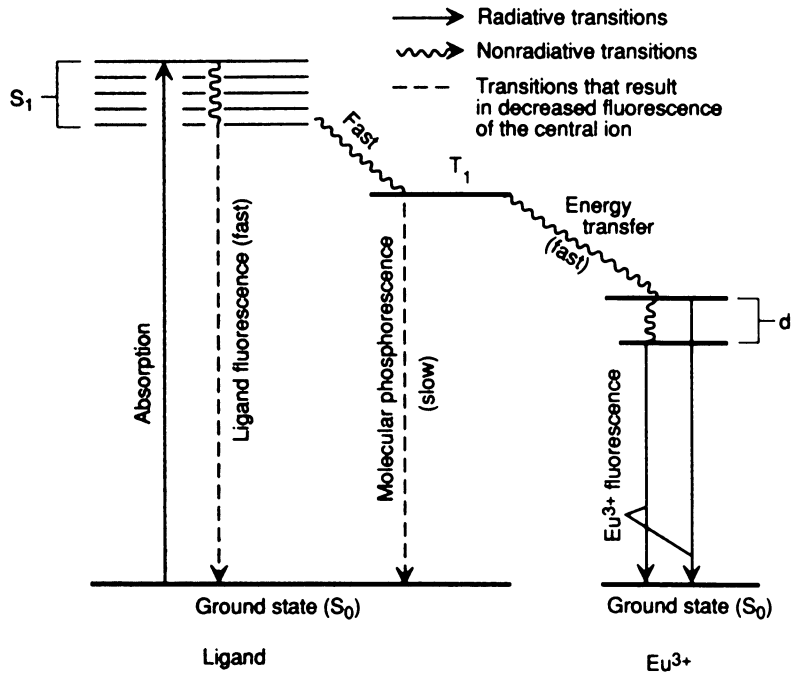


**Figure 2.** Energy transfer mechanism.

The nonradiative transitions $S_1 \rightarrow S_0$, $T_1 \rightarrow S_0$, and $d \rightarrow S_0$ may also occur.

The rationale behind the wide application of this system is as follows. Biotin covalently bound to an antibody, DNA probe, or other molecule is still available for high-affinity interaction with avidin or streptavidin. If the avidin is labeled with a suitable reporter molecule (e.g., an enzyme or a fluorescent compound), the avidin–biotin complex can be used for the quantification of the biotinylated molecule.

Although assay configurations in which the reporter molecule is directly linked to the antibody or to the DNA probe are also successful, the biotin–streptavidin system offers some distinct advantages. First, it is a universal detection system; one streptavidin-based reagent can be applied for any immunoassay or DNA hybridization assay using biotinylated reactants. Second, antibodies and DNA sequences can be easily biotinylated without loss of biological activity. Third, direct labeling of an antibody with a reporter molecule usually results in a 1:1 conjugate. On the other hand, the biotinylation process introduces many biotin molecules per antibody molecule, which in turn bind to more than one labeled streptavidin so that a significant amplification factor is always present. Finally, streptavidin is very stable and usually is not inactivated upon labeling.

The combination of the advantages of time-resolved fluorescence with those of the biotin–streptavidin interaction can be achieved by an appropriate labeling of streptavidin with $Eu^{3+}$ or $Eu^{3+}$ chelators. The most successful strategy for incorporating such metal ions into proteins or DNA probes involves the use of strong aminopolycarboxylic acid chelators (*21*).

The use of an aminopolycarboxylic acid chelator to label a protein is shown in Figure 3. The isothiocyanatophenyl-EDTA is first treated with a protein under conditions that favor multiple chelator incorporation with minimal deleterious effects to the protein affinity, specificity, solubility, or stability. The metal ion is then added in excess to saturate all available binding sites (usually 5–15 per protein), and excess metal ion is removed by dialysis or gel

filtration. (DNA probes with available amino groups are labeled in a similar manner.) These labeled reagents are stable for more than a year. Most $Eu^{3+}$ chelates with aminopolycarboxylic acids are not highly fluorescent because the energy transfer process is not optimized.

### Immunoassay design

Europium chelates can be used as immunological labels in two different assay configurations. In the first type, antibodies or antigens are labeled with $Eu^{3+}$ (*5*, *22*, *23*) and the assay can be either competitive or noncompetitive (Figure 4). In noncompetitive assays (Figure 4a), the antigen (analyte) binds to a solid-phase antibody and a $Eu^{3+}$-labeled detection antibody. After the immunological reaction is completed and excess detection antibody is washed away, an enhancement solution is added and the $Eu^{3+}$ ion dissociates from the labeled antibody at low pH to form a new fluorescent chelate in solution. The fluorescence intensity is proportional to the amount of antigen in the sample.

