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Analytical methodology for immunoassays and DNA hybridization assays – Current status and selected systems * – Critical Review

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Summary

Immunoassay is an established technique which has contributed enormously to biomedical analysis. DNA hybridization (DNA probing) methodology is emerging as the most promising new discipline of laboratory medicine with potential applications in areas such as genetics, pathology, microbiology and oncology. In this review, immunoassay and DNA probing methodologies are considered together because of their many similarities in assay design and labeling systems. Selected labeling systems are described in this paper in order to stress strategies, general principles and future trends. Special attention has been given to systems that introduce linear or exponential amplification. In the author's view, such systems will dominate in future applications. It is anticipated that during the next decade, immunoassay and DNA probing assays will be carried out on completely automated systems.

Contents

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1. Introduction

Analytical methodologies usually rely on the reaction between the analyte of interest and a specific reagent. The analyte concentration can then be determined by measuring either the concentration of the analyte–reagent complex (product) or the amount of residual reagent. In the immunoassay technique, the specific reagent used is an antibody. In DNA probing *, the specific reagent is a complementary nucleotide sequence. These reagents are characterized by two important advantages over conventional analytical reagents, exceptional specificity and very high affinity for the analyte of interest. These characteristics are mandatory for devising sensitive and specific assays.

There is a large variety of analytical designs for immunoassays and DNA probing assays. The labeling–detection systems that are used in these two areas are similar and include radioactive nuclides, fluorescent or chemiluminescent labels, enzymes or combinations of the above. In this review, I will describe a number of analytical detection methodologies that are used for immunoassay and DNA probing. It is anticipated that DNA probing will follow the growth pattern of immunoassay in the last 30 years, with a major impact on clinical biochemistry, microbiology, pathology, hematology and genetics [1–3]. Many believe that DNA-based testing will become

* Although the term DNA probing is used throughout this review, the methods described are equally applicable to RNA probes.

an important independent discipline in the area of laboratory medicine, with its own cadre of trained professionals and technologists [2].

2. Principles, current status and future applications of analytical methods based on immunoassays and DNA probes

2.1. Similarities and differences between immunoassay and DNA probing

These two techniques are suitable for the direct detection of targets of clinical interest. Targets for immunoassay, using polyclonal or monoclonal [4] antibodies include hormones, proteins, metabolites, drugs, tumor products, antigens and antibodies to infectious agents. Targets for DNA probing are sequences of nucleic acids complementary to a nucleic acid probe.

DNA probing is an emerging tool, but the techniques are slow and laborious in comparison to immunoassay [5]. However, a number of commercial products are already available and are discussed in a recent review [6].

It is important to realize the particular strengths of each of these two technologies. In Table I, a partial list of areas of applications of the two technologies is shown and predictions for future developments are made [7]. Antibodies recognize an epitope which consists of about 4–5 aminoacids, which may occur in closely related targets leading to cross-reactivity. This is sometimes desirable; for example a microbiological test may be designed to screen for all strains of a given organism. In DNA-based assays the target is a complementary DNA and the hybridizing probe can be synthesized to react with a unique sequence. Thus, the specificity can be more easily manipulated according to the assay demands.

Current immunoassays detect down to 10^{-13} mol/l of analyte and this is sufficient for many applications involving drugs, metabolites or gene products. An individual cell can produce great amounts of these products constituting an intrinsic amplification of the DNA content. In the area of infectious disease, it is sometimes easier to measure gene products of the infectious agent (e.g. hepatitis B surface antigen) because they are produced in great excess. Alternatively, antibodies against

TABLE I

Applications of immunoassay and DNA probing [a]

Immunoassay	Immunoassay and DNA probing	DNA probing [b]
Endocrinology	Infectious diseases	Genetic disease:
Therapeutic drug monitoring	Cancer	Prenatal diagnosis, carrier detection, susceptibility
Cell typing	Food testing	Latent viral infections
	Plant diagnostics	
	Veterinary diagnostics	
	Forensics	

[a] Modified from ref. [7].

[b] In the areas listed, DNA-probing is likely to establish clear dominance over immunoassay but this does not mean that immunoassay or other testing will not be used at all.

infectious agents can be measured (e.g. hepatitis B surface antibody) because they are also produced in great excess by the host. Exceptional sensitivity is usually needed to directly detect specific genomic sequences of the infectious agent. A useful assay may need sensitivities which are in the order of 10 000–100 000 of target molecules per assay. To solve this sensitivity problem, it is usually necessary to apply a pre-enrichment step (culture), an in-vitro amplification step (polymerase chain reaction) or measure targets that are present in thousands of copies in the infectious agent (e.g. ribosomal or messenger RNA).

It is likely that immunoassay and DNA probing will complement each other. Methods of DNA probing in diagnostic microbiology, pathology and genetics have recently been reviewed [8–14]. DNA probing assays may have important advantages over immunoassay in specific microbiological diagnostic problems such as detection of viroids, which lack any protein component and the detection of certain viruses e.g. cytomegalovirus (CMV) when propagation procedures are very slow and tedious or the Epstein–Barr virus and some papillomaviruses which propagate with difficulty or not all in vitro. In these instances, viral DNA amplification by the polymerase chain reaction (PCR) and DNA probing is a practical tool. Probes can be used to detect group-specific and type-specific viruses as with enteroviruses. An important application of DNA probing is identification of specific gene sequences in bacteria which are linked to pathogenicity. Once the hybridization assays become automated [8], they can be applied to mass screening of samples (e.g. in blood banking) not only for viral and bacterial nucleic acids but also for parasites [5].

2.2. Major labeling systems

Numerous labeling-detection systems have been proposed over the last 30 years (Table II). Commercial systems are available in various formats for both immunoassay and DNA probing. Radioactive labels are gradually being replaced from immunoassay as non-radioactive systems reach or even surpass the detectability of

TABLE II

Major labeling systems

	Examples
1. Radioactive nuclides	^{125}I, ^{32}P, ^{35}S, ^{3}H
2. Fluorescent labels	Fluorescein, Rhodamines, Phycobiliproteins, Rare-earth chelates, Ethidium
3. Luminescent labels	Luminol derivatives, Acridinium esters, Dioxetane derivatives, Bacterial or firefly luciferace
4. Enzymes	Alkaline phosphatase (ALP), Horseradish peroxidase (HRP), beta-Galactosidase
5. Combinations	Enzymes with substrates liberating fluorescent, luminescent or radioactive products, see text.

TABLE III

Possible future applications of ultrasensitive assays

	Examples-comments
1. Measurement of certain analytes at sub-normal concentration ranges	Adrenocorticotropic (ACTH) and antidiuretic (ADH) hormone
2. Assay of more analytes in serum	Hypothalamic releasing hormones, growth factors
3. Discovery of new analytes	New markers of human disease
4. Detection of minute amounts of tumor related products	Early diagnosis and follow-up for relapse after surgery
5. Enumeration of receptor numbers in single cells	Better understanding of receptor-mediated events
6. Assay of nucleic acid sequences, antigens, or antibodies to infectious agents	Human immunodeficiency virus type 1 (HIV-1), Cytomegalovirus, Epstein–Barr virus. Early diagnosis of infected asymptomatic individuals

radionuclides. In DNA probing, radionuclides are still dominant because alternative systems are still less sensitive.

It is now clear that the most sensitive detection systems must rely on amplified designs. Amplification can be chemical, enzymatic-linear or enzymatic-exponential. Systems based on such designs will be described later in this review.

2.3. Usefulness of future more sensitive assay systems

The current practical sensitivity of immunoassay lies in the 10^{-13}–10^{-14} mol/l concentration range which implies that with a typical sample volume of 100 μl, about 10^{-17}–10^{-18} mol of substance ($\sim 6 \times 10^6$–6×10^5 molecules) could be measured. Such sensitivity is adequate for many clinical applications, e.g. in endocrinology, because many hormones or other analytes can be measured directly and without the need for preconcentration. Some possible applications of ultrasensitive assays is shown in Table III. In DNA probing, the ultimate sensitivity would be the detection of a single nucleotide sequence with or without the need of previous amplification. When this is achieved, interpretation of results, in selected cases, may be more confusing than useful because of the chance of finding minute amounts of, for instance, infectious agent sequences in specimens from asymptomatic individuals. Questions then may arise of the importance of the findings and the possible action to be taken. Nonetheless, ultrasensitive methodologies are likely to enrich current knowledge and create novel applications in the field of biotechnology and clinical medicine.

2.4. Current status of immunoassay and future projections

Hundreds of analytes can be measured by immunoassay techniques. Since the original concept [15] developments have included homogeneous immunoassays [16],

'two-site' non-competitive assays [17], novel solid-phases for the easy separation of bound and free label [18], monoclonal antibodies [4] and replacement of ^{125}I with alternative labels [19]. Non-isotopic immunoassays based on fluorescent, chemiluminescent or enzyme labels are commercially available. Future developments in immunoassay will focus on fully automated systems having random access capability and throughputs similar to the well-established clinical chemistry analyzers, enhanced sensitivity, simple assays independent on sample volume (i.e. immunodipstics) [20], multi-analyte assay devices [21] and immunosensors suitable for real time monitoring.

2.5. Current status of DNA probing and future projections

The principles of nucleotide probing methodology rely on the ability of labeled DNA and RNA fragments (probes) to detect, by binding, complementary arrangements of nucleotides (targets). Because such binding can be, under appropriate conditions, extremely strong and specific, the resulting assays can demonstrate excellent specificity and sensitivity. A unique property of nucleic acids is their ability to multiply exponentially by enzymatic catalysis. After amplification by the polymerase chain reaction, the starting material is available in great abundance for further testing.

Although a relatively new technique, DNA probing has already been used in many research applications and more recently for routine testing. It is anticipated that DNA diagnostic laboratories will disseminate quickly during the next decade and become an important part of hospital routine laboratory testing. Clearly, DNA probing is emerging as the most promising new area in laboratory medicine and its impact will be similar to that of immunodiagnostics. The technique is routinely being used in detection of genetic disease [22,27–31] in infectious diseases in humans, animals, food and the environment [9–14], in forensic sciences [23] and in oncology [24–26].

