The evolution of immunoassay technology

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SUMMARY

Since they were first utilized, immunoassays have witnessed phenomenal growth in the range and scope of their applications. A vast array of different labels and assay strategies has been developed to meet the requirements of sensitivity, accuracy and convenience. The development of increasingly sensitive labels and detection equipment has seen a drastic improvement in the sensitivity of immunoassay systems, allowing an ever-increasing range of analytes to be measured accurately. At the same time, simple to use, inexpensive assay systems have been developed with the necessary reliability, accuracy and sensitivity to bring immunoassay technology to much more diverse areas such as home testing, near-patient monitoring, and large screening programmes in developing countries. Recent developments in molecular biology techniques have made possible the production of fusion antibody conjugates, which can lead to further improvements in sensitivity and cost of reagents, as well as possibly revolutionizing the production of monoclonal antibodies. However, dissatisfaction with various aspects of existing immunoassay technology will necessarily lead to the continued development of this already widely diverse subject.

Key words: immunoassay, monoclonal antibodies, diagnostics, parasites.

EVOLUTION OF IMMUNOASSAY TECHNOLOGY

Antibodies have been in use as diagnostic reagents for over 50 years, but the immunoassay, as we understand it today, originated with the work of Yallow & Berson (1959) using anti-insulin antibodies to measure hormone levels in blood plasma. For the next decade, the use of immunoassay remained the almost exclusive domain of biomedical investigators, and utilized primarily radioactive labels. During this period there was little change in the basic technologies employed, although the techniques were further refined and made more 'user-friendly', with improvements made in labelling techniques (Van Weeman & Schuurs, 1971; Stimson & Sinclair, 1974), methods of separating bound and free label and antibody production (Abraham, 1977).

Workers outside the biomedical industry were slow to realize the potential of immunoassays in solving analytical problems, although in the late 1970s and early 1980s there was an increase in applications to which immunoassays were applied, including veterinary medicine, agriculture, the food industry, environmental health and forensic science (Pal, 1978; Collins, 1985; Morgan, 1985; Morris & Bolton, 1985; Morris & Clifford, 1985).

Much of this work was encouraged by the 'rediscovery' of Landsteiner's work (Landsteiner, 1945), on the production of antibodies against low molecular weight haptens and by the work of Kohler and Milstein (Kohler & Milstein, 1975) who reported the successful generation of lymphocyte hybrid cell lines producing antibody against antigenic determinants on the surface of sheep red blood cells. By utilizing these techniques (immunization protocols and monoclonal antibody production by cell fusion) it is possible to raise antibodies to virtually any desired epitope, thereby greatly increasing the applicability of immunoassay techniques to a wide variety of different applications (Eisenbach, 1981; Milstein, 1982).

With the increased usage of immunoassays, developments of immunoassay techniques were rapidly forthcoming. One of the first alternatives was the replacement of the radioisotope labels, which were proving unpopular due to their short half life, potential health hazard and waste disposal problems, with alternative labels (Van Weeman *et al.* 1971; Stimson & Sinclair, 1974), primarily enzyme labels although a whole variety of different labels has now been developed (Soini & Kojola, 1983; Weeks *et al.* 1984*a*; 1984*b*; Hemmila, 1985; Sano, Smith & Cantor, 1992; Petterson, 1993; Lasalle *et al.* 1995; Sekine, Watanabe & Tanaka, 1995; Self & Cook, 1996; Owen, 1997; Bruchez *et al.* 1998) as well as unlabelled assay formats involving biosensors (Karlsson, Michalsson & Mattsson, 1991; Van der Lelie *et al.* 1994; Aizawa, 1994; Morgan, Newman & Price, 1996). The advent of these new labels and the instrumentation to use them has given rise to a whole variety of different immunoassay, technologies and assay formats (Van Weeman & Schuurs, 1971; Stimson & Sinclair, 1974). The driving force behind these developments includes the need for improvements in ruggedness, simplicity, throughput, cost,

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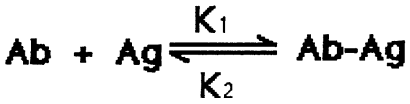


Fig. 1. Schematic of the reversible antibody (Ab) and antigen (Ag) interaction. The antibody combines with the antigen to form the Ab–Ag complex with rate constant K_1 and at equilibrium the complex dissociates to form free Ab and Ag at rate constant K_2.

sensitivity, accuracy or simply the need to evade existing patents. Dissatisfaction with various aspects of current immunoassay performance (speed, sensitivity, reliability, price and automation) will necessarily lead to the continued development of new labels and assay strategies (Kricka, 1994).

Developments in immunoassay technology are suggested to have 3 major targets (Singh, Sharma & Tyle, 1993): (1) assays requiring expensive equipment or reagents and possibly requiring highly trained staff, with the possibility of automation for high-throughput testing. Such tests will only be applicable for use in centralized testing laboratories; (2) near-patient testing for critical patient care. These will not have the same need for automation, expensive equipment and reagents and may be used by individuals with little training; (3) instrument-free tests or self-tests that can be performed by an untrained individual.