Competitive assays with this system can be performed in two different ways. In the first type of assay (Figure 4b), the solid phase is coated with a carrier protein–analyte derivative. The analyte to be measured, in this case triiodothyronine ($T_3$), competes with the immobilized antigen for binding to a limited amount of an analyte-specific antibody labeled with $Eu^{3+}$. After the reaction is completed and excess reagents washed away, the assay is completed by adding the enhancement solution.

In the second type of competitive assay (Figure 4c), the solid phase is coated with an antibody (red) capable of capturing the specific analyte antibody (black). For example, if the analyte-specific antibody is derived from a mouse, the solid-phase antibody is an antimouse antibody derived from a goat or a rabbit. The antigen to be measured, in this case thyroxine, competes with $Eu^{3+}$-labeled thyroxine for binding to a limited amount of a thyroxine-specific antibody. Again, the assay is completed by adding enhancement solution after the reaction is completed and excess reagents washed away. The fluorescence intensity in the competitive assays is inversely related to the amount of antigen in the sample.

In the second type of assay configuration (*1*, *24–26*), a europium chelator, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA), is used as a label. This chelator works best when it is introduced into streptavidin and used in combination with biotinylated antibodies, as shown in Figure 5. BCPDA-labeled streptavidins with a variable load of BCPDA molecules per streptavidin have been prepared (*24–26*).

In the noncompetitive immunoassay using this system (Figure 5a), the analyte of interest is bound to a solid-phase antibody and a soluble biotinylated detection antibody. After the immunoreaction is completed and excess detection antibody is washed away, BCPDA-labeled streptavidin is added and incubated for 30 min. After excess streptavidin is washed away, the amount of fluorescent immunocomplex on the dry solid phase is determined by using time-resolved fluorescence.

In the competitive assay, the immobilized antigen approach is used (Figure 5b). Again, the final step involves BCPDA-labeled streptavidin addition, incubation, and washing prior to detection of the fluorescent immunocomplex on the dry solid phase.

Immunoassays using $Eu^{3+}$ labeling are highly sensitive, and systems with detection limits as low as $10^{-12}$ M have been reported. It is difficult, however, to quantify $Eu^{3+}$ because of exogenous contamination from $Eu^{3+}$ in the environment and on skin surfaces. Assays based on BCPDA operate with excess $Eu^{3+}$, eliminating such contamination