Major efforts are now being concentrated on non-isotopic detection systems because of the cost, short half-life and health hazards associated with the use of ^{32}P and other radionuclides. The non-isotopic detection technologies that are now used in the field of immunoassay (Table II) are also applicable to DNA probing.

2.6. Immunoassay designs

The vast majority of heterogeneous immunoassays, are now performed with three general assay designs (Fig. 1) [32,33]. 'Competitive type' assays are performed with either the immobilized antibody or the immobilized antigen approach. Non-competitive type assays are performed by using the 'two-site' or 'sandwich' principle. Whenever possible, the 'sandwich' type assay is preferred. Only when this principle cannot be applied i.e. when the molecule is of low molecular weight and cannot react simultaneously with two different antibodies, is the competitive assay performed. The theoretical and practical sensitivities obtained with these principles will be considered later.

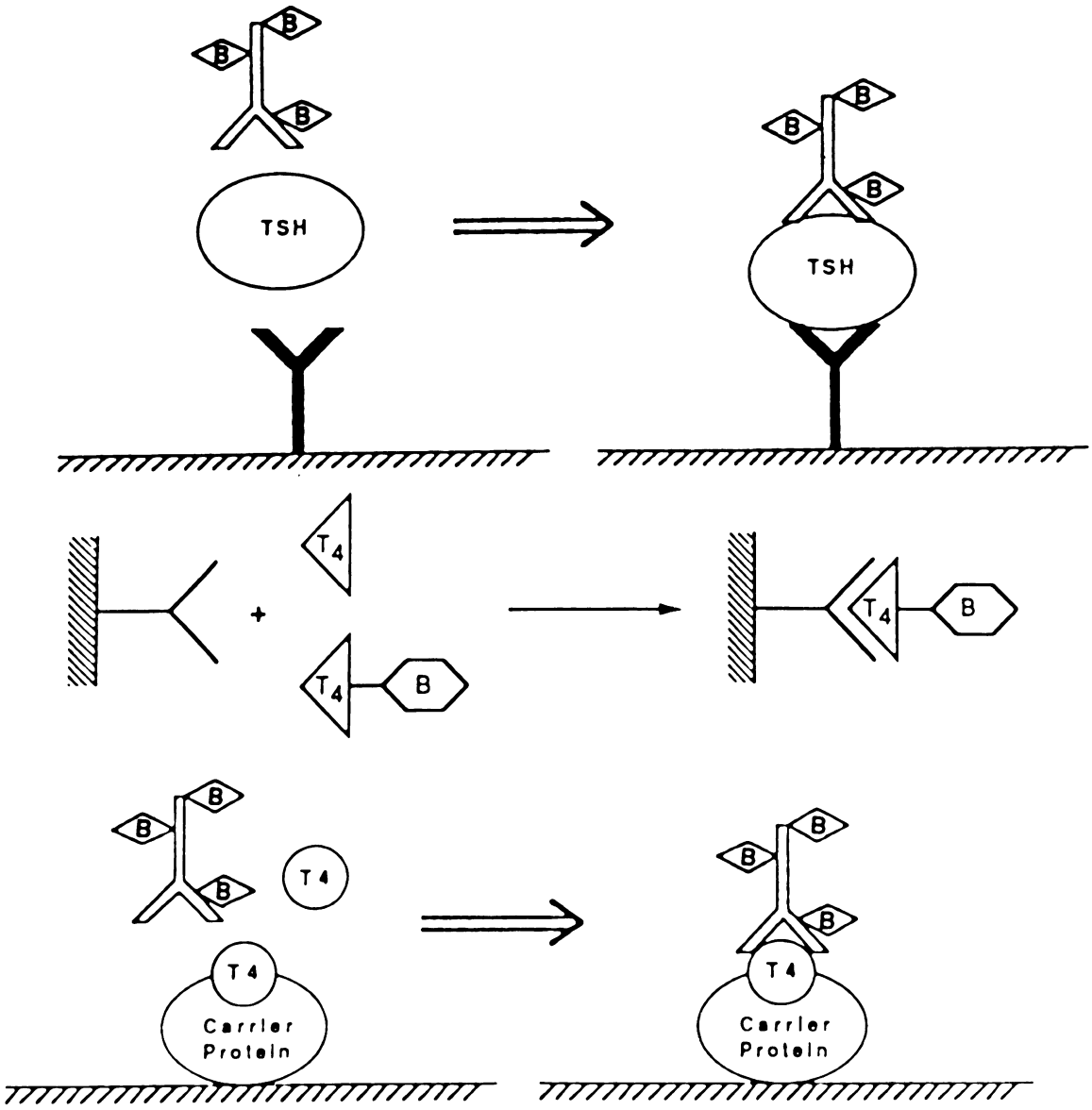

Fig. 1. Heterogeneous immunoassay designs. Upper panel: A 'two-site' non-competitive sandwich methodology for thyroid stimulating hormone (TSH) using two antibodies. One of the antibodies is immobilized on the solid-phase (capture antibody) and the other is labeled (detection antibody). The label is represented by B. In indirect systems, B may be biotin. Excess reagent is removed by washing. Middle panel: Competitive immunoassay using the immobilized antibody approach. B denotes the label that is linked to the antigen, in this case thyroxine (T4). Lower panel: Competitive immunoassay for T4 using the immobilized antigen approach. Solid-phase antigen is a T4-protein conjugate.

In homogeneous immunoassay, 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. Although homogeneous immunoassays are simple to perform and usually completely automated, they suffer from the drawback of not being highly sensitive; the maximum attainable sensitivity not usually exceeding 10^{-9} mol/l. Homogeneous immunoassays are mostly of the 'competitive' type.

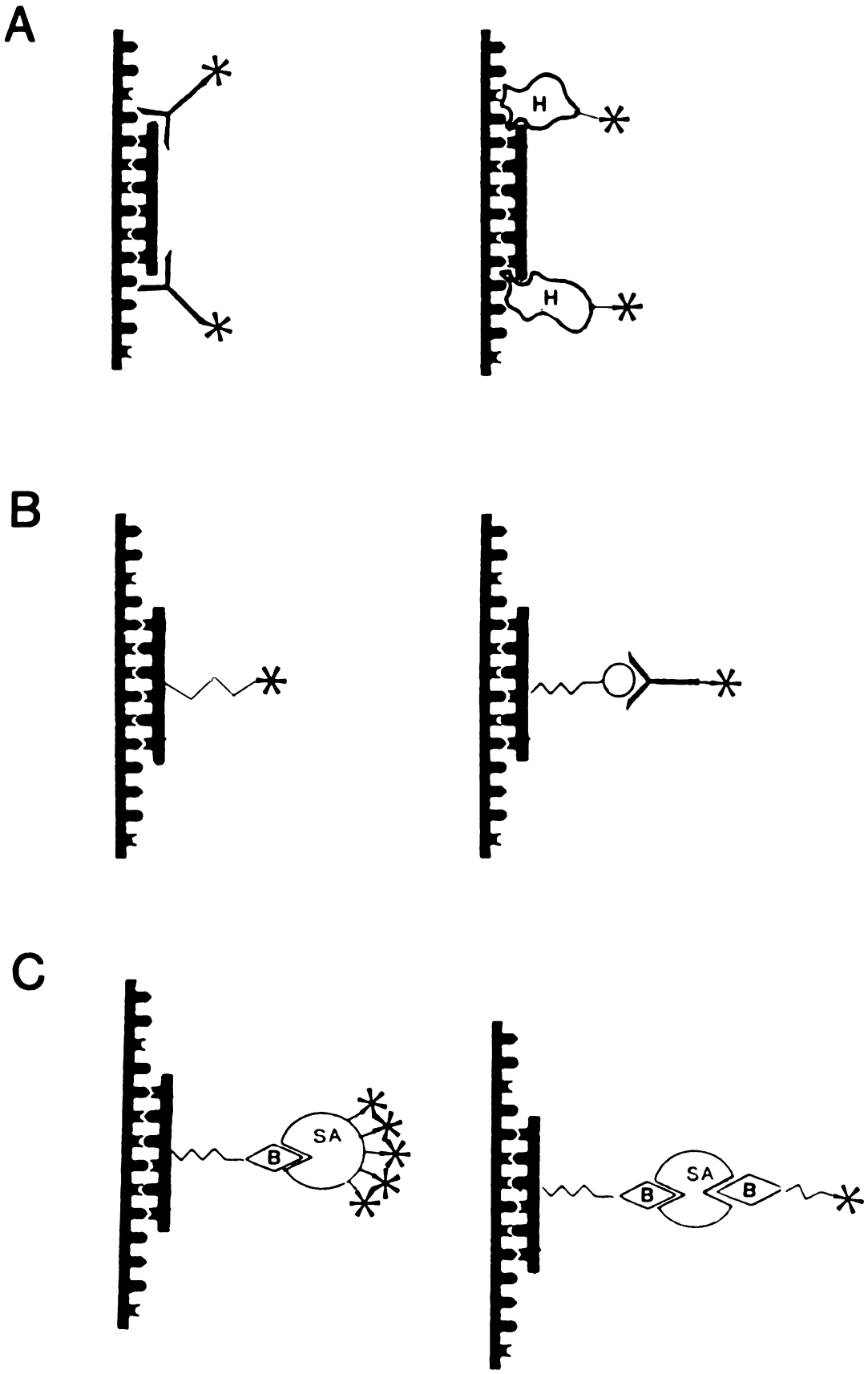

Fig. 2. See figure legend on facing page.

2.7. DNA probing assay designs

Methods of preparing DNA probes are described elsewhere [6,34–36]. These probes can then be labeled and used for hybridization. Two main labeling strategies are used, either direct labeling, with the label covalently attached to DNA; or indirect labeling with a hapten covalently bound to DNA in order to link the probe with a detection system carried by either an antibody or a binding protein specific for the hapten. A binding protein with specificity to double stranded DNA can also be used as the carrier of the label (Fig. 2).

