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Time-resolved fluorometry in nucleic acid hybridization and Western blotting techniques

Time-resolved fluorometry is now used extensively for immunological assays and to a lesser extent in other research areas. In this review I describe applications of time-resolved fluorometry in nucleic acid hybridization and in blotting techniques, including Southern and Western blotting. Clearly, time-resolved fluorometry has potential for playing a major role in techniques other than immunological assays.

1 Introduction

Molecular biology techniques, including Southern, Western and Northern blotting, are widely used for research and, in some instances, for routine testing [1, 2]. Traditionally, these methods employ radionuclide-based detection methodologies and especially ^{32}P , ^{35}S and ^3H nuclides coupled to autoradiography. Although still widely used because of their excellent sensitivity, low background signals, and ease of labeling of probes, these methods have many limitations. Of primary importance is the short shelf-life of labeled reagents, the long exposure times, the possible health hazards, and inconvenience and cost associated with handling and disposing of radioactivity. In addition, the laboratories that use them need a licence.

During the last 10 years, considerable research efforts were made to develop alternative methodologies which could replace radionuclides from many routine applications. These efforts were very successful in the field of immunological assays where the alternative techniques are now dominant. The use of radioimmunoassays is rapidly decreasing. In the field of molecular biology the change is just beginning and will likely continue in favor of the alternative techniques during the next 3–5 years. A close examination between the non-isotopic detection methodologies used in immunological assays and molecular biology techniques reveals that these are based on similar principles [1]. They include probes or antibodies labeled with fluorescent or luminescent molecules or enzymes which catalyze the conversion of substrates to products that emit fluorescent or luminescent light [1, 2]. In this review, I will describe the application of time-resolved fluorometry in nucleic acid hybridization and other blotting techniques. This method has attracted considerable interest for immunological assays [3–5] and is currently used routinely for this purpose.

2 Principles of time-resolved fluorometry

This technique has been reviewed recently [3–5]. The label in such systems is either a lanthanide, *e.g.* Eu^{3+} , or a lanthanide chelator. A fluorescent lanthanide chelate can then be formed by adding either organic chelators as in the DELFIA™ system (LKB-Pharmacia, Turku, Fin-

land) or lanthanide ion as in the FIAgen™ system (CyberFluor Inc., Toronto, Canada). The fluorescent lanthanide chelates possess certain advantages in comparison to conventional fluorescent labels like fluorescein. These include large Stokes' shifts, narrow emission bands and long fluorescence lifetimes. The fluorescence lifetimes of most conventional fluorophores is 100 ns or less; the lifetime of lanthanide chelates is usually around 100–1000 μs . Using a pulsed light source and a time-gated fluorometer, the fluorescence of these compounds can be measured within a time-window, *i.e.* 200–600 μs , after each excitation (Fig. 1). This method decreases the background interference from short-lived fluorescence of natural materials in the sample, cuvettes, optics *etc.*

The chelates used by the DELFIA system are complexes of the type $\text{Eu}^{3+}(\text{NTA})_3(\text{TOPO})_2$, where NTA is naphthyltrifluoroacetone and TOPO is triocylphosphine oxide. The immunological label, Eu^{3+} , is introduced into

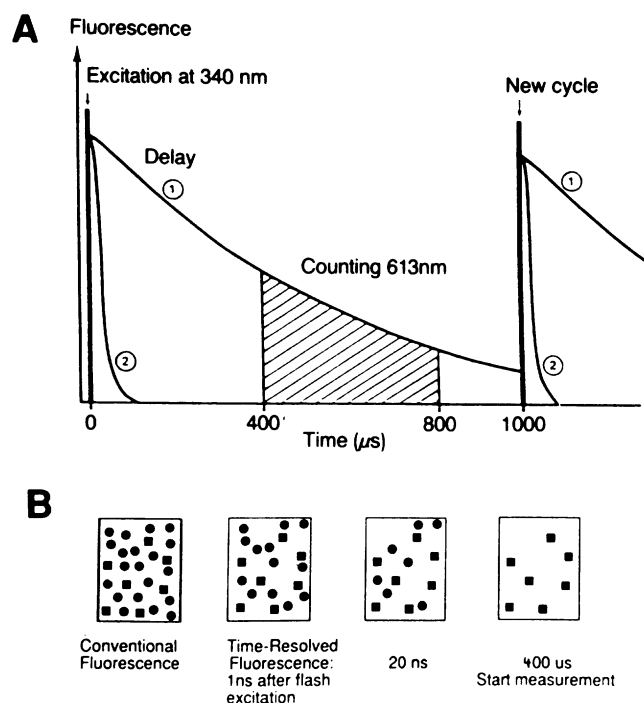


Figure 1. Principle of time-resolved fluorometric measurement. (A) Exponential decay of short-lived (2) and long-lived (1) fluorescence. The latter is quantified in a selected time-window as shown. (B) Events occurring in the cuvette during the time-resolved fluorometric measurement at various time periods. Long-lived and short-lived fluorescence is represented by squares and circles, respectively.

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antibodies or streptavidin by using a strong Eu^{3+} chelator of the aminopolycarboxylic acid type (e.g. an EDTA derivative). Similarly, Eu^{3+} can be incorporated into DNA probes (for details see other sections of this review). Release of Eu^{3+} and recomplexing with NTA and TOPO can be achieved by lowering the pH to around 3.0 (Fig. 2). DELFIA is well-established in the field of nonisotopic immunoassay and is characterized by high sensitivity and broad dynamic range. A drawback of this system is its vulnerability to exogenous Eu^{3+} contamination. The label, Eu^{3+} , can be measured down to about 10^{-13} mol/L using time-resolved fluorescence, and 10^{-17} mol of Eu^{3+} per cuvette can be detected routinely. Analytes can also be measured down to these levels. In DNA applications the detection limit is around 10^{-18} – 10^{-16} moles of probe. This compares to 0.05 – 2×10^{-18} mol of ^{32}P -labeled probe [6].

The FIAgen system uses the europium chelator 4, 7-bis (chlorosulphophenyl)-1,10 phenanthroline-2,9-dicarboxylic acid (BCPDA) as label and is not subject to Eu^{3+} contamination [7, 8]. This system works best when biotinylated antibodies or DNA probes are used in combination with BCPDA-labeled streptavidin. Three streptavidin preparations have been produced so far (Fig. 3), which achieve the following different detection limits: (i) streptavidin (SA) directly labeled with BCPDA [SA(BCPDA)₁₄] for assays with detection limits of 10^{-10} – 10^{-11} mol/L [8], (ii) streptavidin covalently linked to thyroglobulin (TG) carrying approximately 160 BCPDA molecules, [SA(TG)(BCPDA)₁₆₀], for assays with detection limits of 10^{-11} – 10^{-12} mol/L [9]; (iii) the streptavidin-thyroglobulin system described in (b) was activated by an empirical process to yield a reagent suitable for assays with detection limits of 10^{-12} – 10^{-13} mol/L [10]. The best detection limit achieved with the latter reagent for a model alpha-fetoprotein assay was approximately 300 000 molecules per cuvette (5 μL sample volume) [11].

Another new detection system based on time-resolved fluorometry uses alkaline phosphatase as label and a substrate which, when hydrolyzed, forms long-lived, fluorescent complexes with Tb^{3+} and EDTA [12–14]. This system is described in detail below.

