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Strategies for immunoassay

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Abstract: The scope and diversity of immunoassay technology has shown phenomenal growth. Requirements for convenience (whole blood), reliability, simplicity (nonseparation), multiple simultaneous assays, and extreme sensitivity (>zeptomole detection limits) are increasingly demanding. Various strategies have been devised to address these requirements. Many new labels have been tested including laccase, acetate kinase, *Vargula* luciferase, rare earth cryptates, and Pd-coproporphyrin. Whole blood nonseparation immunoassays have been devised based on porous antibody-coated immuno-electrodes. Fusion conjugates provide a reproducible source of bioluminescent conjugates (e.g., firefly luciferase-Protein A). New nonseparation assay strategies use singlet oxygen channeling, phase modulation fluorescence, or dye-sensitized photobleaching principles. Multiple simultaneous assays provide a means of consolidating analytical workload and devising screening tests (eg, strategies based on combinations of labels and spatially separated tests zones). Ultrasensitive chemiluminescent detection reactions for amplifying labels, such as alkaline phosphatase (adamantyl 1,2-dioxetane aryl phosphate substrates) and peroxidase (HRP) (luminol or pyridopyridazine - enhancer (substituted phenol or boronic acid) type detection reagents) have produced significant improvements in sensitivity in sandwich-type assays. Other amplification techniques replicate a bound DNA label directly using the polymerase chain reaction, or replicate a bound peroxidase label indirectly via a catalyzed deposition procedure.

INTRODUCTION

Immunoassay and related ligand binder assay techniques are now widely used in clinical laboratories. The continuing development of this type of technology is focused on improved sensitivity, reliability and assay convenience (e.g., nonseparation assays). This article reviews selected strategies designed to improve assay performance and examines future trends in this type of analytical technology.

NEW LABELS AND CONJUGATES

The original radioisotopic immunoassay labels, such as ^{125} iodine, have been replaced by a variety of nonisotopic labels. Enzyme (horseradish peroxidase, alkaline phosphatase, glucose 6-phosphate dehydrogenase), fluorophor (fluorescein, lanthanide chelates), and chemiluminescent labels (acridinium ester)

predominate, but no one label has all of the attributes of an ideal label - i.e. stable, inexpensive, sensitive and simple measurement method, easy to conjugate to other molecules, a property of the label modulated when conjugate is bound to an antibody. Hence, the search for new labels has continued, and Table 1 lists some of the more recent candidates for an immunoassay label (1). Detection sensitivity has been an overriding consideration because in a sandwich assay the detection limit for the label is the major determinant of overall immunoassay sensitivity. Many of the currently available nonisotopic labels can be detected in attomole amounts (1 attomole = 602,252 molecules) and as little as one zeptomole (10^{-21} moles, 602 molecules) of alkaline phosphatase can be detected by a chemiluminescent assay procedure based on an adamantyl 1,2-dioxetane substrate (2).

Recent work with an acetate kinase label illustrates the sensitivity achievable with an enzyme label and a bioluminescent assay (3). This label is detected in a coupled reaction - first, acetate kinase acts on acetyl phosphate and ADP to produce ATP, and the ATP is detected using a mixture of firefly luciferase - firefly luciferin - Mg^{++}. The detection limit for acetate kinase label is 8.6 zeptomoles (8.6 x 10^{-21} moles) which corresponds to less than 6000 molecules of the enzyme.

TABLE 1. New labels for immunoassay

Acetate kinase	Lanthanide cryptates
Aequorin	Pd-coproporphyrin
Beta-lactamase	Polyacetylene
Europium trisbipyridine cryptate	Samarium (III)
Laccase	*Vargula* luciferase

Fusion conjugates

Components of bioluminescent reactions, such as luciferases and photoproteins are attractive as immunoassay labels because of the sensitivity of bioluminescent reactions. In the past, the preparation of conjugates of firefly luciferase and other luciferases has been problematic because these enzymes are easily deactivated by the chemical reactions used to prepare conjugates with antigens and antibodies. Many of the genes for bioluminescent proteins have been cloned as a result of the intensive interest in the molecular biology of bioluminescence. Splicing of these genes with the genes for other proteins (e.g., protein A, IgG heavy chain) has provided a route to a reproducible supply of active fusion conjugates that retain the biological activity of the bioluminescent protein and the specific binding properties of the protein (4-18). This strategy has been applied to other enzymes and some of these conjugates have been evaluated in immunoassay. Table 2 lists some recent examples of fusion proteins used in immunoassay. The analytical performance of fusion conjugates is promising, for example a metapyrocatechase-protein A conjugate was used in a model immunoassay for bovine serum albumin (BSA) and was linear over the range 1 x 10^{-3} to 1 x 10^{-7} g/mL and the detection limit was 0.7 pmol BSA (17).