Immunoassays in the first category will be limited to large central testing laboratories which are able to afford what can often be very expensive equipment and reagents and which have suitably trained staff to run these systems. However, many applications of immunoassay are in small testing centres or near-patient care where there is not the same need for automation and large throughput or availability of highly trained staff (e.g. near-patient testing and home testing, such as pregnancy tests, etc.). Also a large proportion of the world's population lives in poorer countries where tropical diseases are endemic, therefore there is a great need for further development of effective inexpensive diagnostic systems which fit into categories 2 and 3 for use in those areas, where the more expensive systems are not appropriate.

Immunoassay encompasses a diverse range of techniques for the detection and quantification of the antibody-antigen interaction, with a bewildering array of different labels and detection systems available. The basic principle of immunoassay is based on the reversible reaction between an antigen and its specific antibody (Fig. 1).

Categories of immunoassay

Based on this basic principle immunoassays can be divided into a number of categories: (1) labelled assays – in which either the antibody or the antigen is coupled to a detectable label; and (2) unlabelled assays – where the reaction between the antibody and the antigen is directly measurable, e.g. precipitation reactions or immunosensors.

The labelled assay systems can be further subdivided by whether it is the antibody or the antigen that is labelled and by whether unbound label needs to be removed (heterogeneous assay) or if the system measures bound label without the need to separate bound and free label (homogeneous assay). The two basic categories can be further subdivided as indicated below (see Table 1).

Ligand labelled assays. In these systems the ligand of interest is conjugated to the label (a wide variety of different labels can be employed, radioactive, fluorescent, luminescent or enzyme and reacted with a limited concentration of specific antibody which is normally bound to a solid support. Free, unlabelled ligand in the sample will compete with the labelled ligand for the limited number of available antibody binding sites. Once the bound and free ligand are separated, the bound fraction is measured. The amount of bound label (signal) will be in inverse relationship as less labelled ligand will be bound in the presence of high concentrations of competing ligand in the sample (Fig. 2). The detection limit of these assays is greatly improved by the use of high activity labels, but the sensitivity is ultimately dependent on the affinity of the antibody used (Nakamura, Voller & Bidwell, 1986; Howard *et al*. 1989).

Antibody labelled assays. In these assays the specific antibody is conjugated with the label. This may be desirable for a number of reasons; the antigenic properties of many ligands can be adversely affected by conjugation to the label, or the antigen itself may affect the properties of the label when conjugated. This technique also has the advantage that it allows the standardization of the labelling protocol, as the vast majority of antibodies used for immunoassays can be labelled by the same technique whereas different ligands may require completely different labelling methods. There are two main variants of this method: (1) direct detection: the antigen in the sample is bound/captured onto the solid support and this bound antigen is subsequently detected by the addition of a labelled antibody (Fig. 3a). This gives a direct relationship of signal to levels of antigen; and (2) competition assay: a limited amount of purified antigen/antibody is bound/captured onto the solid support and the binding of a labelled antibody/antigen is made to compete with free antigen in the sample (Fig. 3b) after the removal of free labelled antibody/antigen the signal is detected. This gives an inverse relationship between signal and level of antigen in the sample.

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Table 1. Examples of immunoassay methods and techniques

Immunoassay techniques have come to be widely applied in many aspects of disease diagnosis. The ease of use and sensitivity of these assay formats, combined with a relatively low cost per test make them ideal for large-scale screening programmes, and many of the simpler immunoassay formats have been applied to this purpose. Recently more sensitive immunoassays have been developed utilizing luminescent labels which are witnessing increased usage. Emerging techniques such as immuno-PCR and immunosensors are also being developed and are starting to be applied in some cases.

Assay format	Matrix	Analyte	Reference
Immunoprecipitation	Serum	*Leishmania donovani*	Ghedin *et al.* (1997)
Agglutination	Blood	*Trypanosoma*	Asonganyi *et al.* (1998)
Antibody labelled sandwich assay	Serum/foetal fluids	*Neospora caninum*	Osawa *et al.* (1998)
Antibody capture sandwich assay	Faeces	Gastro-intestinal nematodes	Johnson *et al.* (1996)
Luminescent immunosensor	Serum	Anti-toxin antibody	Marks *et al.* (1997)
Immuno-PCR	Water	*Giardia*	Mahbubani *et al.* (1998)

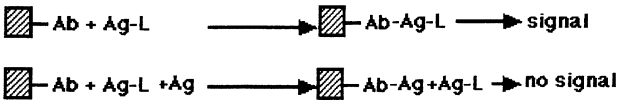


Fig. 2. Ligand (L)-labelled competition assay. Levels of antigen (Ag) in the sample compete with labelled Ag(Ag-L) for binding to limited numbers of specific antibodies (Ab) bound to a solid support. After removal of the unbound ligand the bound label is detected. The more Ag in the sample the lower the signal.

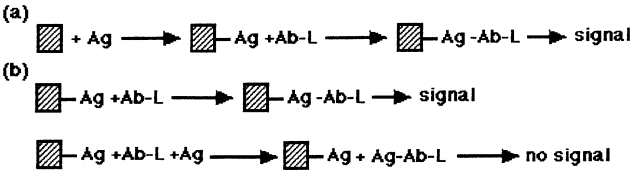


Fig. 3. Antibody labelled assays. (3a) Ag in the sample is coated directly to the plates and then probed with a labelled antibody, after removal of unbound Ab-L the signal is detected, giving readings in direct proportion to the concentration of Ag in the sample. (3b) Purified samples of the Ag are bound to the solid support, and binding of Ab-L for the immobilized Ag is competed for by free Ag in the sample. After removal of the free Ag–Ab-1 complex the signal is detected, giving readings in inverse proportion to the concentration of Ag in the sample.