3 Assay configurations

Time-resolved fluorometry can be used in nucleic acid-hybridization or immunological assays in two different formats. (i) In assays where molecular weight information of the target is not obtained. Such assays include dot or slot blots, dot or spot immunodetection and hybridization assays in microtiter wells or tubes. These types of assays do not involve electrophoresis, they are usually simple to perform and they are quantitative. For these assays, there is no need for the label (e.g. a europium chelate) to be fluorescent. A subsequent step involving Eu^{3+} release and recomplexation may create the fluorescent complex. (ii) In assays where molecular weight information of the target is obtained. Such assays include Western, Northern and Southern blotting and they usually involve an electrophoretic and transfer step at the end of which the target is immobilized at a pre-

cise spot on a nylon or nitrocellulose filter. In such assays, the detectable moiety, e.g. a lanthanide chelate, must be fluorescent in order to reveal the location of the band(s). Examples and useful reagents for such assays will be given below.

3.1 Fluorescence enhancement

Nucleic acids, antibodies or streptavidin can be labeled with Eu^{3+} with use of metal-ion chelators of the aminopolycarboxylic acid-type [15]. Such Eu^{3+} -labeled reagents are practically non-fluorescent. In this case, the Eu^{3+} , after all steps of the assay have been completed, must be released from the labeled reagent and recomplexed with other ligands in order to form fluorescent complexes. This is done in one step by using an 'enhancement solution' which plays the following roles: (i) has a pH of around 2.5–3.0; at this pH the carboxyl groups of the chelator are protonated and are no longer able to bind Eu^{3+} ; thus, Eu^{3+} is released into solution; (ii) contains appropriate ligands which form highly fluorescent complexes with Eu^{3+} . The process is shown in Fig. 2. In one version of an 'enhancement solution' the added organic chelators are naphthoyltrifluoroacetone (NTA) and trioctylphosphine oxide (TOPO). The complex formed has the structure $\text{Eu}^{3+}\text{-(NTA)}_3\text{-(TOPO)}_2$. This complex is in

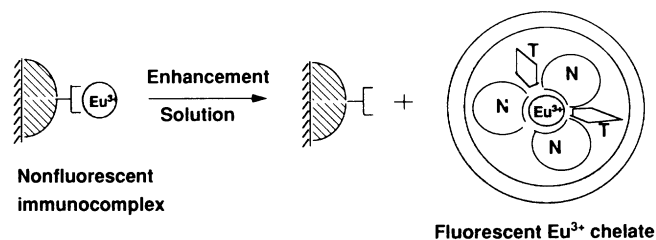


Figure 2. Principle of fluorescence enhancement. A solid-phase non-fluorescent immunocomplex is treated with an enhancement solution. The released Eu^{3+} is complexed at low pH with naphthoyltrifluoroacetone (N) and trioctylphosphine oxide (T) in the presence of Triton X-100 (shown as a double circle). The resulting chelate, in micellar form, is highly fluorescent and can be quantified with time-resolved fluorometry.

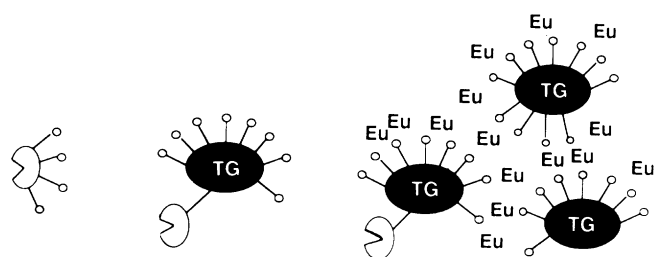


Figure 3. Three streptavidin-based reagents used in the FIAgen system in combination with biotinylated detection antibodies. Left: streptavidin covalently linked to about 14 BCPDA molecules as described [8]. Middle: streptavidin covalently linked to BCPDA-labeled bovine thyroglobulin as described in [9]. The conjugate carries about 140 BCPDA molecules. Right: Streptavidin covalently linked to one molecule of BCPDA-labeled bovine thyroglobulin and noncovalently linked to another two BCPDA-labeled bovine thyroglobulin molecules. This streptavidin-based macromolecular complex (SBMC) is described in [10].

micellar form in the presence of Triton X-100 surfactant. When this complex is formed, Eu^{3+} can be quantified at concentration levels of 10^{-7} – 10^{-13} mol/L using time-resolved fluorometry [16].

3.2 Streptavidin-based macromolecular complex

The streptavidin-based macromolecular complex (SBMC) is a highly sensitive detection reagent labeled with the fluorescent Eu^{3+} -chelate of 4,7-bis (chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA). Its preparation and applications are discussed in detail elsewhere [10]. Briefly, the SBMC is represented by the formula $\text{SA}[\text{TGH}_{3,3}(\text{BCPDA})_{480}]$. The SBMC can be prepared by incubating, at 50–55 °C, a mixture containing the conjugate $\text{SA}[\text{TG}(\text{BCPDA})_{150}]$, BCPDA-labeled thyroglobulin, *i.e.* $[\text{TG}(\text{BCPDA})_{150}]$, and a suitable amount of Eu^{3+} . In this mixture, Eu^{3+} can bind two BCPDA molecules, thus acting as a bridge between $\text{TG}(\text{BCPDA})_{150}$ and $\text{SA}[\text{TG}(\text{BCPDA})_{150}]$. Eu^{3+} is entrapped into the core of the complex and is quantitatively carried during the incubations and washes. The SBMC has a higher BCPDA load than the other two preparations (Fig. 3) and it is a very sensitive detection reagent. An 8- to 26-fold improvement in the detection limit was achieved by using SBMC instead of $\text{SA}[\text{TG}(\text{BCPDA})_{150}]$ in various immunoassays (10). For example, the reported detection limits for an AFP assay, based on two monoclonal antibodies, are 1000, 200 and 12 ng/L when the detection reagents are $\text{SA}(\text{BCPDA})_{14}$, $\text{SA}[\text{TG}(\text{BCPDA})_{150}]$ and $\text{SA}[\text{TG}_{3,3}(\text{BCPDA})_{480}]$, respectively [11].

3.3 Enzymatically amplified time-resolved fluorometry

This system uses alkaline phosphatase (ALP) to label antibodies or streptavidin. The phosphate ester of 5-fluorosalicic acid is used as substrate. The nonhydrolyzed ester and the hydrolyzed ester (free 5-fluorosalicic acid) have different behaviors in Tb^{3+} -EDTA solutions. When 5-fluorosalicic acid is added in an aqueous alkaline solution containing Tb^{3+} -EDTA, a mixed complex is

formed which emits long-lived fluorescence characteristic of Tb^{3+} . 5-Fluorosalicic acid is an appropriate ligand for energy transfer to the metal-ion. An intact hydroxyl group on the 5-fluorosalicic acid molecule is essential for these highly fluorescent mixed complexes to be formed. The phosphate ester of 5-fluorosalicic acid does not cause any increase in the fluorescence.