TABLE 2. Fusion conjugates

alkaline phosphatase - anti-phytochrome single chain antibody (4)
alkaline phosphatase - basic fibroblast growth factor receptor (5)
apoaequorin - IgG heavy chain (6)
bacterial alkaline phosphatase - IgG Fc binding protein (7)
bacterial alkaline phosphatase - synthetic octapeptide (8)
bacterial alkaline phosphatase - anti-HIV 1 gp 41 single chain antibody (9)
bacterial alkaline phosphatase - human proinsulin (10)
beta-galactosidase - interferon-alpha2 (11)
beta-galactosidase - B19-specific oligopeptide (12)
core-streptavidin - single chain antibody (scFv) (13)
firefly luciferase - Protein A (14)
human placental alkaline phosphatase - 4-1BB ligand (15)
marine bacterial luciferase (beta -subunit) - protein A (16)
metapyrocatechase - protein A (17)
protein A - antiphytochrome single chain antibody (4)
Pyrophorus plagiophthalamus luciferase - protein A (18)

WHOLE BLOOD IMMUNOASSAYS

The majority of immunoassays have been optimized for use with blood serum, and few are suitable for other biological specimens such as whole blood or blood plasma. The requirement to separate serum or plasma from cells introduces an extra step into the overall immunoassay procedure. Elimination of this step would streamline the analytical procedure, and reduce the handling of a potentially infectious specimens. Few labels or label detection procedures are unaffected by cells, cell contents (e.g., hemoglobin interference in assay of HRP labels), or other components in plasma. One promising whole blood immunoassay uses a gold-coated microporous membrane (0.2 um pores) that serves as an electrode and as a solid phase. A nonseparatiom whole blood ELISA for hCG (detection limit 2.5 units/L) is performed using the capture antibody immobilized on the membrane and an alkaline phosphatase conjugate. Membrane bound conjugate is distinguished from unbound conjugate in the bulk solution by diffusing an 4-aminophenol phosphate substrate from the back of the membrane-electrode and detecting the 4-aminophenol product by oxidation at the electrode surface (19).

NONSEPARATION IMMUNOASSAYS

The desirable attributes of a nonseparation (homogeneous) immunoassay are speed and simplicity (no need to separate bound from free fractions), and adaptability to automatic chemistry analyzers. Most nonseparation assays are based on a competitive assay design, in which binding of the antibody to a labeled low molecular weight antigen modulates a property of the label. Competitive assays have limited sensitivity and currently available assays (eg, EMIT, CEDIA, TDx) and assays based on phase modulated fluorescence (20), and photobleaching (21) are limited to drugs, and other low molecular weight analytes (eg, thyroxine). A nonseparation assay based on a sandwich design would extend both the sensitivity and size range of these assays. Recently, a luminescent oxygen channeling immunoassay (LOCI) strategy was described (22). It uses two populations of microparticles (250 nm dia) coated with one of a matched pair of monoclonal antibodies. One population of particles is filled with a bromosquaraine

dye and the other with a precursor of a CL molecule (thioxene) together with a fluorophore (europium chelate). An antigen, eg TSH, forms a bridge between two different particles as a result of binding to the matched pair of monoclonal antibodies. Irradiation of the reaction mixture with laser light produces singlet oxygen at the surface of the dye loaded particle, and this reactive species diffuses to the adjacent particle. Here, it reacts to produce a dioxetane that decomposes and transfers energy to the fluorophore which then emits light. This process only occurs between particles captured by a TSH molecule. Any singlet oxygen generated in solution is unlikely to interact with another thioxene-containing particle. A rapid 12 minute assay for TSH based on this principle was shown to have a detection limit of <0.0125 mIU/L.