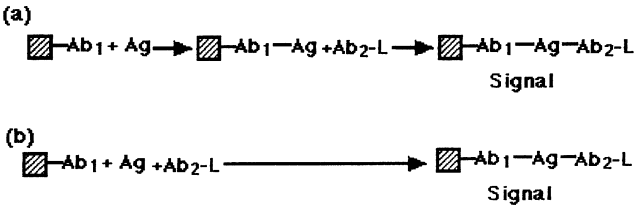


Fig. 4. Sandwich assay. (a) Antibody 1 (Ab_1) is immobilized on the solid support, and the antigen present in the sample (Ag) is captured by this antibody. The captured Ag is detected by the addition of a second labelled Ab(Ab_2-L), which upon removal of unbound labelled antibody gives a signal in proportion to the level of captured Ag. (b) A variation on this technique has both antigen and labelled antibody added simultaneously.

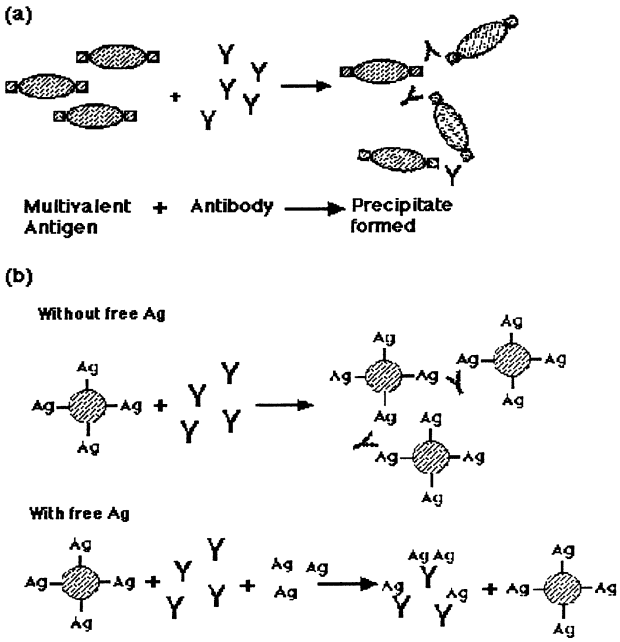


Fig. 5. Immune complex particle assays. (a) The antigen, which possesses multiple antigenic sites is reacted with the antibody to form an immuno-precipitate; both the rate and extent of precipitate formation, which can be detected by eye or by nephelometry or turbidity measurements, is affected by the amount of antigen present in the sample. (b) Particle enhanced immunoprecipitation. Specific antibody (Y) binds to antigen (Ag) coated particles giving rise to precipitate formation. Free antigen in the sample competes for antibody binding and inhibits both the rate and extent of precipitate formation.

Sandwich assays. This assay format ideally utilizes two antibodies, raised to different and spatially separated epitopes on the antigen (although such assays can work using only the one species of antibody if there is a repetitive epitope). The capture antibody is immobilized on a solid support and sample is added; antigen present in the sample will be bound by the immobilized antibody, any unbound sample is removed and the captured antigen is then detected by the addition of a second, labelled

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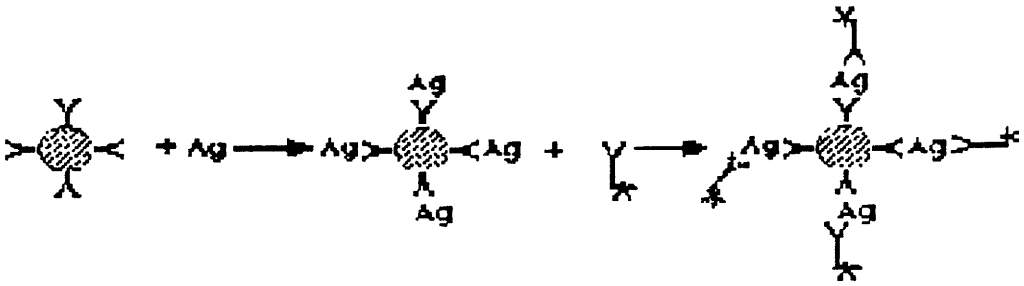


Fig. 6. Particle immunoassay. Beads coated with specific antibody binds antigen in the sample. The antigen is then detected using a second labelled antibody. Separation of bound and free label is facilitated by some property of the bead, e.g. magnetic beads, which allows simple separation.

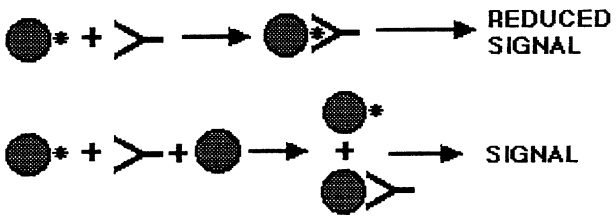


Fig. 7. Homogeneous immunoassays. Antigen is labelled with a ligand, which upon binding of the antibody experiences a loss of activity. In the presence of unlabelled antigen in the sample, antibody binding to the labelled antigen is reduced, giving rise to increased signal in direct proportion to the level of antigen in the sample.

antibody (Fig. 4a). An alternative means of conducting these assays, which makes them both easier to use and more rapid is to eliminate one of the stages by adding the sample and the labelled second antibody simultaneously (Fig. 4b). A major advantage of these assays is that by using two specific antibodies (2 site) the specificity of the assay is improved, as the generation of the signal requires the binding of both antibodies. This assay format allows the development of extremely specific and sensitive assays. However, the antigen being investigated must possess at least two distinct antibody-binding sites and this format cannot normally be used for the measurement of small molecules (e.g. steroids).