For direct labeling, the most widely used labels are ^{32}P, ^{35}S, ^{3}H and ^{125}I, the fluorescent compounds fluorescein, tetramethylrhodamine and ethidium and the enzymes alkaline phosphatase, horseradish peroxidase and bacterial or firefly luciferase. For indirect labeling, antibodies to DNA:DNA or DNA:RNA are used and second antibodies can be applied carrying a suitable reporter [37,38]. Alternatively, histones or other proteins carrying a reporter can be used [39–42]. In one of the most widely used procedures for indirect labeling, the vitamin biotin is covalently attached to the probe. Avidin or streptavidin carrying enzymes, fluorescent probes, or other labels are then used to link the probe to the reporter system [43–59]. Alternatively, biotin antibodies are used carrying either the reporter or other intermediate bridge systems (e.g. second antibody, protein A etc) to complete a detectable complex [60]. Other haptens have been used instead of biotin with anti-hapten antibodies carrying reporter molecules. Some of these haptens include dinitrophenyl [61], ethidium [62], *N*-2-acetylaminofluorene [63–65], *N*-2-acetylamino-7-iodofluorene, sulfone [66], 5-bromodeoxyuridine [67] and digoxigenin [68]. For more details see a recent review [69].

The solid-phases used in DNA-based assays are similar to those used in immunoassay and include nitrocellulose, polypropylene, polystyrene (microtiter plates), cellulose [70], latex particles [71], magnetic spheres [72–74] and nylon [75]. The 'sandwich' type heterogeneous immunoassays (Fig. 1) are very similar to the 'sandwich' DNA hybridization assay as described in [76]. The competitive-type heterogeneous immunoassays are very similar to the strand DNA probe displacement assays [77,78]. The homogeneous DNA hybridization assays described in [79]

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Fig. 2. The most widely used assay designs for DNA probing. A: After hybridization of the target sequence (usually immobilized on a solid-matrix) with the probe, antibodies against DNA:DNA or DNA:RNA duplexes are added. These antibodies can carry a reporter molecule (RM, ——*) or the RM can be carried by a second antibody (not shown). Instead of antibody, a histone (H) which carries a RM can also be applied. In these and the assays described below, excess probe and excess reagents that are added are washed out under carefully controlled conditions. The signal generated by the reporter can either be visualized on films or measured by appropriate instrumentation. B: The specific probe is directly labeled with the RM. A great variety of RM can be used as described in the text. The probe may also carry a low molecular weight substance (hapten, ——○, right panel) or biotin, B (C, left panel) which is then reacting with a specific antibody or a binding protein (e.g. streptavidin, SA) carrying RM. Alternatively, (C, right panel) streptavidin can be used unlabeled and biotinylated RM are added which link to the hybridized complex as shown.

(energy transfer principle) are similar to the one used for immunoassay many years ago [80]. The CEDIA(™) homogeneous immunoassay system [81] has also been used for DNA probing with appropriate modifications [82]. Also, the enzyme-channeling principle has been applied successfully for DNA probing [83]. Because of its wide utilization in immunoassay and DNA probing, the biotin–streptavidin system will be described briefly under a separate heading.

3. Amplification strategies and background signal minimization

3.1. The biotin-streptavidin system

Among the known non-covalent molecular interactions, the avidin or streptavidin–biotin interaction is unique because of its specificity and exceptional affinity. The affinity constant of the biotin–avidin interaction is around 10^{15} l/mol and is among the highest values reported; the affinity constants for antigen-antibody interactions is at best around 10^{11}–10^{12} l/mol. The avidin-biotin system can be used in the field of immunoassay and DNA probing in a number of different ways, based on the principle that biotin can be easily covalently linked to proteins, DNA or other molecules without altering their binding or other biological activity. Active biotin analogs suitable for conjugation (biotinylation) having a variety of reactive groups are commercially available [84]. The conjugation chemistries involved are simple and efficient. In one variation of the possible assay designs, biotinylated antibodies or probes are used (Fig. 2) and avidin or streptavidin is the carrier of the reporter molecule. Alternatively, avidin or streptavidin is used unlabeled to link a biotinylated reactant (antibody or probe) with a biotinylated reporter molecule (Fig. 2). This latter design takes advantage of the property of avidin or streptavidin to bind more than one biotin molecule (theoretically up to four could be bound).

This versatile system has been used in many other different applications e.g. in immunohistochemistry, flow-cytometry, protein blotting, protein purification, nuclear medicine etc. [85–88]. The most serious limitation of this system is that streptavidin and avidin show higher non-specific binding than antibodies. Aside from this, a number of advantages are associated with the system (Table IV). Streptavidin has advantages over avidin, in that it is not a glycoprotein and it has an isoelectric point of 7 compared with 10 for avidin, both factors leading to reduced non-specific binding.

TABLE IV

Advantages of the biotin–(Strept)avidin system

1. Universal detection system
2. Antibodies labeled easily and without inactivation with biotin
3. Amplification
4. Streptavidin very stable, not usually inactivated upon labeling
5. Biotin-streptavidin interaction very strong

3.2. Other amplification schemes

On many occasions, the signal generated by a reporter is not enough to be measured and an amplification step may prove efficient in solving the problem. In general, there are at least, three ways of amplifying a system. a. *Chemical.* In this approach, the density of labels per detection reagent can be greatly increased by derivatizing the detection reagent with a tail carrying many reporters [89–91]. Alternatively, controlled polymerization of the reporter can be used and it has been employed successfully with ALP [92,93]. The biotin–streptavidin interaction also constitutes a versatile amplification system which was used in combination with the 'tail' approach. We have added a tail on streptavidin which could carry as many as 450 fluorescent europium chelates [91]. This amplification, in combination with the 10-fold amplification of the biotin moieties that can be conjugated to an antibody, could yield a total amplification factor of 10^3–10^4. b. *Enzymatic amplification* is extremely powerful and under optimized conditions, detectabilities as low as 600 molecules per assay have been reported [94]. The only problem is that the amplification is linear and in order to achieve high factors, the incubations need to be prolonged to sometimes impractical levels. c. *Exponential amplification.* Systems that offer either exponential amplification of analyte numbers before assay (polymerase chain reaction) [95–97] or exponential amplification of the signal after a binding reaction occurs (replicase system) [98–100] have been proposed. The advantage here is that in relatively short times, extremely high amplification factors can be achieved.

An interesting way of increasing the density of labels used in DNA probing has been recently reported by Ureda et al. [101]. They formed a controlled network of nucleic acid hybrids composed of the target fragment, several oligonucleotide probes, branched DNA amplifiers and alkaline phosphatase (ALP)-labeled oligonucleotides. The network could incorporate about 300 ALP molecules per target sequence. Approximately 50 000 molecules of double-stranded DNA could then be detected. Another amplification strategy applied mostly in immunoassay is based on the use of liposomes [102].

Although in theory, all these amplification schemes could reach the ultimate detectability, the major problem is that once a non-specific effect occurs (e.g. non-specific binding of labeled reagent), it also triggers the amplification loop thus leading to amplification of noise at the same time. It is clear that new methods for the removal of non-specific binding and better understanding of its nature are needed before the full potential of amplifications can be realized. A new method for background removal in DNA probing assays is discussed in some detail below.

3.3. Background signal rejection by reversible target capture

This technique was originally developed by Morrissey et al. [103] and works as follows [103–106]. A biological sample is first dissolved in a guanidinium isothiocyanate (GuSCN) solution which lyses cells, and dissolves macromolecular complexes. Nucleic acid probes are then added as described in the legend of Fig. 3 to