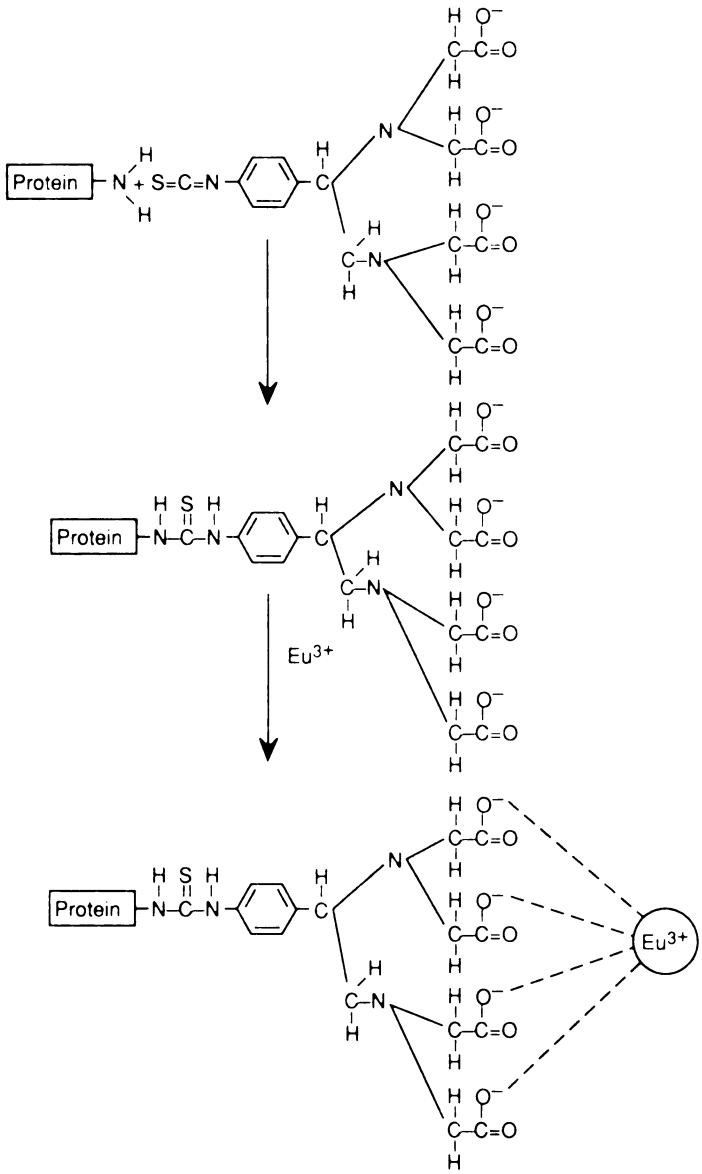


**Figure 3.** Labeling of a protein with isothiocyanatophenyl-EDTA, one of the commonly used aminopolycarboxylic acid derivatives.

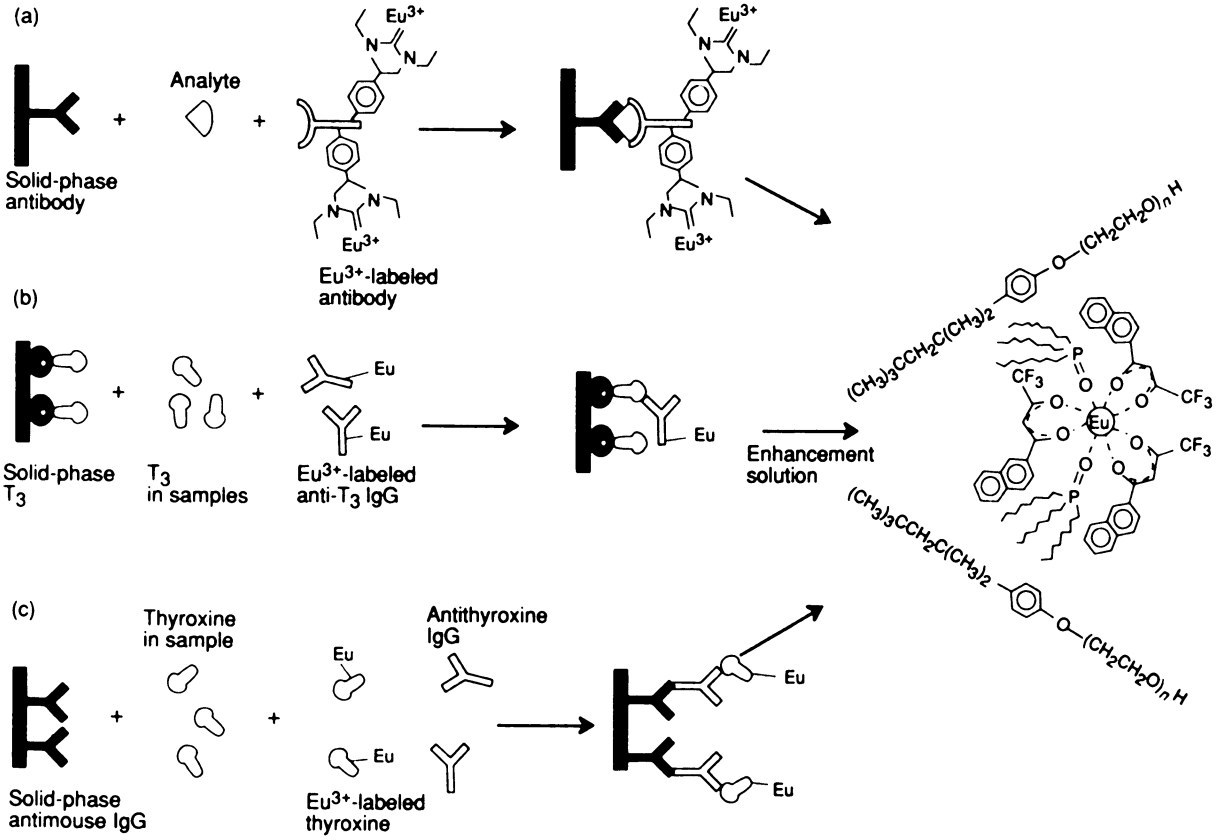


**Figure 4.** Immunoassays using $Eu^{3+}$-labeled reagents.

(a) Noncompetitive assay for an analyte (yellow) using a solid-phase immobilized antibody (red) and an $Eu^{3+}$-labeled detection antibody (blue), (b) competitive assay using the immobilized antigen approach, and (c) competitive assay using the immobilized antibody approach.

problems. In addition, BCPDA-based systems can be used to study the spatial distribution of the analyte of interest (e.g., when the analyte is separated on solid electrophoretic media) because the final measurement is taken on the solid phase and not in solution.