A 'sandwich-type' immunoassay protocol with this system would be based on solid-phase capture antibodies and ALP-labeled detection antibodies or biotinylated detection antibodies in combination with ALP-labeled (strept)avidin. After completion of the immunoreaction, the substrate solution is added. During the incubation period, the enzyme catalyzes the hydrolytic cleavage of the ester, producing 5-fluorosalicic acid. An alkaline Tb^{3+} -EDTA solution is then added (developing reagent) and the fluorescence of Tb^{3+} in solution is measured with a time-resolved fluorometer. The CyberFluor 615TM Immunoanalyzer or the Arcus^R time-resolved fluorometer may be used for the fluorescence measurements. This method is sensitive [13, 14] and, in addition, extraction is not needed for fluorescence development and contamination is not a problem because excess Tb^{3+} is used.

3.4 Methodologies involving Eu^{3+} labeling and dissociation

As already mentioned, Eu^{3+} is not fluorescent by itself but it becomes fluorescent when complexed to certain organic chelators. Syvanen *et al.* [17] developed dot-blot procedures using Eu^{3+} as the detectable moiety. In these assays, the target was immobilized on nitrocellulose; the probe was labeled with the hapten 7-iodo-*N*-acetoxy-*N*-2-acetylaminofluorene (AAIF) or sulfone. After hybridization, the hybrid was reacted with anti-hapten rabbit or mouse antibodies followed by reaction with secondary anti-rabbit or anti-mouse antibodies labeled with Eu^{3+} . Similarly, sandwich hybridizations were performed using a capture-immobilized probe and labeled detection probe. The final step involved the addition of enhance-

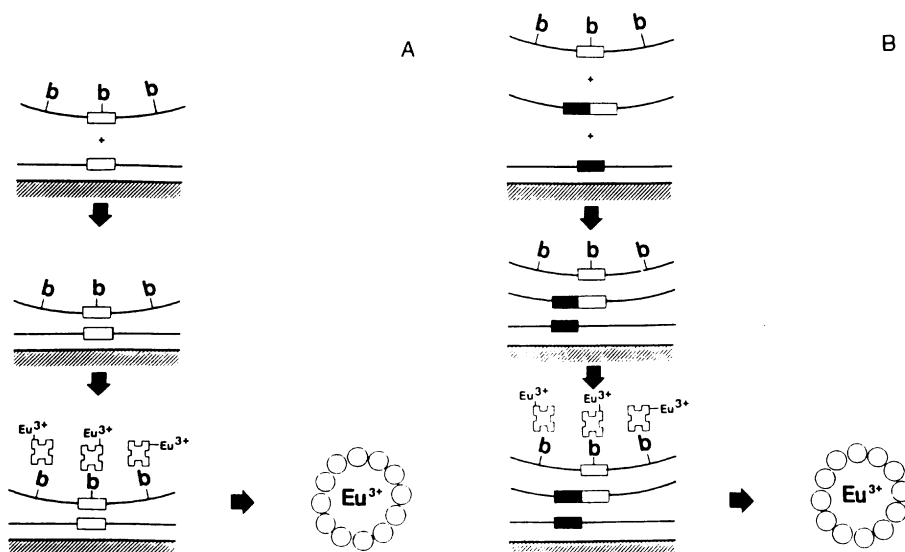


Figure 4. (A) Dot blot hybridization. The sample DNA is immobilized on a solid support. A biotinylated DNA probe is allowed to hybridize to its target sequences. The bound biotin-probe is detected by Eu -labeled streptavidin. The Eu -fluorescence is developed by adding enhancement solution. (B) Sandwich hybridization. The sample is added simultaneously with the biotinylated DNA probe to the hybridization reaction. The captive probe, which has been attached to the solid support before the assay, and the biotinylated probe will hybridize to the target DNA. The bound biotin-probe is detected by using Eu -labeled streptavidin as above. Reprinted from [22] by permission.

ment solution which dissociates the bound Eu^{3+} and recomplexes it in solution as shown in Fig. 2. For convenience, the nitrocellulose filter containing target DNA was cut to a small circle which was fitted into microtiter wells in order to perform all of the assay steps. The above assay is quantitative and was able to detect up to 20 pg of a DNA target, corresponding to 5×10^5 molecules (approximately 1 amol). Using the sandwich hybridization technique, the authors succeeded in detecting adenovirus type 2 DNA in nasopharyngeal aspirates.

Dahlen [18] used Eu^{3+} -labeled streptavidin as the detection reagent and polystyrene microtiter wells as the solid-phase carrier of target DNA. The probe was biotinylated (Fig. 4). Using adenovirus type 2 DNA, he achieved a detection limit of 10 pg (approximately 0.5 amol, or 2.5×10^5 molecules). In another, improved version of the procedure, involving sandwich hybridization (see Fig. 4), Dahlen *et al.* [19] used a microtiter well-immobilized capture probe, a biotinylated detection probe and Eu^{3+} -labeled streptavidin. With pBR322 as the target, the authors were able to detect up to 4×10^5 molecules. The method was successfully applied to detect the presence of the β -lactamase gene in uropathogenic *Escherichia coli* strains.

More recent advances involve the direct labeling of DNA probes with Eu^{3+} . This is feasible if aliphatic amino groups are available on the DNA for labeling. Amino groups on cytosines can be generated by the transamination reaction as described by Viscidi *et al.* [20]. The labeling of DNA is then achieved by reaction with an isothiocyanate analog of a europium chelate available from Pharmacia (Fig. 5). The labeled DNA is represented in Fig. 6. Such labeled probes can be used for hybridization with the target DNA which is usually immobilized on nitrocellulose filters. The final measurement step is done in tubes containing the nitrocellulose filters, by adding

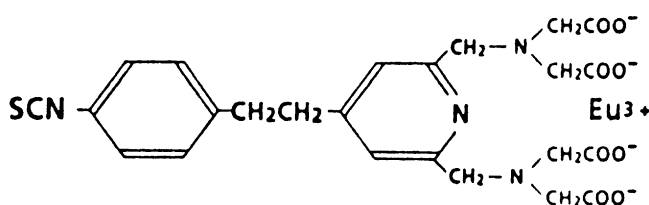


Figure 5. The structure of the Eu chelate for DNA labeling.

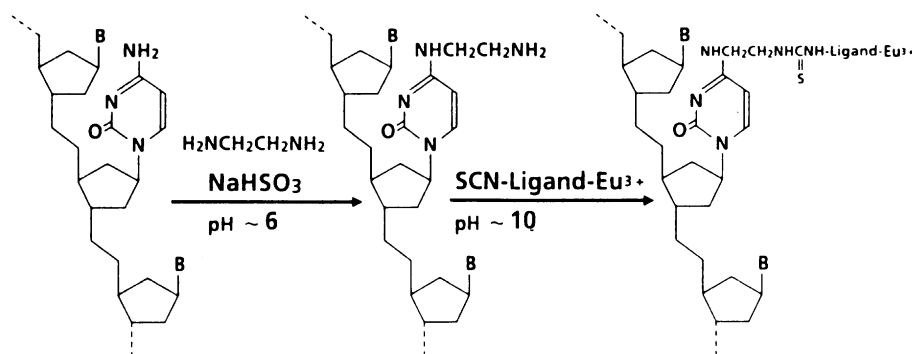


Figure 6. Chemical labeling of DNA with a europium chelate. In the first reaction the cytosine bases in DNA are transaminated in the presence of sodium bisulfite and ethylenediamine. In the second reaction the modified DNA is reacted with an isothiocyanate derivative of the europium chelate. Reprinted from [22] by permission.

enhancement solution to extract Eu^{3+} as shown in Fig. 2. Model assays using adenovirus DNA as target have shown that sensitivities in the range of 10–100 pg could be achieved with either the Eu^{3+} -labeled probe or a ^{32}P -labeled probe. Comparable results were also obtained when actual clinical samples were tested.