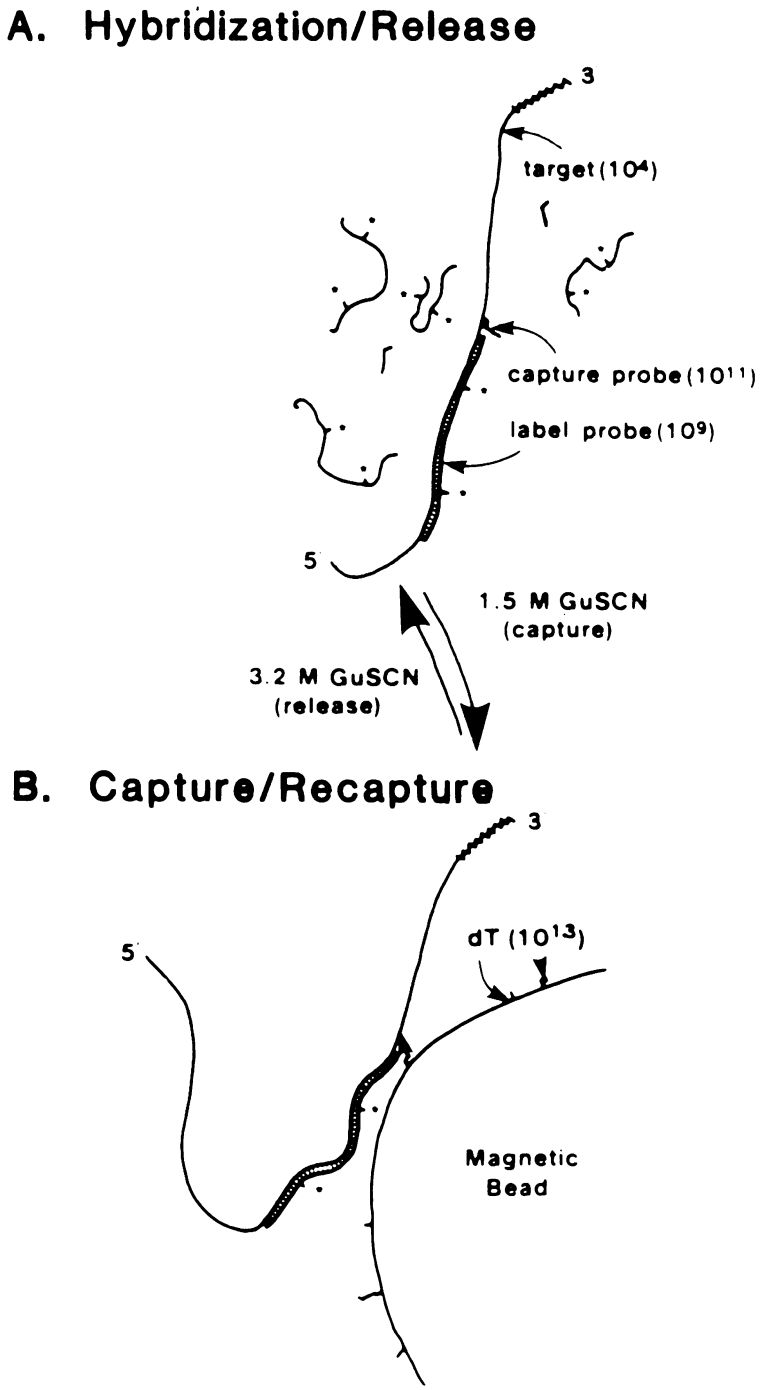

Fig. 3. Schematic of reversible target capture (RTC). Label probes and capture probes are mixed with RNA or DNA targets. Label probe-target hybrids are formed during hybridization for 17–24 h in 4 mol/l GuSCN at 37°C. Capture probes were then allowed to hybridize with the label probe-target complexes during a 10 min incubation in 1.5 mol/l GuSCN at room temperature (22°C). The ternary hybrid thus formed is shown in A. Beads coated with oligo(dT) are added and the hybrids captured during an incubation of 10 min at 37°C in 1.5 mol/l GuSCN through (dA):(dT) interactions (B). The bulk of unreacted label probe and cell debris is washed away and hybrids released from beads in 3.2 mol/l GuSCN. The solution is diluted again to 1.5 mol/l GuSCN, and hybrids recaptured on fresh beads. Numbers in parentheses indicate number of molecules of each nucleic acid species in the hybridization mixture. Reprinted by permission from Reference 106.

form a ternary complex between target, label probe and capture probe. Beads coated with oligo-deoxythimidine (dT) are then added to capture the ternary complex which is washed to remove unreacted probes and cell debris. The ternary

complex is then released from the beads and recaptured on new beads by adjusting the GuSCN concentration. Every cycle, which involves washing and fresh bead addition, achieves a 10^2–10^3-fold reduction of background noise coming from non-specifically bound label probe, with a recovery of specific signal of 50–90%. Three cycles are enough to drive the noise to undetectable levels. This method was used to measure as few as 4000–15000 target molecules [104,106]. It has general applicability and shows promise of solving some of the background signal problems which are associated with DNA probing.

4. Theoretical sensitivity and possibilities for detecting single analyte molecules

4.1. Theoretical sensitivity of immunoassay

There is a need for ultrasensitive immunoassay methodology which could be able to detect routinely a few hundreds of molecules per assay (Table III). To devise such techniques, the labels used to monitor the extend of the immunological reaction must be detected at extremely low concentrations. One of the major problems of radioisotopes is that their specific activity, defined as the number of detectable events per unit time per number of labeled molecules, is much lower than the specific activity of currently used non-isotopic labels. For example, in order to detect one disintegration of ^{125}I per sec (dps), one must have available 7.5×10^6 labeled molecules. For ^{3}H, which has longer half-life, 5.6×10^8 labeled molecules must be present in order to observe one dps.

In contrast, one detectable event can be generated shortly after triggering of a single chemiluminescent label. Fluorescent labels can be utilized even more efficiently because the excitation-emission cycle (generating 1 photon per molecule) can be repeated many times during a very short period (e.g. 10^8 times per sec when short-lived fluors are used). Enzymes are also attractive because, depending on their turnover number, they can generate many product molecules per enzyme molecule.

Ekins et al. [107,108] have analyzed the differences between competitive and non-competitive immunoassay designs and found that the non-competitive principles have potential for far better sensitivity. Competitive immunoassays are able to reach detection limits of 10^{-14} mol/l or about 10^{-18} mol of analyte ($\sim$ 600000 molecules) per 100 μl sample. These limits are affected mainly by antibody affinity and experimental errors rather than by the nature of the label. Non-competitive immunoassays, using ^{125}I also have a detection limit of about 10^{-14} mol/l. By contrast with competitive assays, non-competitive assays using fluorescent, chemiluminescent or enzyme labels have a potential sensitivity 10^3-fold lower allowing detection of 600 molecules per assay (100 μl sample). This limit has been reported in practice [94].

The limiting factor in sensitivity with non-competitive assays is background noise. Background signal is that observed when analyte is not present in the assay and may be due to signal arising from the label itself because of the non-specific adsorption of the labeled reagent used or from other sources, e.g. the instrument, the solid support (e.g. some solid-phases fluoresce), cuvettes or serum components. The

magnitude of the background signal, the precision of its measurement and the slope of the calibration curve are the factors determining sensitivity.

The conclusions mentioned above can be extended to the field of DNA probing. The problems arising from background signals due to non-specific binding of the labeled probes has been addressed repeatedly.

4.2. Detection of single analyte molecules

The ultimate analytical goal is to detect single analyte molecules [109–111]. The approaches taken to achieve this goal will be considered below. Radioisotopes are not suitable as labels for single molecule detection because of their low specific activity i.e. very low frequency of 'detectable events'. Two months are needed to record 50 disintegrations that will be released by 100 ^{125}I molecules. Similarly, other labels that do not produce measurable number of events within short periods of time are also not suitable. It appears that the most suitable labels for single molecule detection are the fluorescent compounds. It is possible to generate 10^8 photons per sec per fluorescein or phycoerythrin molecules by exciting them with high energy lasers of the appropriate frequency. This happens because a measuring cycle (excitation-emission) can be as short as 10 nsec and the fluorescence quantum yield of these fluors is close to unity. This photon flux can be measured relatively easily. However, during specific signal measurement, non-specific signal transduction (e.g. scattered light or signal originating from impurities in solvents) must be meticulously minimized. On the other hand, it has to be kept in mind that after prolonged excitation, many fluorescent molecules undergo photodestruction. Thus, by increasing the number of measuring cycles, the emitted light diminishes exponentially due to photodestruction [111]. An approach to solve this problem is to have a measuring flowing stream that regenerates continuously the molecules exposed to the excitation beam.

When these precautions were taken, three molecules of fluor were measured [111]. However, because of the regeneration, a more realistic claim would be about 26 000 fluorescent molecules detected per sec. Instead of using a flowing stream, it is also possible to measure the total number of photons emitted by a fluor before complete photodestruction. This is ~ 40 000 or 100 000 photons per molecule for fluorescein or phycoerythrin, respectively [111]. Thus, the signal originating from one molecule could be measured provided that the measuring efficiency is high enough and background signal from scattering is successfully rejected.

To detect a single or a few labeled molecules using a fluorometric system the following criteria must be met (a) the product of the emission rate of the label and the detection efficiency of the instrument must be greater than the dark count rate of the photomultiplier during the observation interval. In practice, this criterion is only met when the excited-state lifetime of the fluor is less than 10 μs. For this reason, long-lived fluorescence emitting labels (e.g. europium chelates) or chemiluminescent labels are not suitable for single molecule detection unless amplification is introduced by other means (e.g. enzymes); (b) the fluorophores used must be photostable otherwise the emission will diminish exponentially with time with loss

of signal and (c) the fluorescence emitted by the single molecule should be greater than the background fluorescence or the signal originating from scattered light.

In most efforts for measuring very low numbers of molecules by fluorometric detection, lasers are the excitation sources of choice because of their very high intensity and their ability to focus on very small volumes. It can be calculated that if a substance can be detected at levels as low as 10^{-12}–10^{-13} mol/l, then, use of extremely low volumes, in the order of 10^{-9}–10^{-12} l, is a way of measuring single analyte molecules. Many such approaches have been reported [112–115] and find application in flow cytometry and high performance liquid chromatography. However, for many other applications such small volumes are not useful. The usual sample volumes applied in the field of biochemical sciences today lie between 10^{-6}–10^{-3} l (1–1000 μl). Methods with potential detectabilities down to 10^{-16}–10^{-18} mol/l could be very useful because analyte molecule numbers as low as 600–60000 in 1 ml serum sample could then be measured.

Another way of measuring extremely low numbers of analytes in reasonable volume sizes is to apply a reaction between the analyte and a labeled specific reagent, e.g. an antibody or a DNA probe and then use a detection system which can amplify the label signal e.g. an enzyme substrate. Alternatively, the number of analytes of interest can first be amplified (nucleic acids are amenable to such manipulation) and then measured with a binding assay. Such principles will be outlined later in this review.

5. Selected highly sensitive detection systems for immunoassay and DNA probing

Below I will describe in some detail a number of detection systems that are currently used with success or they show promise for the future. Special emphasis is given to systems that offer exceptional sensitivity.

5.1. High-sensitivity enzyme-linked immunosorbent assay (HS-ELISA)

This detection technique was introduced in 1980 by Shalev et al. [116] and represents a successful combination of enzymes and fluorogenic substrates. In typical immunoassay designs, alkaline phosphatase acts as the primary label in heterogeneous formats. It catalyzes the conversion of the substrate 4-methylumbelliferyl phosphate (non-fluorescent) to 4-methylumbelliferone (highly fluorescent; excitation wavelength 365 nm, emission wavelength 455 nm). The reaction involved is shown in Fig. 4. The use of the fluorogenic substrate increased sensitivity over colorimetric detection by a factor of 16- to 39-fold. Further improvements in

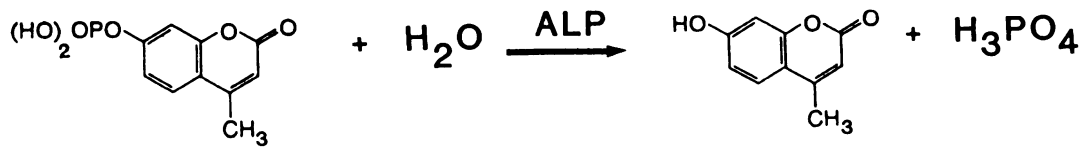

Fig. 4. 4-Methylumbelliferyl phosphate (4-MU-P) is hydrolysed by alkaline phosphatase to 4-methylumbelliferone (highly fluorescent) and inorganic phosphate.

maximizing the antigen binding surface area and prolonging the incubation times resulted in overall sensitivity improvements of the order of 10^5– to 10^6-fold. This HS-ELISA assay was able to measure down to 24000 molecules of analyte in a model system. The principles of HS-ELISA have been adapted to completely automated immunoassay systems with success. Such systems include the IMx® analyzer (Abbott Diagnostics, Abbott Park, IL, USA) and the Stratus® analyzer (Baxter Healthcare Corp., Miami FL, USA).