Immune complex/particle assays. These assays involve the direct detection of immune complexes either by eye (observation of precipitate formation) or by more quantitative methods (nephelometry and turbidity measurements). The basic principle of these assays is the reaction of a specific antibody with a multivalent antigen which leads to the formation of a visible precipitate (Fig. 5a) or the reaction can be enhanced by labelling the antibody or antigen with particles or marker compounds (Garland *et al*. 1986; Borque, Olivan & Iguaz, 1995: Fig. 5b).

A number of variations on this basic principle have been developed including a dual particle assay where both the antigen and specific antibody are bound to beads, using antibody-coated beads and a multivalent antigen. A whole range of different particles have been utilized e.g. latex beads, erythrocytes, colloidal dyes and metal solutions, where immunoprecipitation leads to the formation of coloured complexes which can be detected by colorimetry or absorbance readings (Van Weeman & Schuurs, 1971; Stimson & Sinclair, 1974).

Various techniques can be used to increase the sensitivity of these assays e.g. nephelometry and turbidity, which utilize the light scattering properties of the antigen-antibody complexes. These instruments have the advantage that they can also provide kinetic measurements of precipitate formation, which is advantageous as the rate of formation of the complex as well as its concentration can be important. The use of such devices allows rapid, sensitive and quantitative assays to be developed. Although these assays are still not as sensitive as most other types of immunoassay, their ease of use makes them readily applicable for a number of purposes (Rosseneu *et al*. 1981; Garland *et al*. 1986; Borque *et al*. 1995; Hayes, Jourdan & Herzog, 1996).

Another variation on these assays is where the antibody is bound to beads, normally magnetic beads, the antigen in the sample is allowed to react with these beads; the bound antigen is then detected by the addition of a second labelled antibody (Fig. 6). One major advantage of this type of assay is that the solid support i.e. beads, can bind large amounts of antibody thus increasing the sensitivity and range of the assay, and the separation of bound from free label can be improved, again leading to increased sensitivity.

Homogeneous immunoassays. In these assays the activity of the label is modified by the binding of antigen and antibody, thus removing the need to separate the bound and free label prior to detection (Fig. 7). The fact that there is no separation step means that the methodology of these assays can be made much simpler, more rapid and more easily automated than heterogeneous (separation of bound/free label) assays.

In an ideal homogeneous assay the binding of antibody and antigen would give 100% enhancement or inhibition of the label. In practice, however, this is rarely the case, which means that these assays are

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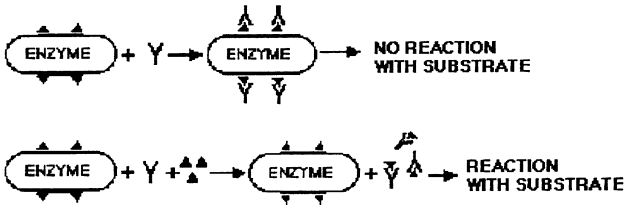


Fig. 8. The enzyme is conjugated to the antigen, and when the complex binds antibody the enzyme cannot react with its substrate giving a reduced signal. In the presence of free antigen in the sample antibody binding to the enzyme antigen complex is reduced, allowing enzyme and substrate to react giving signal in proportion to the level of antigen in the sample.

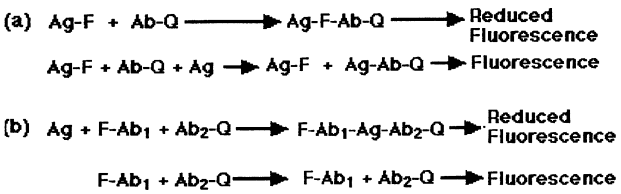


Fig. 9. (a) The antigen (Ag) is labelled with a fluorescent compound (Ag-F) and the antibody is labelled with a quenching agent (Ab-Q). If there is no Ag present in the sample then Ab-Q binds to Ag-F leading to reduced fluorescence. Increasing levels of free Ag in the sample lead to increased fluorescence. (b) For large multivalent antigens two labelled Ab can be used, one labelled with quencher and the other with fluorescent label. Increasing levels of Ag in the sample lead to reduced levels of fluorescence.

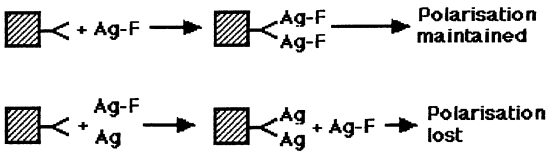


Fig. 10. Antibody is immobilized on the solid surface and binding of the fluorescent labelled antigen (Ag-L) is competed for by free antigen (Ag) in the sample. The more free antigen in the sample the greater the loss of polarization.

generally less sensitive than heterogeneous assays (Gosling, 1990), although the added simplicity means they are still widely used (Dosch *et al*. 1998).