Other investigators have also labeled probe DNA using the transamination reaction and the europium chelate of Fig. 5. In these studies, λ DNA was used as probe and target, immobilized on nitrocellulose filters or polystyrene microtiter wells [21]. The detection limit was approximately 0.15 amol of target DNA (approximately 10^5 molecules), comparable to that achieved with ^{32}P -labeled probes.

The ability to label DNA with biotin and Eu^{3+} makes possible the use of solution hybridization of target with two probes labeled with biotin and Eu^{3+} , respectively. After collecting the hybrid on an immobilized streptavidin matrix, detection is performed with the use of enhancement solution (Fig. 7). This elegant method was used to detect HPV 16 DNA, down to 10^6 molecules [22].

Enzymatic Eu^{3+} -labeling of DNA probes is also possible using the classical techniques of nick translation or random primer [22]. For this, a dCTP analog that carries Eu^{3+} is used in the reaction mixture (Fig. 8). This nucleotide, 2'-deoxycytidine 5'-triphosphate (Eu -dCTP) is commercially available. The use of such Eu^{3+} -labeled probes is the same as described elsewhere in this review.

Very short probes or polymerase chain reaction (PCR) primers can also be labeled with Eu^{3+} by incorporating amino groups anywhere in the probe sequence during phosphoramidite synthesis. These could then be reacted with the isothiocyanate analog of the Eu^{3+} chelator of Fig. 5. For PCR, Eu^{3+} is incorporated at the 5'-end of one of the primers.

Oser *et al.* [23] labeled DNA probes with Eu^{3+} using a different method. A plasmid-cloned probe was made single-stranded by limited exonuclease digestion. The double-stranded part of the probe, including plasmid sequences, was enriched with SH-groups by UV light cross-linking with a thiol-containing psoralen derivative. This modified probe was then covalently linked to diethylenetriamine pentaacetic acid (DTPA)-labeled polylysine using a chemical cross-linker. This procedure yields DNA that has a single-stranded region, available for hybridization,

and a double-stranded region, labeled with DTPA. These probes were used in dot-blot protocols, and after hybrid formation, Eu^{3+} was added to label the hybrid through the DTPA chelating group. Eu^{3+} quantification was performed after extraction with the enhancement solution. Target DNA was detected down to the 5 pg level (6×10^5 molecules, 1 amol) and the method is quantitative. As with all methods relying on Eu^{3+} extractions, it is not suitable for Southern, Northern, Western, colony, or *in situ* hybridizations. In a newer version of this method [24], the single-stranded probe was tailed with a double-stranded A–T polymer which was then crosslinked to a psoralen derivative carrying amino groups. The amino groups were then used to covalently link a fluorescent terbium chelate (Fig. 9). However, no successful application of such probes has as yet been published.

Sund *et al.* [25] used polyvinylamine and polyacrylic acid as carriers of many chelator groups of the aminopolycarboxylic acid-type which were saturated with Eu^{3+} . These Eu^{3+} -labeled polymers were then linked to short probes, approximately 50 nucleotides long. When used in hybridization experiments with the principle of Eu^{3+} quantification as described above, about 200 pg of adenovirus 2 DNA could be detected.

The polymerase chain reaction (PCR) technique allows approximately 10^6 -fold amplification of specific regions of DNA in a few hours. When combined with PCR, nonisotopic hybridization techniques have no sensitivity limitations. Dahlen *et al.* [26] applied PCR to amplify

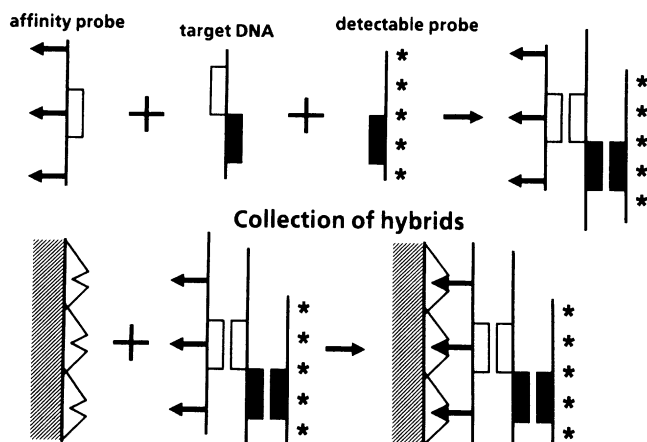


Figure 7. Schematic presentation of the solution hybridization. First two different probes, one labeled with an affinity label (*e.g.*, biotin) and the other labeled with a detectable label (*e.g.*, europium), hybridize with the target DNA in solution. In the next step, the formed hybrids are collected onto an affinity (*e.g.*, streptavidin) matrix. Reprinted from [22] by permission.

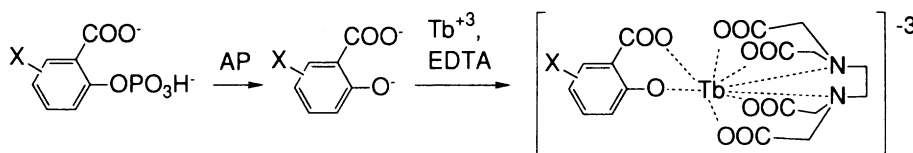


Figure 10. EALL (enzyme amplified lanthanide luminescence) detection of alkaline phosphate using substituted salicyl phosphate substrates. After dephosphorylation by alkaline phosphatase, the luminescent ternary complex is formed between the product salicylic acid, terbium ion, and EDTA. For membrane applications, the substituent is a branched alkyl group in the 5-position, while for solution applications, the substituent is 5-fluoro.

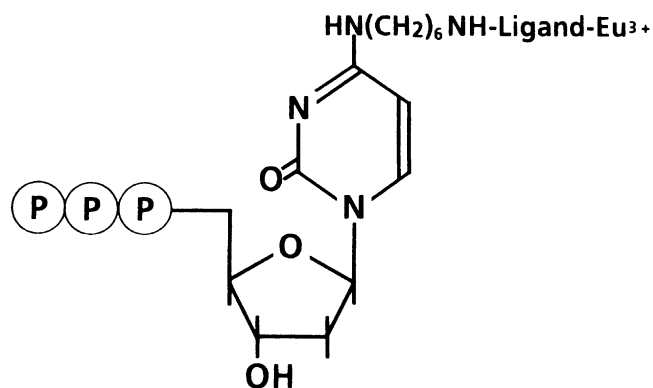


Figure 8. Eu-labeled dCTP for enzymatic labeling of DNA. Reprinted from [22] by permission.