Multiple fluorescence labeling can be used to improve the sensitivity of these assays, and proteins with BCPDA loads as high as 450 have been described. With such proteins, the detectability of an immunoassay for $\alpha$-fetoprotein was lowered to ~300 000 molecules per assay (27).

### DNA hybridization

Nucleotide probing methodology relies on the ability of labeled DNA and RNA fragments (probes) to detect, by binding, complementary sequences of nucleotides (targets). Although nucleic acid hybridization is a relatively new technique, it has already been used in many research applications and recently for routine testing (28). DNA probing techniques have been used to test for the presence of genetic disease, for both carrier detection and prenatal diagnosis; to detect pathogenic organisms such as viruses, bacteria, and par-

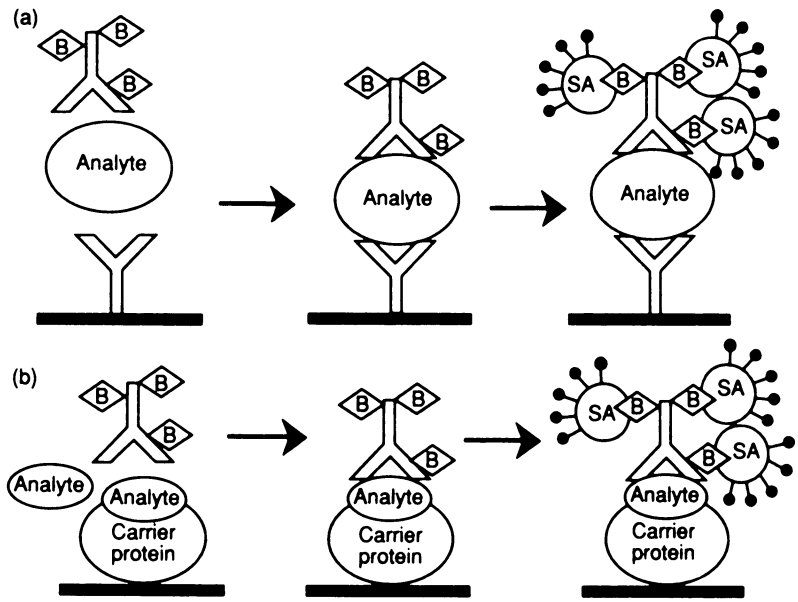


**Figure 5.** Immunoassays using 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA)-labeled streptavidin (SA) in the presence of excess $Eu^{3+}$ and biotinylated (B) antibodies.

(a) Noncompetitive assay using a solid-phase immobilized antibody and a soluble biotinylated detection antibody and (b) competitive assay using the immobilized antigen approach. The BCPDA–$Eu^{3+}$ complex is represented by —●.

asites in humans, animals, food, and the environment; to establish the identity of a particular person in crime cases and paternity disputes; and to test for mutation, activation, amplification, and expression of oncogenes.

Nucleic acid hybridization assays are expected to be used even more often in the future as improved probes are developed and new genes are cloned. Because of the cost, short half-life, and health hazards associated with the commonly used radionuclides, major efforts are now under way to develop sensitive nonisotopic detection systems for these assays.

Nucleic acid hybridization assays are performed in several different ways (Figure 6). The target sequence is immobilized on a solid support such as nitrocellulose or nylon by either direct spotting or capillary transfer of the nucleic acids previously separated by electrophoresis on agarose gels (Southern blotting).

After the probe is added under carefully controlled conditions, the hybrid is detected by using labeled antibodies or binding proteins (histones) or by having the probe itself carry one of the detection systems shown in Table I. In indirect systems, the probe is linked to a low molecular weight substance (hapten), which bridges the probe with a hapten-specific antibody carrying the detection system. In a variation of this method, biotin is used as hapten and streptavidin carrying the detection system is used. Alternatively, streptavidin is used as a bridge to link the biotinylated probe with a reporter molecule system (e.g., an enzyme).

The signal generated by the detection system can be assessed by viewing the colored insoluble products resulting from enzymatic action on the membrane directly or after exposure to films (radionuclide or chemiluminescent labels) or UV radiation (fluorescent labels). In some cases, instruments are used to obtain quantitative information.

$Eu^{3+}$-labeled nucleic acids along with hapten- or biotin-labeled probes have been used with $Eu^{3+}$-labeled antihapten antibodies or streptavidin, respectively (*29*, *30*). Work is under way in our laboratory to examine the capabilities of BCPDA-labeled streptavidin for nucleic acid hybridization assays.