This technique is really only applicable to small antigens/haptens e.g. drugs and hormones, in which antibody binding can directly affect the label. A wide range of different approaches to the development of homogeneous assays have been used. Many of these involve coupling the antigen to the label, which is normally an enzyme, such that binding of the antibody to the labelled enzyme prevent the interaction of the enzyme and its substrate, thus reducing the level of signal generated. The presence of free antigen in the sample will compete for the binding of the antibody, releasing enzyme to react with the substrate and thereby generating a signal in

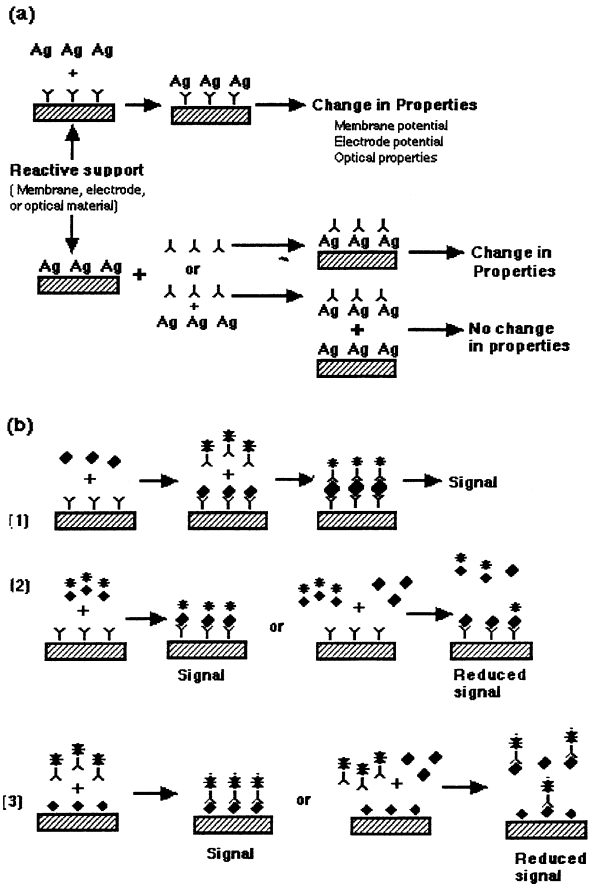


Fig. 11. (a) Indirect immunosensors. Either antibody (Y) or antigen (Ag) is bound to the solid matrix to form a sensor device. This sensor should be sensitive in its surface characteristics to give a measurable response upon immuno-complex formation. Electrode, membrane, piezoelectric or optically active surfaces may be employed. (b) Indirect (labelled) immunosensors. The sensor is coated with either antibody (Y) (1 and 2) or antigen (◆) (3). Antigen in the sample is captured by the antibody (1) and this captured antigen is then detected by the addition of a suitably labelled second antibody, or the binding of labelled antigen or antibody (2 and 3) is competed for by the presence of unlabelled antigen in the sample, giving a reduction in the signal generated in proportion to the level of antigen in the sample. The presence of the bound label can be measured by various means (potentiometric, amperometric or optical properties), often without the need to separate bound and free label, with only the product of the label close to the sensor surface (bound) being measured.

direct relationship to the level of antigen in the sample (see Fig. 8) (Kohl *et al*. 1997).

Homogeneous assays have also been developed using fluorescent labels (Burd, 1981; Li *et al*. 1981; Ullman *et al*. 1996; Matveeva *et al*. 1997), which follow much the same principle (as shown in Fig. 8) as that used for enzymes. In fluorescent quenching immunoassays two labels are involved, a fluorescent marker and a compound/molecule which, when in close proximity to the fluorescent label will limit

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(quench) the fluorescence emitted (Matveeva *et al.* 1996; Mathis, 1997). This can be carried out in two ways (Fig. 9).

Fluorescent polarization immunoassay relies on the planar orientation of light waves, achieved by the use of a polarization filter. This polarized light causes excitation of the fluorescent label which then emits light of a higher wavelength. If the fluorescent label is held in place (by the antibody) then the emitted light maintains the polarization. However, if the label is free to rotate (unbound) then the polarization is lost (Fig. 10).

As with most homogeneous immunoassays, FPIA is simple and precise. However the dynamic range of these assays is quite small (Singh *et al.* 1993) and the technique is not readily applicable to molecules over 20000 daltons. Even so these assays have been developed and used for a number of different analytes (Bogusz *et al.* 1990; Stamp *et al.* 1991; Kivisto, 1992; Hanseler & Keller, 1994; Burger *et al.* 1996; Matveeva *et al.* 1997; Eremin, 1998).

Immunosensors

Immunosensors are devices which comprise an antibody or antigen coupled to a reactive surface, which responds to the binding of the complementary antigen or antibody (e.g. by changes in optical or electrical properties). Immunosensors can be divided into two main categories: indirect immunosensors, which utilize a separate labelled species to facilitate detection after binding e.g. fluorescent or enzyme labels (Fig. 11a), or direct immunosensors in which the reaction between an antibody and its corresponding antigen give rise to changes in the reactive surface which can then be measured (e.g. potential difference, current, mass, heat or optical properties) (Fig. 11b) (Aizawa, 1994; Connolly, 1995; Lu, Smyth & O'Kennedy, 1996; Gizeli & Lowe, 1996; Morgan *et al.* 1996; Berggren & Johansen, 1997; Turner, 1997; Si, 1997; Polzius *et al.* 1997; Tiefnauer *et al.* 1997; Santandreu *et al.* 1998). Although indirect immunosensors are less influenced by non-specific binding phenomena, the fact that direct immunosensors can carry out real time monitoring of the sample is a distinct advantage.