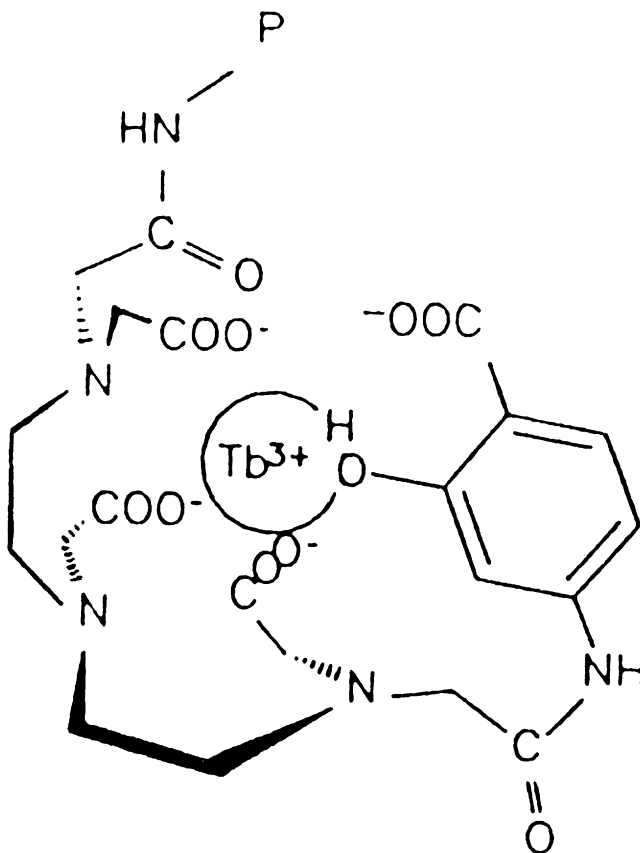


Figure 9. Presumed structure of Tb-DTPA-pAS attached to the primary amino group of one psoralen moiety in an oligonucleotide (P = psoralen moiety). For Tb fluorescence emission the salicylate moiety absorbs light and transfers the energy to the Tb^{3+} ion. The light conversion process is responsible for the delayed fluorescence. Reprinted from [24] by permission.

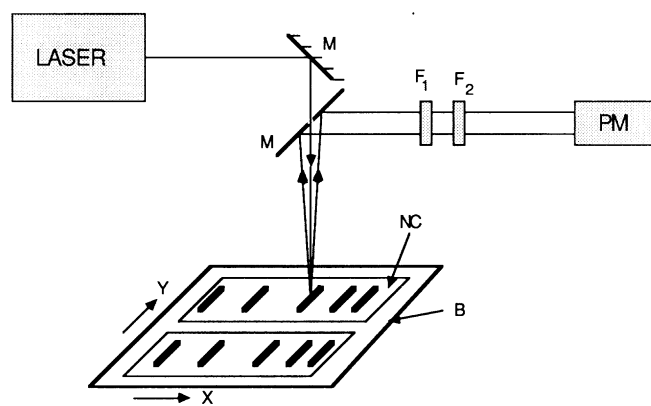


Figure 11. Simplified optical diagram of the CyberFluor 615™ Immunoanalyzer working as a high resolution time-resolved fluorometric scanner. The excitation source is a nitrogen laser emitting 20 pulses of light (337.1 nm) per s, lasting approximately 3 ns. M, mirrors, one with a hole in the middle; F1, F2 are liquid and interference filters, respectively. Other lenses, mirrors and filters and other components have been eliminated for simplicity. The electronic circuitry used for the time-resolved measurement is not shown. The metal base (B) used to position the nitrocellulose membranes (NC) is an exact replica of a microtitration plate. The metal base movement is in the X and Y direction as shown and also described in the text. Components are not shown to scale. Reprinted from [29] by permission.

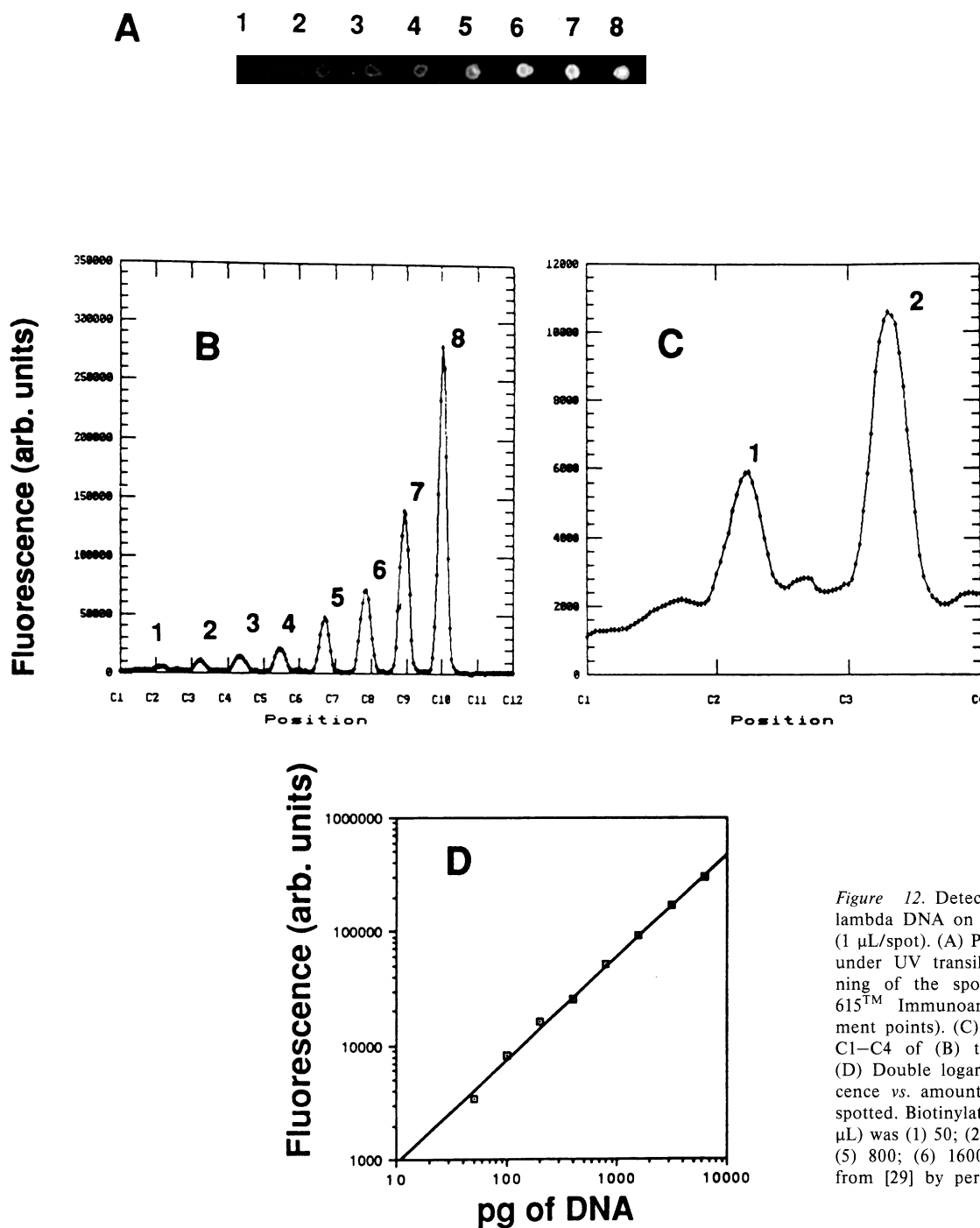


Figure 12. Detection of biotinylated lambda DNA on spots with the SBMC (1 µL/spot). (A) Photograph of the spots under UV transillumination. (B) Scanning of the spots with the modified 615™ Immunoanalyzer (200 measurement points). (C) Scanning of positions C1-C4 of (B) to improve resolution. (D) Double logarithmic plot of fluorescence vs. amount of biotinylated DNA spotted. Biotinylated DNA spotted (pg/1 µL) was (1) 50; (2) 100; (3) 200; (4) 400; (5) 800; (6) 1600; (8) 3200. Reprinted from [29] by permission.