5.2. Ultrasensitive enzyme radioimmunoassay (USERIA)

This assay was introduced by Harris et al. in 1979 [94]. The basic idea was to use a radioactive enzyme substrate to take advantage of the enzymatic amplification. The enzyme is alkaline phosphatase and the substrate ^{3}H-adenosine monophosphate (^{3}H-AMP) which is split to ^{3}H-adenosine and inorganic phosphate. Because both ^{3}H-AMP and ^{3}H-adenosine are radioactive, a separation step is needed to remove the unreacted substrate. In the cholera toxin assay, the method could detect down to 600 molecules per cuvette which is close to the theoretical limit of 'non-competitive' type assays. Under the same conditions, a classical ELISA methodology was 10^4-fold less sensitive. USERIA, although extremely sensitive, has not found commercial application because it relies on radioactivity and needs a separation step.

5.3. Enzymatic cycling

This assay was originally proposed by Johannsson et al. [117]. In a typical 'two-site' immunoassay system, alkaline phosphatase (ALP) is used as the label (Fig. 5). The substrate nicotinamide adenine dinucleotide phosphate ($NADP^+$) is dephosphorylated by bound ALP to produce NAD^+. NAD^+ activates a secondary

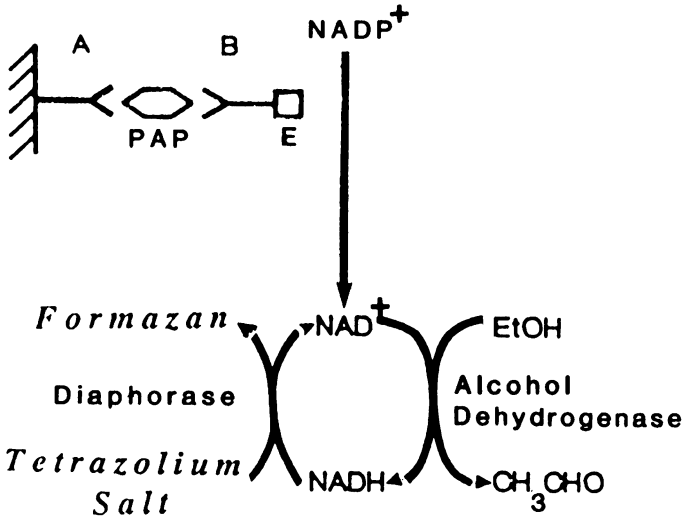

Fig. 5. Schematic of the alkaline phosphatase (E) signal amplification. Formazan represents the coloured end product. The assay configuration is a 'two-site' sandwich assay for prostatic acid phosphatase (PAP), EtOH = ethanol; CH_3CHO = acetaldehyde. Total amplification is 3.6×10^6 per min, derived from 60 000 molecules of NAD^+ per min per ALP molecule and 60 molecules of formazan per min per NAD^+.

enzyme system which comprises a redox cycle driven by alcohol dehydrogenase and diaphorase. In this cycle, NAD^+ is reduced to form NADH and ethanol is oxidized to acetaldehyde. The NADH, in the presence of diaphorase, then reduces a tetrazolium salt, iodonitrotetrazolium violet to form an intensely coloured soluble formazan dye, and NAD^+ is regenerated. The rate of reduction of the tetrazolium salt is directly proportional to the concentration of NAD^+ originally formed by the bound enzyme. This system is very sensitive even with spectrophotometric detection because of the very high amplification introduced (amplification over 10^6 per min). The practical application of this assay for TSH yielded a detection limit of 0.0013 mU/l which is equivalent to ~ 280 000 molecules per assay cuvette. Potentially, the reagents are applicable for any assay where ALP is the primary label. The detection reagents are now commercially available.

5.4. Time-resolved fluorescence

This technique has been reviewed recently [118,119] and it will only be discussed here briefly. The label in such systems is either Eu^{3+} or a europium chelator. A fluorescent europium chelate can then be formed by adding either organic chelators as in the DELFIA® (LKB-Pharmacia, Turku, Finland) or Eu^{3+} in the FIAgen® system (CyberFluor Inc., Toronto, Canada). The fluorescent europium chelates (and some other lanthanide chelates) possess certain advantages in comparison to conventional fluorescent labels like fluorescein, i.e. large Stokes shifts, narrow emission bands and long fluorescence lifetimes. The fluorescence lifetimes of most conventional fluorophores is 100 nsec or less; the lifetime of lanthanide chelates is 100–1000 μsec. Using a pulsed light source and a time-gated fluorometer, the fluorescence of these compounds can be measured in a window of 200–600 μsec after each excitation (Fig. 6). This method decreases the background interference from short-lived fluorescence of natural materials in the sample, cuvettes, optics etc.

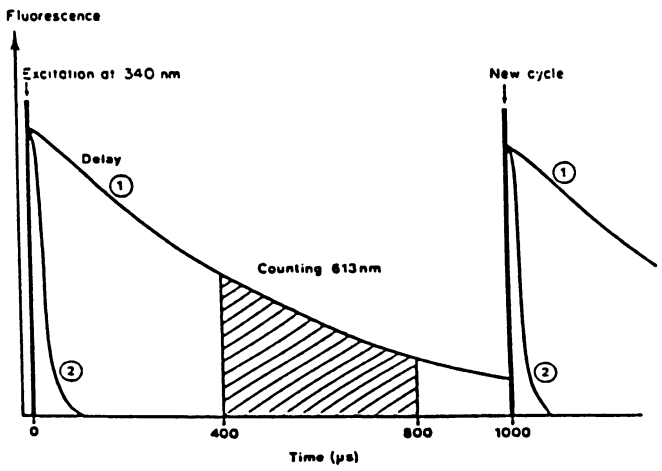

Fig. 6. Measurement principle of time-resolved fluorescence. The cycle time is 1 ms and pulsed excitation less than 1 μs occurs at the beginning of each cycle. The delay time after the pulsed excitation is 400 μs and the actual counting time within the cycle has the same duration. The total measurement time per cuvette is 1 s. Curve 1 presents the fluorescence of the europium chelate and curve 2 the background fluorescence (actual decay time less than 1 μs). Reprinted by permission from ref. [118].

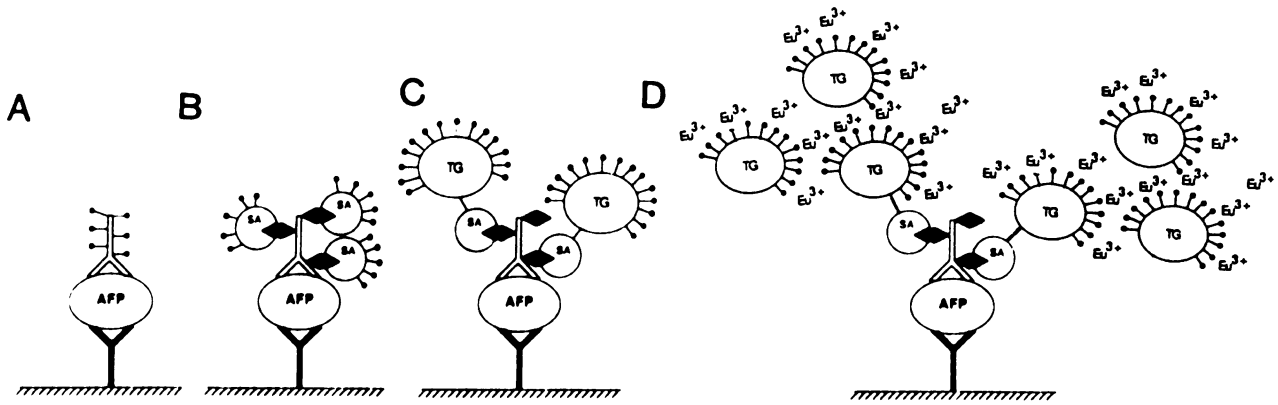

Fig. 7. A 'two-site' immunoassay for alpha-fetoprotein (AFP) using biotinylated (◆) detection antibodies (panels B, C, D) or BCPDA labeled antibodies (panel A). In panel B, streptavidin (SA) is directly labeled with BCPDA (—●). In panel C, SA is covalently linked to thyroglobulin (TG) labeled with BCPDA. In panel D, excess BCPDA-labeled TG is complexed to SA-conjugated TG through Eu^{3+} ions. All assays work with excess Eu^{3+}.

The chelates used by the DELFIA system are complexes of the type $Eu(NTA)_3(TOPO)_2$ where NTA is napthoyltrifluoroacetone and TOPO is triocylphosphine oxide. The immunological label, Eu^{3+}, is introduced into antibodies or streptavidin by using a strong Eu^{3+} chelator of the ethylenediaminetetraacetic acid (EDTA) type. Similarly, Eu^{3+} can be incorporated into DNA probes. Release of Eu^{3+} and recomplexing with NTA and TOPO can be achieved by lowering the pH to around 3.0. DELFIA is well-established in the field of non-isotopic immunoassay and is characterized by high sensitivity and broad dynamic range; it is vulnerable to exogenous Eu^{3+} contamination. The label, Eu^{3+}, can be measured down to 10^{-13} mol/l using time-resolved fluorescence and 10^{-17} mol per cuvette can be detected routinely. Analytes can also be measured down to these levels and in DNA applications, the detection limit is around 10^{-18}–10^{-16} mol of probe. This compares to $0.05–2 \times 10^{-18}$ mol of ^{32}P labeled probe [8].