ADVANCES IN IMMUNOASSAY TECHNOLOGY

Today, immunoassays are the predominant analytical techniques for quantitative measurements. They are able to operate over a wide dynamic concentration range, utilize a wide range of biological fluids (blood, urine, saliva, culture media, etc.) and employ a diverse range of different assay formats (ELISA, fluoroimmunoassay, evanescent wave biosensors) to provide, sensitive, inexpensive and specific assays. Since the earliest immunoassays using radiolabels were developed many changes have been made to the design of the assay format. All of these changes have been aimed at improving the utility of the assay (ease of use, throughput and automation), the sensitivity, accuracy and specificity of the assay and to increase the range of analytes which can be monitored.

There are a number of areas in which improvements to immunoassay can arise. Improved methods of antibody production and purification will have an obvious effect on the performance of any immunoassay, as all such assays are dependent upon an antibody and the production of higher affinity antibodies with improved specificity will have a great influence on the performance of the assay. Other areas in which improvements can be made is in the choice of label and detection equipment. Originally immunoassays employed radiolabels, but other detection systems rapidly became available such as enzymes, fluorescent and luminescent labels, light scattering, immunosensors and unlabelled assay systems, along with improved detection equipment. The choice of solid support used and the method of antibody or antigen attachment can have a major influence on the performance characteristics of the assay, as well as the efficiency of separating bound and free label in heterogeneous assays.

Antibody production

Originally, immunoassays utilized polyclonal antibodies raised against the antigen as the detecting reagent. A major drawback to the use of polyclonal antibodies is the heterogeneous nature of the antisera produced which will contain many antibodies of varying affinities and specificities, recognizing different epitopes on the immunogen, and also non-specific, cross-reacting antibodies which will adversely affect the performance of the assay. Despite these drawbacks, useful assay systems were developed and polyclonal antibodies are still used at some stage in many immunoassays today.

With the advent of hybridoma technology (Kohler & Milstein 1975), it became possible to produce antibody which is homogeneous and constant in its properties and binds to a single well defined epitope (or very closely similar epitopes); with the correct immunisation procedure it is possible to produce antibodies to almost any imaginable antigen (Eisenbach, 1981; Milstein, 1982). This basic technology has been improved over the years, particularly by the preselection of antigen-binding B cells and by screening with antigen coated filters (Casali *et al.* 1986; Gherardi, Pannal & Milstein, 1990), and the use of semi-solid media for the simultaneous selection and cloning (Davis, 1986).

A limit to the ability to produce the ideal antibody reagent is the ability to devise suitable screening methods for positive clones of suitable affinity and specificity, and ideally the screening method should

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resemble as closely as possible the format of the final test (Collins, 1985). It is quite useful if an estimate of the affinity of the antibodies tested can be obtained in early screening although this need not be too precise and should not be too time-consuming. Some workers recommend that determination of antibody titre should be used as a measure of affinity (Van Heyningen, Brock & Van Heyningen, 1983). However this is heavily influenced by cell concentration and antibody secretion rate, such that clones present at low cell numbers and secreting antibody slowly may be de-selected even though they are producing high affinity (and therefore potentially useful) antibody. Thus, some people recommend the use of Scatchard analysis to give an indication of affinity (Soos & Siddle, 1982) although this method can be slow and time-consuming. The advent of immunosensors which are able to carry out real time analysis of antibody antigen interactions opens up the possibility of quickly and efficiently screening clones for the affinity of the antibody they produce (Malmborg & Borrebaeck, 1995; Haimovich *et al*. 1998; Morton & Myszka, 1998).

One area in which some people believe polyclonal sera have an advantage over their monoclonal counterparts is in their avidity, with polyclonal sera exhibiting superior 'affinity' to monoclonal antibodies. One reason for this is the wide diversity of antibodies present in polyclonal sera (>1000 different antibodies are likely to contain at least one of very high affinity) but also because antibodies can display co-operative binding. This is dependent upon antibody bivalency and has been attributed to the formation of heterocyclic complexes. It is also possible that the binding of one antibody to this epitope can stabilize neighbouring epitopes and lead to enhanced binding of the relevant antibody. This phenomenon of improved avidity can be seen in mixtures of monoclonal antibodies (Ehrlich, Moyle & Moustafa, 1983; Ehrlich & Moyle, 1986).

Such benefits prompted people to examine the possibility of making bi-specific monoclonal antibodies by the fusion of two previously established hybridomas to produce antibodies displaying the binding characteristics of both the other antibodies in the one molecule (one antigen binding region of the antibody recognizing the epitope for antibody A and the other recognizing that of antibody B) or by the process of antibody engineering (Cao *et al*. 1995; Ruf & Carayon, 1995; Self & Cook, 1996; Coloma & Morrison, 1997; Kreutz & Suresh, 1997; Manzke *et al*. 1998). One potential application of these antibodies in immunoassays is to produce antibodies which recognize a specific antigen epitope and a label e.g. an antibody was produced which recognized horseradish peroxidase and substance P, which gave improved sensitivity and reduced backgrounds in competition assays and immunocytochemistry (Suresh, Cuello & Milstein, 1986).