target DNA and then hybridized it with two probes in solution. One was biotinylated and the other was labeled with Eu^{3+} . The sandwich hybrids were then collected onto streptavidin-coated microtiter wells and bound Eu^{3+} quantitated by extraction in enhancement solution (Fig. 7). The probe used was synthesized to contain a 5'-tail of 35–40 diaminohexane-modified deoxycytidines which were subsequently reacted with an isothiocyanate-derivative of a Eu^{3+} chelator saturated with Eu^{3+} . This assay was able to quantify reliably down to about five molecules of HIV-1 DNA and linearity was up to 500 copies.

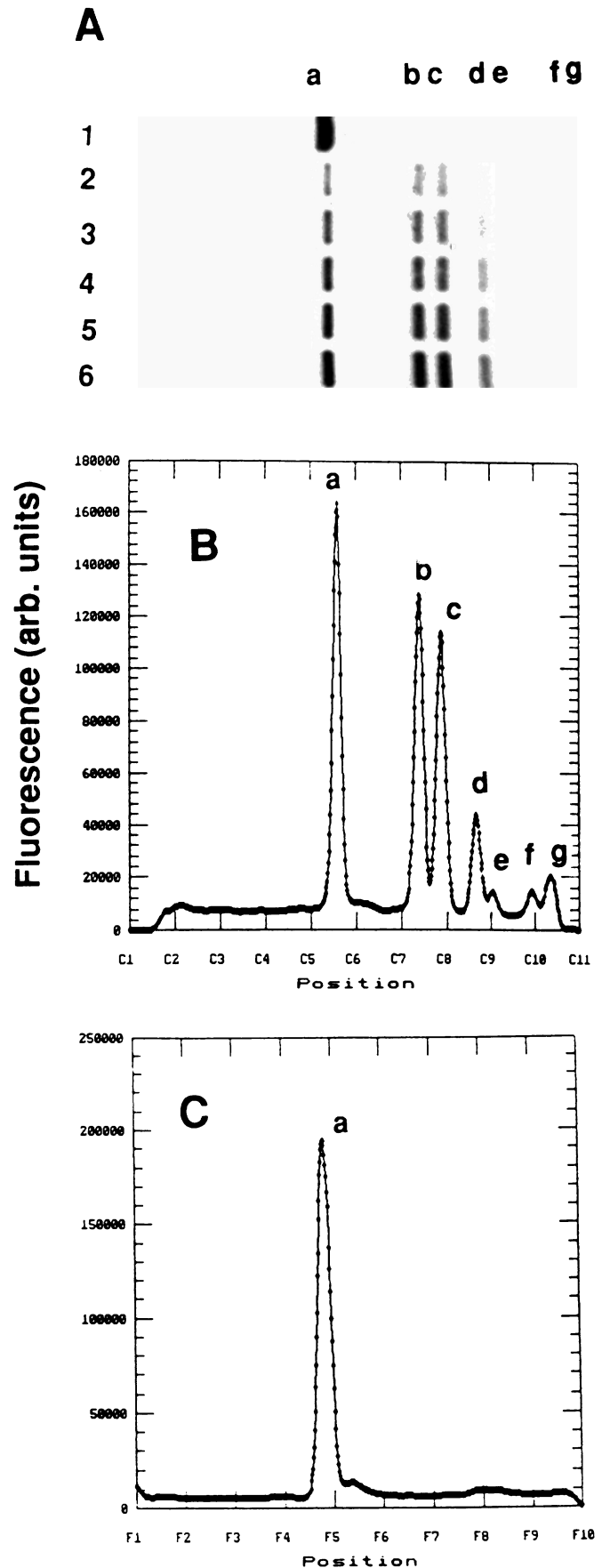
Alternative strategies involve PCR product detection without hybridization. For this, one primer can carry Eu^{3+} and the other biotin. After affinity collection with a streptavidin matrix, Eu^{3+} quantification is performed as shown in Fig. 5 [22]. Bush *et al.* [27] used the transcription-based amplification system (TAS) and the self-sustained sequence replication reaction (3SR) to amplify rRNA from *Escherichia coli*. The amplified RNA was detected by hybridization with a probe labeled with an unspecified rare-earth chelate and a capture polystyrene bead. The hybrids were separated and washed. Then, the beads were concentrated and their fluorescence was quantified. This method was able to detect one bacterial cell or 100 copies of rRNA.

3.5 Enzymatically amplified time-resolved fluorescence

Enzyme substrates have been synthesized for alkaline phosphatase, xanthine oxidase, β -galactosidase and glucose oxidase which have the property of forming fluorescent complexes with lanthanides, only after the enzymatic conversion to products [12]. Among these substrates, 5-fluorosalicyl phosphate (FSAP), a substrate of ALP proved to be the most useful as a detection system. The measurement principle is shown in Fig. 10. 5-Fluorosalicyl phosphate is split by ALP and the released 5-fluorosalicylate forms highly fluorescent complexes at high pH, with Tb^{3+} and EDTA. Other derivatives of 5-FSAP that were also synthesized have the ability to precipitate on filters, thus showing promise as detection reagents for Southern, Northern and Western blotting. One such reagent is 5-tert-octylsalicyl phosphate (TOSAP) [28].

The above method can quantify approximately 0.6 amol of ALP and was used for developing highly sensitive immunoassays [13, 14]. The method has been used for DNA hybridization experiments in microtiter wells. The detection limit, with pBR322 as target and probe (biotinylated) was approximately 3 pg. Using TOSAP, similar detection limits were obtained in dot-blot experiments with pBR322 as target and probe. In both cases, ALP was linked to the probe with a streptavidin-ALP conjugate.

Figure 13. (A) Hybridization experiments after Southern blotting of various amounts of enzyme-digested (lanes 2–6) or undigested (lane 1) linearized plamid pBR328 from 0.8% agarose gels. The probe was biotinylated linearized pBR328 plamid. The total amount of DNA (in ng) in lanes, was (1) 160; (2) 5; (3) 10; (4) 20; (5) 40; (6) 80. The length of each fragment is (in base pairs) (a) 4907; (b) 2176; (c) 1766; (d) 1230; (e) 1033; (f) 653; (g) 517. (B) Time-resolved fluorometric scanning of lane 6 (700 points), indicating the seven bands. (C) as in (B) but for lane 1. Reprinted from [29] by permission.