MULTIPLE SIMULTANEOUS IMMUNOASSAYS

There are a number diagnostic situations in which several immunoassay tests are required that are then interpreted as a group (e.g., Triple test for detection of Downs syndrome). The notion of performing these tests simultaneously is compelling on economic grounds and time efficiency. The two most effective strategies have been to use discrete test zones (e.g., spatially separated test zones on a membrane) or a combination of different labels (23). A technical hurdle in the latter strategy is to identify labels (one per analyte) that can be measured simultaneously or serially without compromising the properties of the individual labels. Fluorescent lanthanide chelates that have different fluorescence emission wavelengths and kinetics have proved effective for this task. This type of simultaneous multianalyte testing has been applied to detection of myocardial infarction via testing of serum for myoglobin and carbonic anhydrase III using a combination of europium and samarium labeled antibodies (24).

ULTRASENSITIVE DETECTION AND AMPLIFICATION REACTIONS

Ultrasensitive detection reactions for enzyme labels such as HRP have centered on CL assays. A range of new enhancers for the enhanced chemiluminescent assay for HRP have been discovered and tested including aryl boronic acids (25, 26), and substituted acetanilides (27). In addition new substrates for this reaction have been synthesized such as 7-phenyl-8-hydroxypyrido[3,4d]pyridazine-1,4(2H,3H)dione (28). The number of new methods for detection of alkaline phosphatase has increased dramatically in recent years. The most popular substrates for immunoassay applications are chemiluminescent adamantyl 1,2-dioxetane aryl phosphate derivatives derivatives (e.g., AMPPD^{R}, CDP-*Star*^{TM}) (2). These substrates have excellent sensitivity (detection limit,1 zmol) and light emission intensity and kinetics can be modulated by choice of substituents and enhancer molecules.

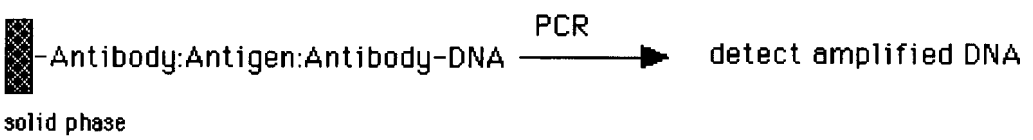

Fig. 1 Immuno-PCR assay strategy for an antigen (PCR, polymerase chain reaction)

It was perhaps inevitable that the exquisite sensitivity of the polymerase chain reaction (PCR) would be exploited for detection of a DNA label in a sandwich immunoassay (29). The principle of Immuno-PCR is illustrated in Figure 1. It is a conventional sandwich assay design except that a piece of double stranded DNA (size range 261 bp - 2.67 kb) is the label. After completion of the immunological steps the DNA label is amplified in a PCR reaction using labeled (^{32}P) or unlabeled nucleotides. In a 25 cycle PCR reaction, each bound DNA label is amplified by a factor of more than a million-fold. The amplified DNA is then electrophoresed and detected by staining with ethidium bromide or by autoradiography if ^{32}P labeled nucleotides were used in the PCR mix. Detection limits of less than 600 molecules have been reported (Table 3) and the Immuno-PCR strategy produces a 10^4 - 10^5 increase in sensitivity compared to a conventional ELISA assay (29-34).

An alternative method of achieving label replication is provide by the catalyzed reporter deposition assay (CARD) strategy (35). This is an ELISA assay in which

TABLE 3. Immuno-PCR reactions

analyte	detection limit, molecules	ref
beta-galactosidase	6,022,520 (10 amol)	30
bovine serum albumin	580	29
human chorionic gonadotropin	6,022,520 (10 amol)	30
thyrotropin	602,252 (0.1 amol)	30
tumor necrosis factor alpha	21,687,456 (0.625 pg)	31
human proto-oncogene ETS1	5,780	32
anti-apolipoprotein E	1,881 (0.5 fg)	33
gastric cancer associated antigen McAb MG7	22,885,576,000 (38 fmol)	34

an HRP label oxidizes a biotinylated tyramine substrate to produce a radical species that reacts nonspecifically with protein in the immediate vicinity of the HRP label. The "deposited" biotin groups are incubated with an avidin-HRP conjugate and the net effect is that a single HRP label is surrounded by many peroxidase molecules. Increases in sensitivity of 10-fold over a conventional ELISA are obtained using the CARD technique.