Antibody engineering has emerged recently as a possible approach to produce monoclonal antibodies without the use of hybridoma technology (Winter & Milstein, 1991). Here antibody genes are cloned directly from the lymphocytes of immunized animals, then expressed in bacterial systems and the antibody products are then screened for antigen binding. Another very useful possibility of antibody engineering is the production of antibody-enzyme conjugates by recombinant technology rather than by chemical conjugation. This can avoid many of the potential problems, loss of activity of the label or immunoreactivity of the antibody, associated with chemical conjugation (Lindblach, Mossblach & Bulow, 1993; McCormack *et al*. 1996). Although this approach avoids hybridoma technology and the culturing of animal cells, it still requires the initial injection of animals and is very expensive. However, in the future it may be possible to bypass the animals altogether and make antibodies completely *in vitro* (Winter & Milstein, 1991). Whether or not *in vitro* production of antibodies ever becomes a useful diagnostic technology or not, one aspect of antibody engineering that should have a great influence on immunoassay is the modification of antibody affinity and specificity. It is possible to alter existing monoclonal antibodies such that they display altered binding characteristics and produce these altered antibodies in bacterial systems in large quantities, rather than immortalised cells. This technique can potentially be used to increase the affinity and specificity of hybridoma-produced monoclonal antibodies, thus improving their applicability to immunoassays (Gandecha *et al*. 1994).

Choice of label

The most commonly used labels for immunoassays were radioactive labels, utilizing I^{125}-, C^{14}-, H^{3}-labelled antibody or antigen. Radiolabels have a distinct advantage in that the incorporation of the label has minimal effects on the immunogenicity of the labelled species, making them ideal for the labelling of small ligands. However, the low specific activity of these species (even I^{125} will only release less than 1/100000 of its activity in a 1 min counting period) coupled with the short half-life (and hence shelf-life) of some labels and the regulatory pressures regarding storage and waste disposal make them a poor choice for convenient use. Although they are still in common usage today (Deberg *et al*. 1998; Lewis *et al*. 1998; Lynas *et al*. 1998; Mei *et al*. 1998; Mistry *et al*. 1998).

The move towards non-radioactive detection started in the 1970s with enzyme labels coming to the fore. Although they initially lacked the sensitivity of the radiolabels, they quickly replaced radiolabels in many laboratories because of their ease of use, lack of disposal problems and the availability of relatively

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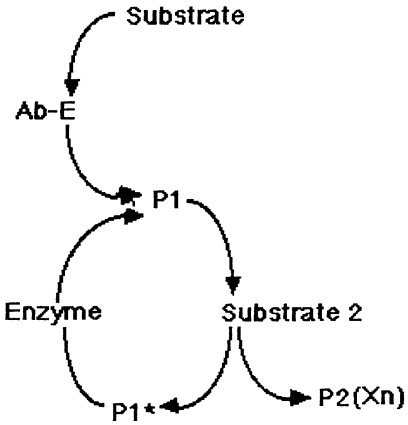


Fig. 12. The substrate reacts with the enzyme on the bound antibody to produce a product P1. This product then reacts with the amplification substrate (substrate 2) to produce detectable product P2. The P1 used in this reaction is then recycled to be used in production of further molecules of P2 leading to increased signal.

inexpensive detection equipment. Nowadays there is a bewildering array of different labels available, enzymes, fluorescent and luminescent labels, a wide variety of different solid particles as well as the development of unlabelled assays and immunosensors.

Enzyme labels are still currently by far the most common labels in use and a vast range of different enzymes have been applied to immunoassays (Oellerich, 1984; Gosling, 1990; Kricka, 1994; Hage, 1995; Schmidt, 1997). However horseradish peroxidase (HRP) and alkaline phosphatase (AP) remain the most commonly used enzymes.

The sensitivity of an enzyme label will depend to a large extent on the turnover rate of the enzyme and the level of enzyme labelling that can be achieved without affecting either the immunogenicity or enzyme function and the choice of substrate used. A number of chemiluminescent substrates have now been developed for both HRP and AP which give enhanced sensitivity, and a number of highly sensitive immunoassays have been developed (Bronstein *et al*. 1989, 1991; Edwards *et al*. 1990; Nishizono *et al*. 1991; Kobos, 1995; Paffard *et al*. 1997; Suzuki, 1998).

Another method which is widely used to increase the sensitivity of enzyme immunoassays is enzyme amplification, which can provide 100-fold increases in the measurable signal for a given amount of enzyme. In these assays the product (P1) generated by the enzyme label is utilized by an amplification solution to produce a second product (P2), P1 is then recycled (often by an enzyme), thus each molecule of P1 produced by the label can lead to the production of multiple (often over 100) molecules of P2 therefore generating increased signal (Fig. 12).

This approach has been utilized in many assays systems, producing assays with sensitivities in the low picomolar range or even lower (Bates, 1987, 1996; Obzansky *et al*. 1991; Dhahir, Cook & Self, 1992; Ishizuka *et al*. 1992; Cook & Self, 1993; Hansen, Gundstrup & Mikkelsen, 1993; Ghindilis *et al*. 1995). However one potential drawback to this technique is the large potential effect of non-specific binding to give high background values.