## Homogeneous time-resolved immunoassays

In homogeneous immunoassays, one fraction of the label (bound or free) can be quantified in the presence of the other by taking advantage of a physicochemical difference usually associated with the presence of the antibody. In time-resolved fluorescence immunoassays, two general possibilities have been reported for devising homogeneous immunoassays. In a thyroxine ($T_4$) assay, $T_4$ is conjugated to a fluorescent europium chelate. This $T_4$ conjugate, when it binds to a specific $T_4$ antibody, emits fluorescence but with greatly reduced intensity (*31*). In this assay, $T_4$ and a fixed amount of $T_4$ conjugate compete for binding to the antibody, and the fluorescence intensity is inversely related to the amount of $T_4$ in the sample.

Another approach exploits the change in the fluorescence lifetime of an analyte–europium chelate conjugate when bound to an analyte-specific antibody. Homogeneous phase-resolved immunoassay techniques based on fluorescence lifetime selectivity have also been reported (*32*, *33*).

In many clinical situations, certain serum analytes are usually measured in pairs (e.g., thyroxine–thyrotropin ($T_4$–TSH), lutropin–follitropin (LH–FSH), and folate–vitamin $B_{12}$), and assays to measure both at the same time are extremely useful. For example, an assay for LH and FSH can be based on the fact that the a-subunits are identical and the b-subunits are different (*34*). A monoclonal antibody specific for the a-subunit can be immobilized in a solid-phase microtitration well, whereas an antibody specific for the b-subunit of LH is labeled with $Eu^{3+}$ and an antibody specific for the b-subunit of FSH is labeled with $Tb^{3+}$. After $Eu^{3+}$ and $Tb^{3+}$ are extracted with the same chelating solution, the two rare-earth ions can be easily quantified because their emission lines at 614 nm ($Eu^{3+}$) and 544 nm ($Tb^{3+}$) do not overlap.

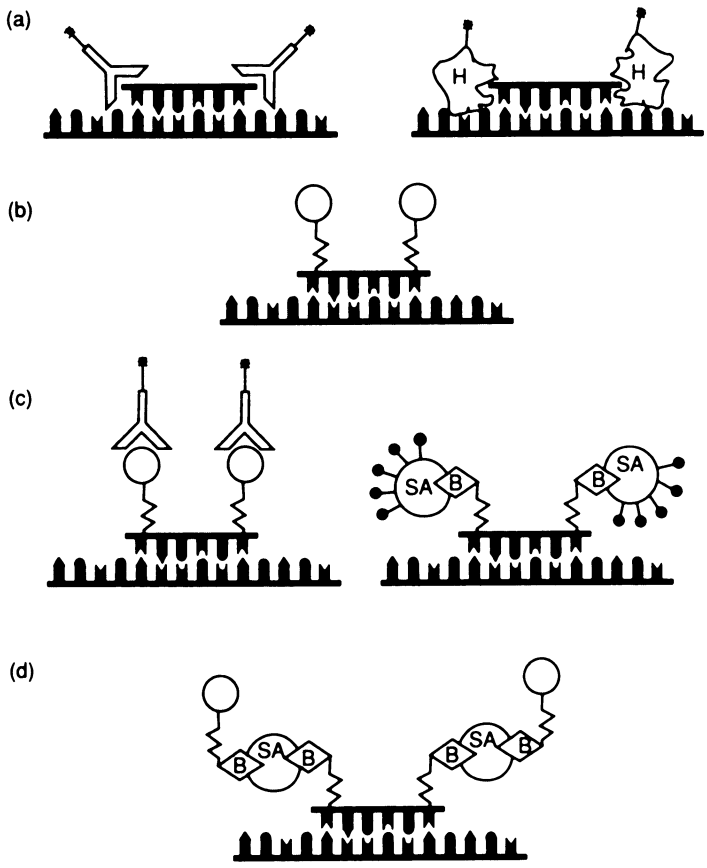


**Figure 6.** Assay designs in DNA hybridization.

(a) The DNA probe (red) is used unlabeled and the target-probe hybrid is detected by using either labeled antibodies against the double-stranded DNA or histones (H); (b) the DNA probe is directly labeled with a reporter molecule (yellow); and (c) the DNA probe is conjugated to a hapten molecule, and antihapten-labeled antibodies are used to detect the hybrid. In a variation of this technique, the probe is biotinylated (B) and labeled streptavidin (SA) is used as the detection reagent; (d) unlabeled SA is used to bridge a biotinylated probe with a biotinylated reporter molecule.

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