The FIAgen system [118,89–91] uses the europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) as label and is not subject to Eu^{3+} contamination effects. This system works best when biotinylated antibodies are used in combination with BCPDA-labeled streptavidin. Three streptavidin preparations have been produced so far (Fig. 7) which achieve different detection limits as follows: streptavidin (SA) directly labeled with BCPDA [$SA(BCPDA)_{14}$] for assays with detection limits of 10^{-10}–10^{-11} mol/l; streptavidin covalently linked to thyroglobulin (TG) carrying ~ 160 BCPDA molecules [$SA(TG)(BCPDA)_{160}$] for assays with detection limits of 10^{-11}–10^{-12} mol/l and the same streptavidin-thyroglobulin system activated by an empirical process for assays with detection limits of 10^{-12}–10^{-13} mol/l. The best detection limit achieved with the latter reagent for a model alpha-fetoprotein assay was ~ 300 000 molecules per cuvette (5 μl sample volume).

A number of papers demonstrate the feasibility of using Eu^{3+} as label in DNA probing applications [119–122]. In one report [122], haptenized probes were used and second antibodies labeled with Eu^{3+}, directed against the anti-hapten antibod-

ies were utilized. A detection limit of 20 pg of DNA ($\sim 5 \times 10^5$ molecules) was obtained. In another report [120], biotinylated probes were used in combination with Eu^{3+}-labeled streptavidin. The sensitivity achieved was ~ 10 pg of DNA. Sund et al. [121] used the 'tail' approach described under 'Amplification Strategies' to synthesize DNA probes multiply labeled with Eu^{3+}. The detection limit reported was about 200 pg of DNA, an order of magnitude worse than with the indirect techniques.

5.5. Enhanced luminescence

The phenomenon of luminescence (bioluminescence and chemiluminescence) has been used to devise analytical systems in many different ways. In this review, only chemiluminescence approaches that proved to be successful for practical applications will be described. For more information on bioluminescent and other chemiluminescent assay configurations, see refs. [123–125].

Luminol and isoluminol have been tried as luminescent labels for immunoassays but only with limited success because the light output was generally poor and the duration of the light output very short. Recently, a series of compounds was discovered that enhance the light output from the oxidation of luminol and allow optimization of the reaction to give a prolonged output of light (many minutes) of high intensity [126,127]. This discovery was commercialized under the name 'Amerlite®' for immunoassays and under the name 'ECL gene detection system' for DNA applications [128,129] (Amersham International, Arlington Heights, IL, USA). This enhanced luminescence detection system works as follows (Fig. 8). The primary label is horseradish peroxidase and is conjugated to either an antibody (as shown in Fig. 1) for 'non-competitive' immunoassays or the analyte of interest for 'competitive' immunoassays. The label is then detected by adding the substrate (luminol), the oxidant (H_2O_2) and an enhancer (e.g. firefly luciferin or benzothiazoles). The emitted light is measured with a luminometer. For DNA applications, the system

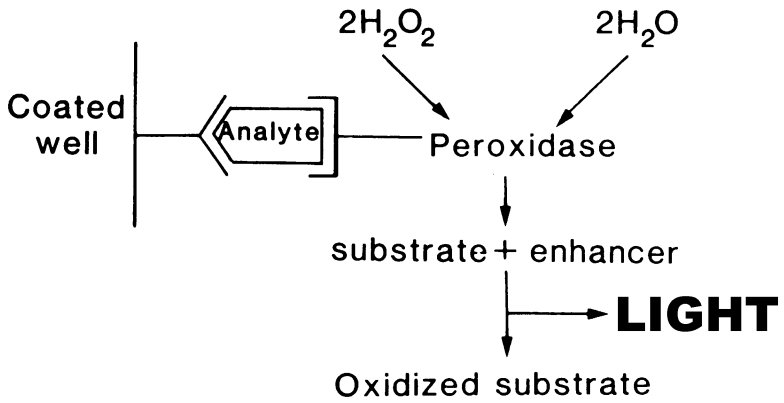

Fig. 8. Application of enhanced luminescence for immunoassay (shown in Figure) or DNA probing. The antibody or probe is labeled with horseradish peroxidase. In DNA probing, hybridization takes place to a target sequence immobilized on a solid support. The detection reagent contains hydrogen peroxide, luminol (substrate) and an enhancer. In DNA probing, the light emitted is detected on a special film in less than 1 h. In immunoassay, the light emitted is quantified by using a luminometer.

can be used with Southern, Northern, Western colony plaque and dot blots. Horseradish peroxidase can be introduced into DNA probes by using glutaraldehyde. After hybridization, the HRP-labeled probe can be detected exactly as described for immunoassay (Fig. 8). A special photographic film is used to obtain a hardcopy in a manner similar to that of autoradiography. This system combines ease of labeling, stability of probes, detection within 1 h and excellent sensitivity. It is claimed that this system is suitable for single copy gene detection in Southern blots. As little as 1 pg of DNA can be detected.

5.6. Chemiluminescence using acridinium esters

Acridinium esters produce a flash of light when oxidized in alkaline conditions with hydrogen peroxide (Fig. 9). When used as labels, they can be detected at levels of 10^{-18} mol/cuvette [130]. The advantages of acridinium esters (AE) over luminol and isoluminol derivatives [131] are that AE have a higher quantum efficiency, they do not suffer from serious quenching effects when linked to proteins or haptens, they do not need an enzyme during the oxidation process and the step of releasing the label before the final measurement is taken is not needed. Activated acridinium esters, *N*-hydrosuccinimide derivatives, are commercially available and they are used to produce labeled immunoreactants, e.g. antibodies, antigens and DNA probes. Acridinium ester-based luminescent immunoassays are now commercially

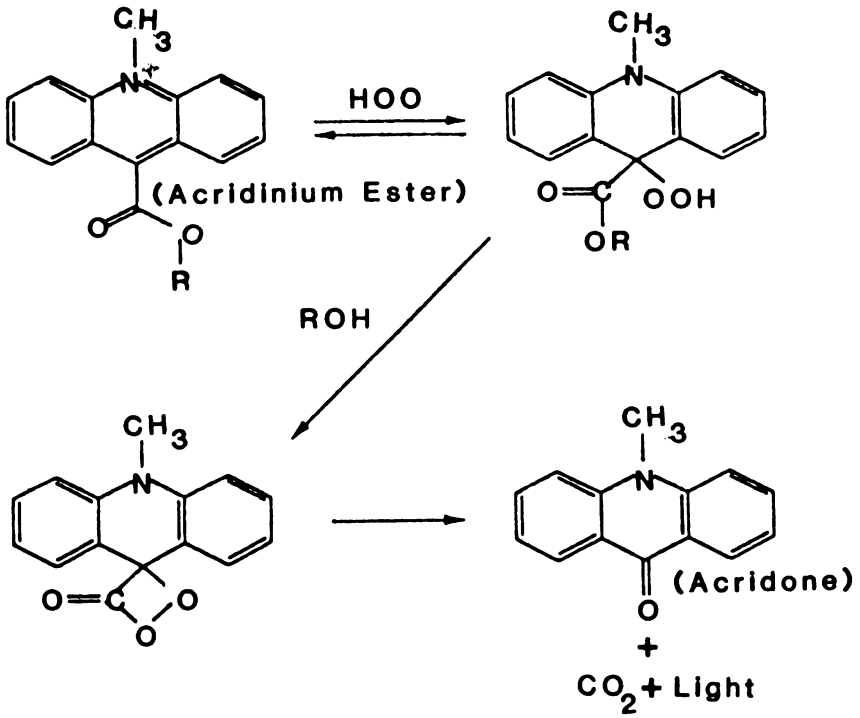

Fig. 9. Chemiluminescent reaction mechanism for methyl acridinium esters. The chemiluminescent reaction begins with attack by hydroperoxy anions on the 9 position of the acridinium ring. Under alkaline conditions, a cyclooxetane ring intermediate is formed, followed by rapid conversion to an excited *N*-methylacridone, which emits light upon relaxation to the ground state. (Reprinted by permission from ref. [134].)

available [132,133]. These assays are either of the 'competitive' or 'non-competitive' design and they use either plastic or magnetic particles for solid-phase separation. In one of the most sensitive immunoassays for TSH that has been reported [132], the label is an acridinium ester. This assay can measure TSH down to 0.002 mU/l. Established and novel procedures for further improving the applications of acridinium esters in immunoassay by using liposomes have been reported [102]. Liposomes offer very high amplification factors but leakage is still a problem. Acridinium esters are now entering the DNA probing field with excellent prospects [134,135]. Some technical advances are: the development of methodology to introduces protected alkylamine linker arms at any location within synthetic DNA probes; the preparation of *N*-hydroxysuccinimide (NHS) derivatives of acridinium esters which can be used to easily label an $-NH_2$ containing probe and methodology that is analogous to the 'homogeneous immunoassay' design and does not require separation of free and bound probe before the final analysis. A homogeneous DNA probing assay using an acridinium ester as label depends on elimination of unhybridized excess probe by selective hydrolysis so that the products released do not chemiluminesce. The hybridized probe is protected from hydrolysis and this assay is called 'hybridization protection assay'. With this method, which has the advantages of simplicity and speed, target sequences in the 10^{-16}–10^{-17} mol range ($\sim 6 \times 10^6$–6×10^7 molecules) can be detected. In a heterogeneous assay design, with the same reagents and excess probe removal after hybrid adsorption to magnetic particles, the sensitivity was further improved, due to more effective background reduction. This sensitivity was equal to $\sim 6 \times 10^{-18}$ mol of target sequences ($\sim 3.6 \times 10^6$ molecules). The maximum sensitivity of the labeled probe itself, is around 5×10^{-19} mol ($\sim 3 \times 10^5$ molecules).