Southern blot experiments were also successfully performed with this method (28). The fluorescent bands could be examined by eye under UV irradiation or photographed with a specially designed time-resolved fluorometric camera. In principle, this method can find applications in any blotting protocol because the final product is fluorescent and immobilized on membranes. Biotinylated or digoxigenin-labeled DNA could be used in combination with any protocol involving solution or membrane hybridization. However, no applications have been reported except from those mentioned above.

3.6 Methodologies based on BCPDA

The streptavidin-based macromolecular complex (SBMC) described in Section 3.2 (Fig. 3) can be applied to solution-based and membrane-based techniques. For example, biotinylated DNA could be detected on spots. By using a scanning time-resolved fluorometer (Fig. 11) detection limits of approximately 10 pg were achieved [29]. The results were quantitative (Fig. 12). Successful Southern blots were also performed (shown in Fig. 13).

The obtained bands were sharp and could be seen under UV light as well as photographed or scanned.

Recently, Western blot analysis and spot immunodetection was also reported using this reagent [30]. Five pg or more of target protein could be detected with this method (Fig. 14). Because of the use of the scanning time-resolved fluorometer for measurement, the results were quantitative and reproducible. A representative Western blot analysis of mouse IgG with this reagent is shown in Fig. 15. Clearly, the method has potential for other applications involving blotting or filter hybridizations.

Recently, we were able to prepare PCR primers, labeled with BCPDA and amplify cytomegalovirus DNA with them [31]. The PCR product, labeled with BCPDA, was quantified in agarose gels soaked in a Eu^{3+} solution, using scanning time-resolved fluorometry. In addition, the same PCR products could be blotted to nitrocellulose and quantified by scanning the membrane or visualized by using anti-BCPDA antibodies coupled to alkaline phosphatase.

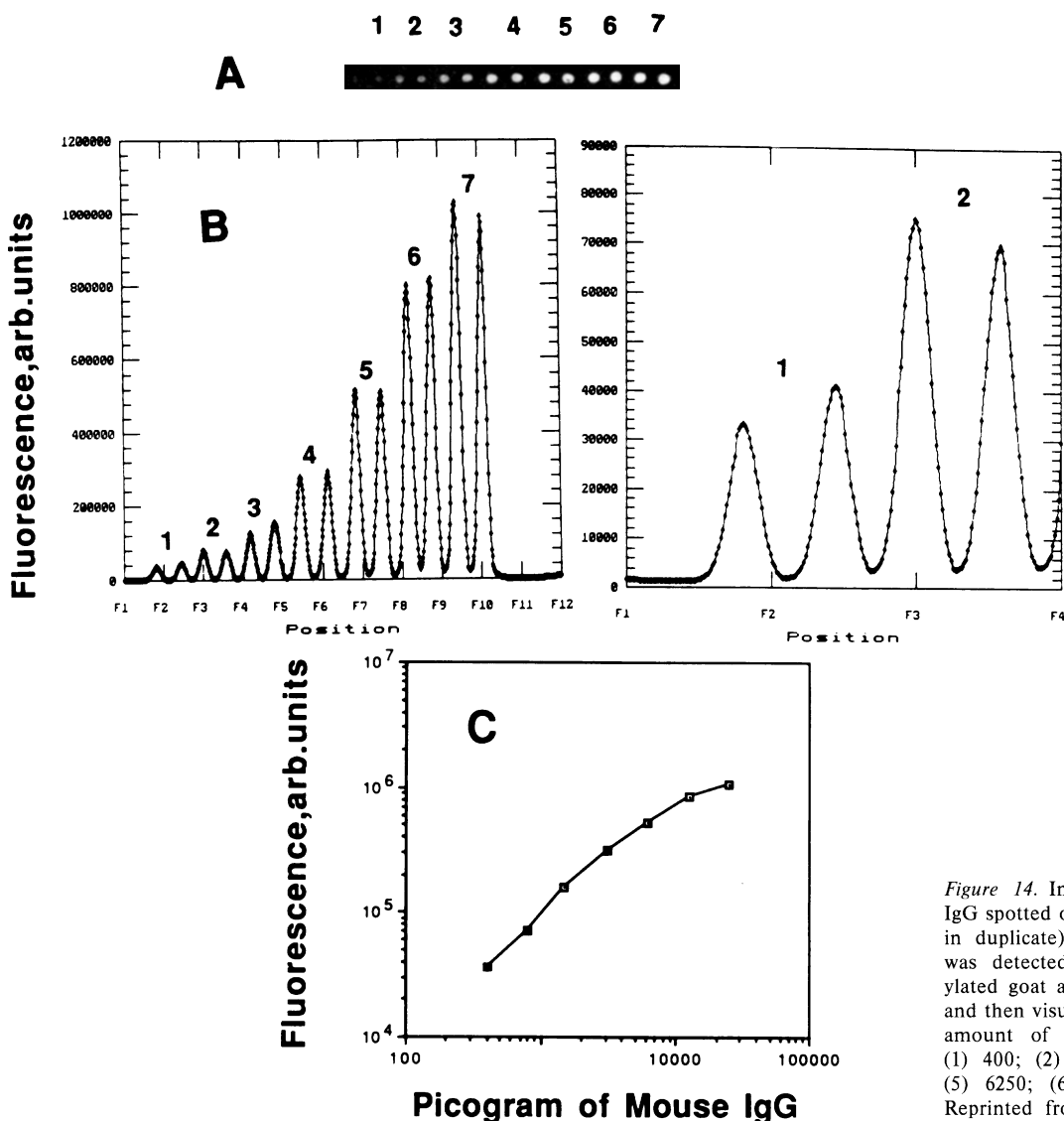


Figure 14. Immunodetection of mouse IgG spotted on nitrocellulose (1 μL /spot, in duplicate). Immobilized mouse IgG was detected by reaction with biotinylated goat anti-mouse immunoglobulin and then visualized with the SBMC. The amount of spotted protein (pg) was (1) 400; (2) 800; (3) 1500; (4) 3100; (5) 6250; (6) 12500; and (7) 25000. Reprinted from [30] by permission.

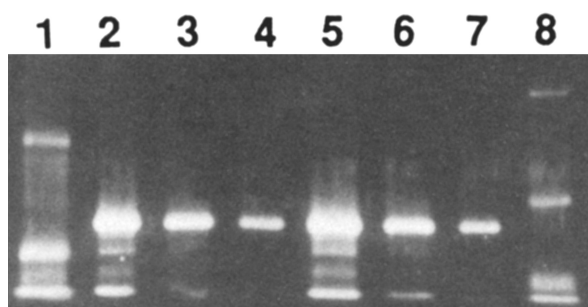


Figure 15. Western blot analysis of mouse IgG ($M_r \sim 160\,000$). Lanes (1) and (8) contain 1 μg of biotinylated M_r markers from Pierce and Sigma, respectively. Lanes (2), (3) and (4) contain 1000, 250 and 60 ng of mouse IgG, respectively. Lanes (5), (6) and (7) are duplicates of lanes (2), (3) and (4) respectively. Reprinted from [30] by permission.