The signal from an alkaline phosphatase label can be amplified by an FADP-based technique. The label dephosphorylates FADP to produce FAD and this reactivates apo amino acid oxidase which in turn produces peroxide, and this is detected by a CL HRP detection reaction (36). The detection limit for alkaline phosphatase is 0.4 amol in an assay that takes 5 min to complete.

CONCLUSIONS

The scope and diversity of immunoassay technology continues to expand. Promising new labels include aequorin, acetate kinase and DNA. Sensitivity improvements have been achieved via coupled enzyme cycling reactions and by combining an immunoassay with PCR detection for a DNA label. Detection of less than 1000 molecules has now been achieved in a range of assays. Despite this considerable progress, many analytical challenges remain including whole blood, nonseparation immunoassays (particularly for large molecules). The recent microparticle-based LOCI assay points the way to simple nonseparation assays for large molecules such as TSH.

REFERENCES

1. L.J. Kricka. *Clin. Chem.* **40**, 347 (1994).
2. I. Bronstein *et al. Bioluminescence and Chemiluminescence: Current Status,* p. 73, Wiley, Chichester, (1993).
3. S. Murakami *et al. Bioluminescence and Chemiluminescence. Fundementals and Applied Aspects*, p.427, Wiley, Chichester, (1994).
4. A. Gandecha *et al.. Prot. Express. Purif.* **5**, 385 (1994).
5. D. Aviezer *et al. J. Biol. Chem.* **269**,114 (1994).
6. J. Casadei *et al. J. Biolumin. Chemilumin.* **4**, 346 (1989).
7. C.L. Wang *et al. Protein Eng.* **7**,715 (1994).
8. A. Witkowski *et al. Anal. Chem.* **65**,1147 (1993).
9. J. Kohl *et al. Ann. N.Y. Acad. Sci.* **646**,106 (1991).
10. C. Lindbladh *et al. Biochem. Biophys. Res. Commun.* **149**, 607 (1987).
11. A. Peterhans *et al. Anal. Biochem.* **163**, 470 (1987).
12. T.F. Schwarz *et al. J. Clin. Microbiol.* **29**,431 (1991).
13. S. Dubel *et al. J. Immunol. Methods* **178**, 201 (1995).
14. E. Kobatake *et al. Anal. Biochem.* **208**, 300 (1993)
15. K.E. Pollok *et al. Eur. J. Immunol.* **24**, 367 (1994).
16. C. Lindbladh *et al. J. Immunol. Methods* **137**,199 (1991).
17. E. Kobatake *et al. Anal. Biochem.* **186**,14 (1990).
18. C. Oker-Blom *et al. BioTechniques* **14**, 800 (1993).
19. C. Duan and M.E. Meyerhoff. *Anal. Chem.* **66**, 1369 (1994).
20. A.J. Ozinskas *et al. Anal. Biochem.* **213**, 264 (1993).
21. S. Bystryak *et al. Anal. Biochem.* **225**, 127 (1995).
22. E.F. Ullman et al., *Bioluminescence and Chemiluminescence. Fundementals and Applied Aspects*, p.16, Wiley, Chichester (1994).
23. L.J. Kricka. *In-Service Train. Contin. Ed.* **10**, 5 (1992).
24. J. Vuori *et al. Clin. Chem.* **37**, 2087 (1991).
25. O. Nozaki, *Bioluminescence and Chemiluminescence. Fundementals and Applied Aspects*, p.52, Wiley, Chichester (1994).
26. L.J Kricka and X. Ji. *Clin. Chem.* **40**, 1828 (1994).
27. T. Kissel *et al. Clin. Chem.* **41**, S57 (1995).
28. M. Ti *et al. Biochem. Biophys. Res. Commun.* **193**, 540 (1993).
29. T. Sano *et al. Science* **258**, 120 (1992).
30. R.C. Ebersole *et al. Clin. Chem.* **41**, in press (1995).
31. P.P. Sanna *et al. Proc. Natl. Acad. Sci. U.S.A.* **92**, 272 (1995).
32. H. Zhou *et al. Nucleic Acids Res.* **21**, 6038 (1993).
33. T. Sano *et al. Science* **260**, 698 (1993).
34. J. Ren *et al. Chung-Hua Chung Tsa Chih* **16**, 247 (1994).
35. M.N. Bobrow *et al. J. Immunol. Methods* **125**, 279 (1989).
36. M. Fisher *et al. Anal. Biochem.* **227**, 73 (1995).