5.7. Chemiluminescent enzyme substrates

It has already been mentioned that radioactive and fluorogenic enzyme substrates have been used very successfully. Enzyme substrates yielding chemiluminescent products have also great potential. A chemiluminescent enzyme substrate for alkaline phosphatase or β-galactosidase has recently drawn much attention [136–139]. This substrate (with either phosphate or galactose at the hydroxyl present on the benzene ring) releases a chemiluminescent product in the presence of either ALP or β-galactosidase, as shown in Fig. 10. This chemical system has been used to devise a number of highly sensitive immunoassays [137]. An even more sensitive detection system results after the addition of fluorescent micelles formed from cetyltrimethylammonium bromide and a fluorescein derivative (Fig. 11). The enhanced system reportedly detects < 1000 molecules of ALP [140,141].

The application of the system for DNA probing is straightforward. ALP-conjugated probes are used in a conventional way or indirect labeling is used under the principles described in Fig. 2. These systems can detect single copy genes in Southern blots of human genomic DNA [142]. The signal can be quantitated by a luminometer or visualized on photographic films. In one report the detection limit was improved by a factor of 25- to 100-fold in comparison to colorimetric ALP

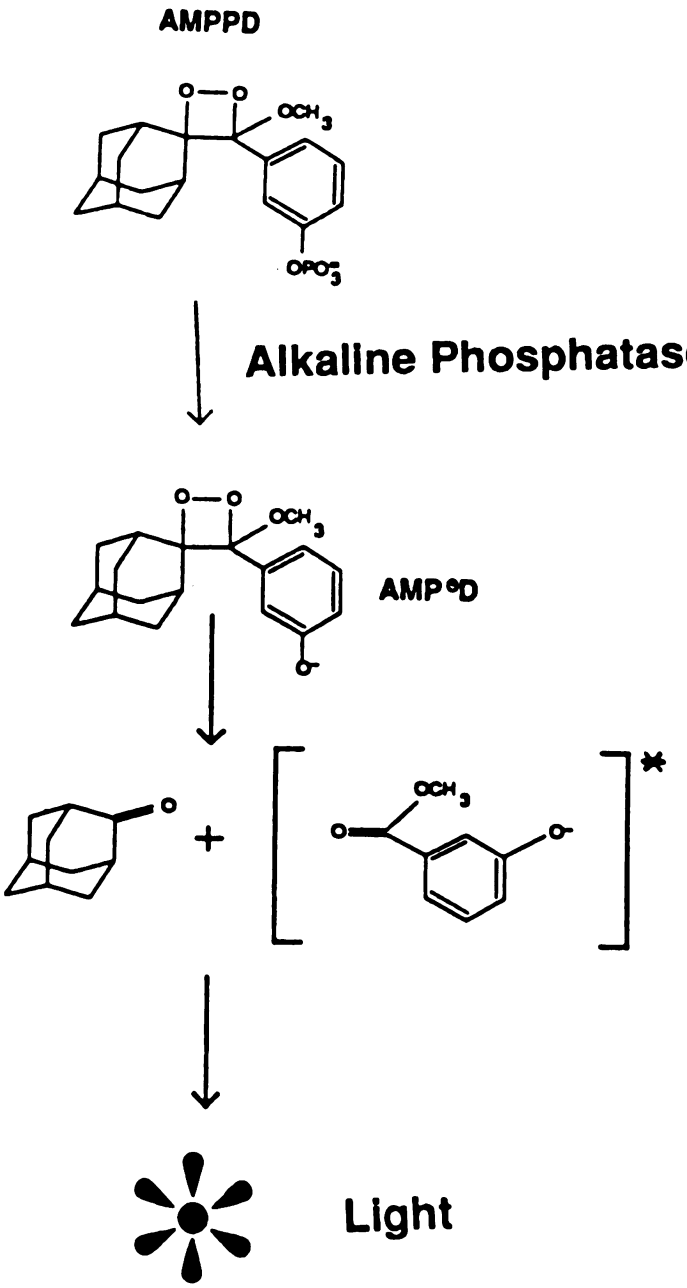

Fig. 10 Adamandyl-1,2-dioxetane phosphate (AMPPD) is a substrate for ALP. The intermediate product of hydrolysis fragments into adamantanone and the excited state of methyl meta-oxybenzoate anion which is the light emitter.

detection. In this report, after 4 h hybridization, $\sim 1.4 \times 10^4$ copies of Herpes Simplex virus I were detected with the chemiluminescent substrate [139]. Ureda et al. used a modified system which allowed the incorporation of 60–300 molecules of ALP per probe [101]. This was achieved by using single stranded probes which could hybridize and branch with each other in a controlled fashion. After the branching, an alkaline phosphatase containing probe is hybridized to multiple sites thus incorporating many ALP moieties. ALP could then be detected with AMPPD as shown in Fig. 10. This assay principle was applied to measure as few as 50 000 molecules of double-stranded DNA. This assay is performed in microtiter plates in a way similar to ELISA and is amenable to automation.

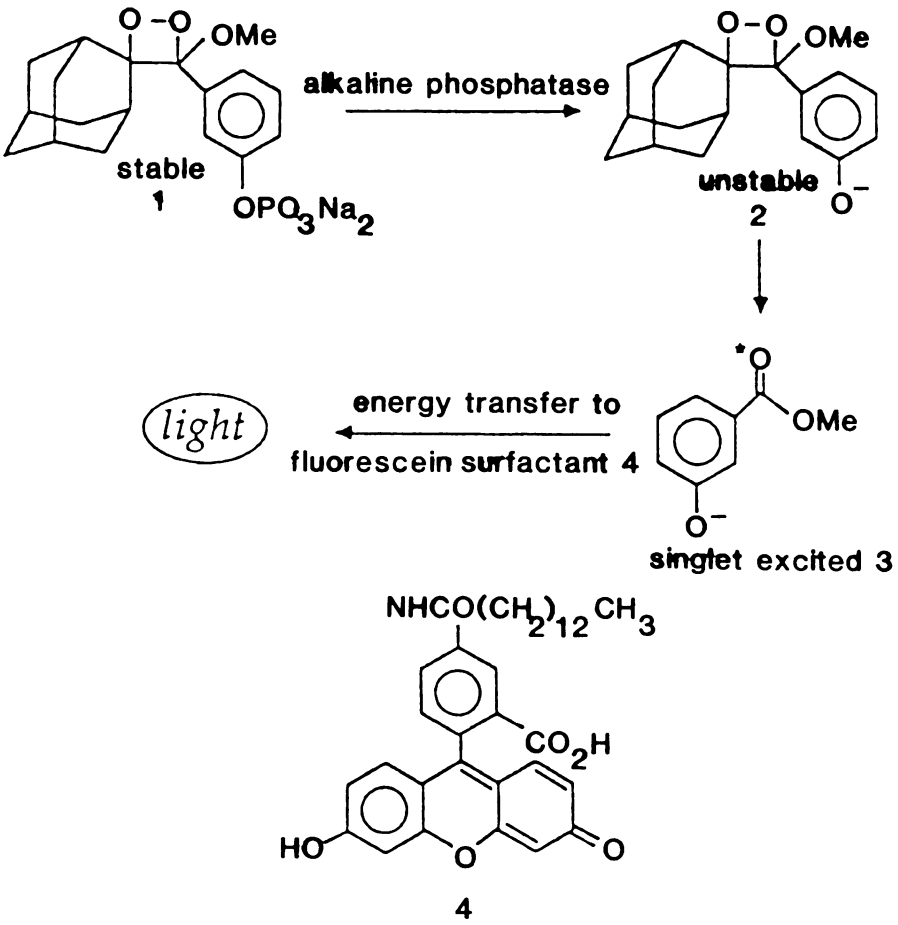

Fig. 11. Enhanced system based on the substrate AMPPD (see Figure 10) in the presence of fluorescent micelles formed from cetyltrimethylammonium bromide and 5-(*N*-tetradecanoyl-aminofluorescein [4]. (Reprinted by permission from ref. [141].)

5.8. Exponential amplification systems

Analytical methodologies having exceptional sensitivity using immunoreactants or DNA probes will undoubtedly become available during the next decade. One of the measuring approaches that should have success in solving sensitivity problems is amplification. Chemical amplifications similar to the one described by our group [89–91] or Ureda et al. [101] have potential but such amplification usually does not exceed a factor of 10^4. Enzymatic amplification can afford higher factors but the amplification introduced varies linearly with time, thus, the practicality of an assay is limited if exceptionally long incubation times are used for each measurement. An alternative strategy has been introduced recently and is based on exponential rather than linear amplification. This technique can afford amplification factors of 10^6 or more within minutes to hours. In the classical polymerase chain reaction approach [95–97], a target sequence of nucleic acid is amplified by the enzyme Taq polymerase using a repeating cycle protocol. At the end of the cycling protocol (usually ~ 20–40 cycles are used), the target sequence is accumulated and can be used for a variety of purposes. In a different but related system, the template is a specific RNA piece, about 218 nucleotides long (Fig. 12), which can be exponentially replicated by an RNA polymerase (called Qβ replicase) isolated from Qβ phage infected