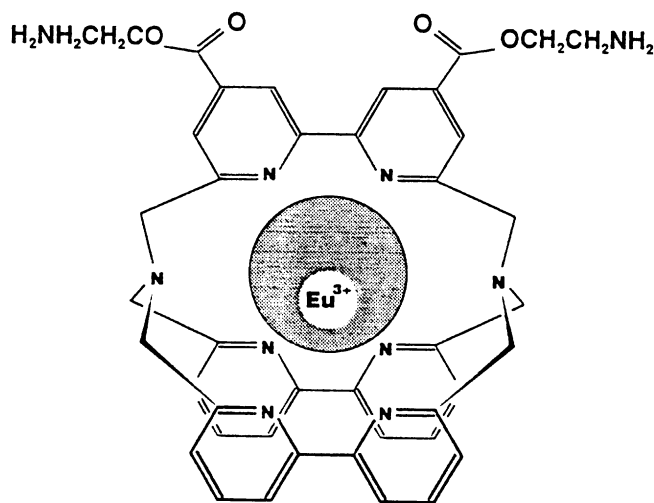


Figure 16. Molecular structure of europium (III) trisbipyridine cryptate. Reprinted from [33] by permission.

3.7 Streptavidin labeled with a europium cryptate

Prat *et al.* [32] have developed a streptavidin-based reagent labeled with a europium trisbipyridine cryptate. This fluorescent reagent (Fig. 16) is theoretically useful for any type of membrane-based application but its usefulness was demonstrated only with dot-blot assays. When biotinylated pBR322 plasmid was used as probe, to hybridize with immobilized pBR322 on nitrocellulose filters, the detection limit achieved was approximately 5 pg (2 amol). When M13 DNA was used in a similar experiment, the detection limit was 500 pg (200 amol). In both cases the signal was monitored with a home-made, time-resolved fluorometer. This system has potential for Southern, Western and Northern blot analysis and has the advantage of quantification. More recently, this fluo-

rescent label was used for the detection of polymerase chain reaction products fixed on solid supports [33].

4 Conclusions

Time-resolved immunofluorometry is an established nonisotopic immunoassay technique which is now frequently used for nucleic acid hybridization and Western blotting. Clearly, there is a need to develop new lanthanide chelates which are fluorescent by themselves and could be quantified without any need for metal-ion extraction. When such chelates become available, time-resolved fluorescence-based assays using Southern, Western and Northern blotting as well as colony and plaque lifts, *in situ* hybridizations, immunohistochemical assays, flow-cytometric assays and DNA sequencing assays may become commercially available.

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5 References

- [1] Diamandis, E. P., *Clin. Chim. Acta* 1990, 194, 19–50.
- [2] Diamandis, E. P., *Clin. Biochem.* 1990, 23, 427–443.
- [3] Diamandis, E. P., *Clin. Biochem.* 1988, 21, 139–150.
- [4] Diamandis, E. P. and Christopoulos, T. K., *Anal. Chem.* 1992, 62, 1149A–1157A.
- [5] Diamandis, E. P., *Clin. Chem.* 1991, 37, 1486–1491.
- [6] Landegren, U., Kaiser, R., Caskey, C. T. and Hood, L., *Science* 1988, 242, 229–237.
- [7] Evangelista, R. A., Pollak, A., Allore, B., Templeton, E. F., Morton, R. C. and Diamandis, E. P., *Clin. Biochem.* 1988, 21, 173–178.
- [8] Diamandis, E. P. and Morton, R. C., *J. Immunol. Methods* 1988, 112, 43–52.
- [9] Diamandis, E. P., Morton, R. C., Reichstein, E. and Khosravi, M. J., *Anal. Chem.* 1989, 61, 48–53.
- [10] Morton, R. C. and Diamandis, E. P., *Anal. Chem.* 1990, 62, 1841–1845.
- [11] Christopoulos, T. K., Lianidou, E. S. and Diamandis, E. P., *Clin. Chem.* 1990, 36, 1497–1502.
- [12] Evangelista, R. A., Pollak, A. and Gudgin-Templeton, E. F., *Anal. Biochem.* 1991, 197, 213–224.
- [13] Christopoulos, T. K. and Diamandis, E. P., *Anal. Chem.* 1992, 64, 342–346.
- [14] Papanastasiou-Diamandi, A., Christopoulos, T. K. and Diamandis, E. P., *Clin. Chem.* 1992, 38, 545–548.
- [15] Lovgren, T. and Pettersson, K., in: Van Dyke, K. and Van Dyke, R. (Eds.), *Luminescence Immunoassay and Molecular Applications*. CRC Press, Boca Raton 1990, pp. 233–250.
- [16] Hemmila, I., Dakubu, S., Mikkala, V.-M., Siitari, H. and Lovgren, T., *Anal. Biochem.* 1984, 137, 335–343.
- [17] Syvanen, A.-C., Tchen, P., Ranki, M. and Soderlund, H., *Nucleic Acids Res.* 1986, 14, 1017–1028.
- [18] Dahlen, P., *Anal. Biochem.* 1987, 164, 78–83.
- [19] Dahlen, P., Syvanen, A.-C. and Hurskainen, P., *Mol. Cell. Probes* 1987, 1, 159–168.
- [20] Viscidi, R. P., Connelly, C. J. and Yolken, R. H., *J. Clin. Microbiol.* 1986, 23, 311–317.
- [21] Hurskainen, P., Dahlen, P., Ylikoski, J., Kwiatkowski, M., Siitari, H. and Lovgren, T., *Nucleic Acids Res.* 1991, 19, 1057–1061.
- [22] Lovgren, T., Hurskainen, P. and Dahlen, P., in: Kricka, L. J. (Ed.), *Nonisotopic DNA Probe Techniques*, Academic Press, New York 1992, pp. 227–262.
- [23] Oser, A., Roth, W. K. and Valet, G., *Nucleic Acids Res.* 1988, 16, 1181–1196.
- [24] Oser, A., Collasius, M. and Valet, G., *Anal. Biochem.* 1990, 191, 295–301.

- [25] Sund, C., Ylikoski, J., Hurskainen, P. and Kwiatkowski, M., *Nucleosides Nucleotides* 1988, 7, 655–659.
- [26] Dahlen, P., Iitia, A. J., Skagins, G., Frostell, A., Nunn, M. F. and Kwiatkowski, M., *J. Clin. Microbiol.* 1991, 29, 798–804.
- [27] Bush, C. E., Van den Brink, K. M., Sherman, D. G., Peterson, R. W., Beninsing, L. A. and Godsey, J. H., *Mol. Cell. Probes* 1991, 5, 467–472.
- [28] Gudgin-Templeton, E. F., Wong, H. E., Evangelista, R. A., Granger, T. and Pollak, A., *Clin. Chem.* 1991, 37, 1506–1512.
- [29] Christopoulos, T. K., Diamandis, E. P. and Wilson, G., *Nucleic Acids Res.* 1991, 19, 6015–6019.
- [30] Diamandis, E. P., Christopoulos, T. K. and Bean, C. C., *J. Immunol. Methods* 1992, 147, 251–259.
- [31] Chan, A. W., Diamandis, E. P. and Krajden, M., *Anal. Chem.* 1993, 65, 159–163.
- [32] Prat, O., Lopez, E. and Mathis, G., *Anal. Biochem.* 1991, 195, 283–289.
- [33] Lopez, E., Chypre, C., Alpha, B. and Mathis, G., *Clin. Chem.* 1993, 39, 196–201.