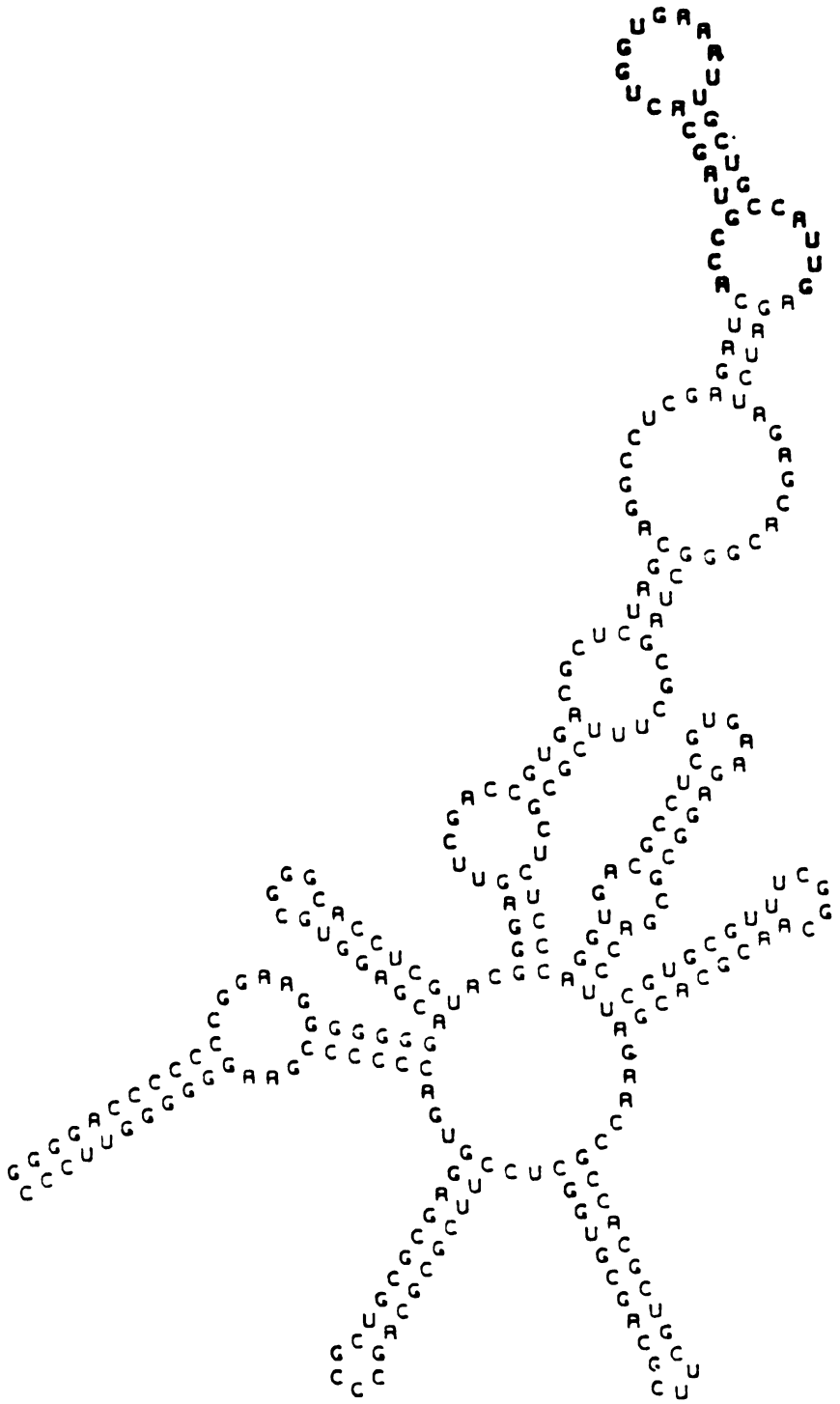

Fig. 12. A replicable human immunodeficiency virus (HIV-1) hybridization probe. Bold letters represent the 30-nucleotide long probe sequence that is complementary to nucleotides 4622–4651 in the pol gene of HIV-1 genomic RNA. This sequence was inserted into a replicatable RNA piece to obtain a product (shown above) that can serve two functions: to hybridize specifically to complementary target sequences and retain the ability to be exponentially amplified by Qβ replicase. (Reprinted by permission from ref. [100].)

Escherichia coli. The latter system has been proposed as a potentially powerful detection technique for both immunoassay and DNA probing applications [98–99]. Amplification as high as 10^9 can be achieved within 30 min. The system works as follows (Fig. 13) [100]: The specific RNA piece could be used as a reporter on a

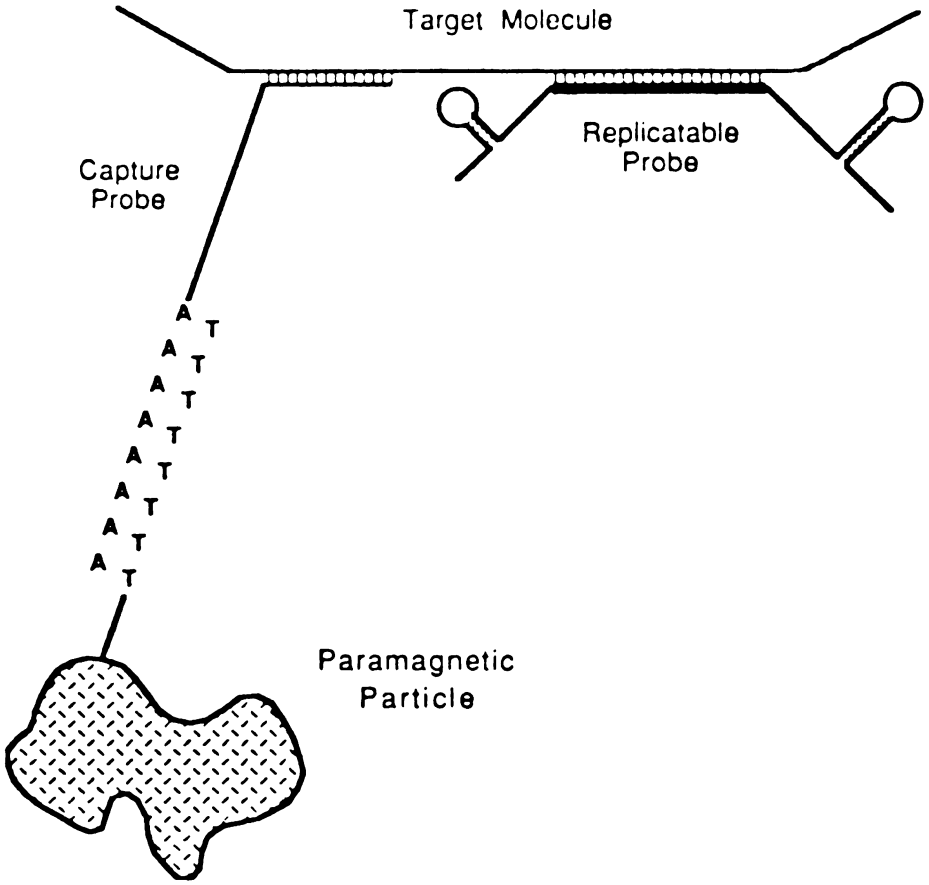

Fig. 13. Hybridization assay. The target molecule is shown bound to specific probe carrying a replicatable sequence (as shown in Fig. 12) and to another probe which in non-covalently linked to a paramagnetic particle (solid-phase). After excess probe is removed by washing, the hybrid is detected as described in the text. The above system can be used in combination with the 'reversible target capture' technique for background signal minimization. (Reprinted by permission from ref. [100].)

specific antibody or DNA probe. More specifically, the probe could be embedded within the sequence of a replicable RNA [99]. After the binding reaction with the ligand or target sequence takes place and excess reagent containing the reporter is washed away, Qβ replicase is added along with excess nucleotides and appropriate buffers and salts to exponentially amplify the reporter which could then be conveniently quantified by a classical technique (e.g. absorption or fluorescence spectroscopy). This approach is very attractive and has the potential for single molecule detection. The approach is limited by non-specific binding of the molecule carrying the reporter, since this binding is also amplified. Methods to remove non-specific binding must be devised if the benefit from such amplifications is to be realized.

The practical demonstration of the abilities of this technique have been published recently [100]. A recombinant replicatable RNA containing an inserted HIV-1 probe sequence was synthesized and used as shown in Fig. 13. The method of reversible target capture was used to reduce background. The detection limit achieved was ~ 10000 target molecules of HIV-1 mRNA. Novel methods to further reduce the background signal of the replicase system and to devise homogeneous assays have also been proposed recently [143].

6. Conclusions

In the field of immunoassay, non-isotopic systems are steadily replacing the tradional radioactive labels. The current challenge is successful automation, especially on analyzers that have random access capabilities. Another objective is to further improve the current detection limits so that more analytes will become accessible. In DNA probing, radioisotopes are still dominant because they offer better sensitivity. The ultimate sensitivity for detection of specific sequences, is one target molecule [8,101]. This is theoretically possible using an exponential amplification step prior to hybridization. Currently, the best procedures detect a few thousand molecules without the need for amplification.

It is difficult to objectively compare current detection techniques in the field of DNA probing. Many investigators report sensitivities in conventional units (ng or pg of target) and not in molar units. There is no universal target sequence for checking detection limits in DNA probing experiments independent of the detection system used and other factors e.g. nature of target, probe, conjugation chemistries, hybridization conditions, solid-phases etc. become the limiting factor. Many investigators report the ultimate detection limit of a system under conditions of zero non-specific binding. In a recent publication, a number of detection techniques were critically compared [144] and it was shown that the detection limit of a label often deteriorates when the label is introduced to the probe or used in actual hybridization assays.

DNA will become the ultimate marker of many human diseases and DNA diagnostics will come to dominate in genetic, malignant and infectious disease, as well as in forensic applications and agriculture. Currently, many DNA based assays are slow and cumbersome and progress to simplify and automate these assays is needed. Radioisotopic detection should give way to accurate, rapid, sensitive and economical non-isotopic detection systems. Currently, many problems can be solved by following amplification with the polymerase chain reaction with a non-radioactive detection technique. It is anticipated that with the evolution of extremely sensitive systems, PCR amplification may become less necessary thus simplifying and shortening procedures.

One of the limitations for devising ultrasensitive methodologies is the background signals observed during measurements. Concentrated efforts to understand and solve the non-specific binding problems of the labeled reagents used will contribute to the development of more sensitive techniques. The challenge of measuring single or a few analyte molecules still exists. However, progress in this field is evident [109–111] and successful reports on visualizing molecules with microscopic techniques are not infrequent [145–146]. It is anticipated that our ability to visualize and manipulate single molecules will contribute significantly in diverse areas including DNA sequencing [147].

Progress in the areas mentioned above will undoubtedly lead to more economical, safe and efficient use of the current immunological techniques and to the wide dissemination of DNA-based assays which will help diagnose, monitor and understand the nature of infectious, malignant and genetic disease.

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