Fluorescent and luminescent labels

A second group of non-isotopic immunoassays that has recently seen much development for application in immunoassay are fluorescent and luminescent labels, with a wide range of different fluorescent and luminescent compounds being developed for use (Soini & Kojola, 1983; Oellerich, 1984; Weeks *et al*. 1984*b*; Hemmila, 1985; Gosling, 1990; Batmanghelich *et al*. 1991, Batmanghelich *et al*. 1992; Woodhead & Weeks, 1991; Kricka, 1994; Maeda *et al*. 1995; Hage, 1995; Keith, 1997; Schmidt, 1997; Zoha, Ramnarain & Allnutt, 1998; Barthellus, 1998; Steijger *et al*. 1998; Yakunin & Hallenbeck, 1998; Gerardi, Barnett & Lewis, 1999). Fluorophores such as fluorescein have been widely used in immunodiagnostics, although a number of drawbacks to these compounds is apparent e.g. poor separation of emission from excitation, interference from background fluorescence and fluorescence quenching (Hemmila, 1985).

Lanthanide chelates and related compounds have been widely used as labels for immunoassay, and their long fluorescence decay times have allowed their use in time resolved immunofluorescence to provide extremely sensitive assay systems (Soini & Kojola, 1983; Hemmila, 1985; Papanastasioudi-amandi, Christopoulos & Diamandis, 1992; Qin *et al*. 1996; Hemmila, Mukkala & Tukalo, 1997). The use of time resolved fluorimetry does however require the use of dedicated equipment which can be expensive.

Luciferases from several bioluminescent species have seen much use as labels for immunoassay since the early 1980s (Jablonski, 1985; Kricka, 1991; Huang *et al*. 1996; Murakami *et al*. 1998). One of the major problems associated with the use of luciferase was the difficulty in preparing conjugates that did not deactivate the luciferase. Advances in the molecular biology of the bioluminescent organism have resulted in the cloning of the genes for many luciferases, giving a reproducible source of these reagents and allowing the production of conjugates by recombinant techniques (Meighen, 1991; Lindblach, Mosbach & Bulow, 1991; Kobatake *et al*. 1993; Billiald *et al*. 1997).

More recently work has concentrated on aequorin, a bioluminescent compound isolated from jellyfish, this has a detection limit of about 1 attomole aequorin (Kricka, 1994), and recombinant aequorin has been used in the production of highly sensitive bio-

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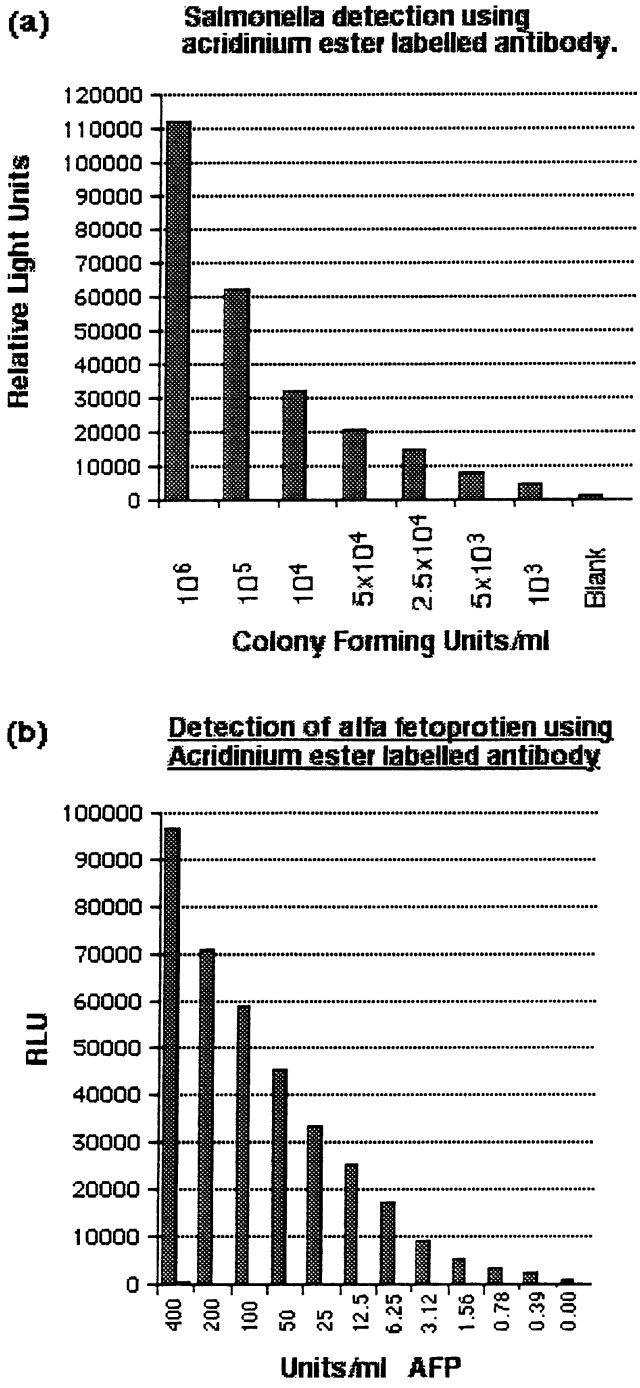


Fig. 13. (a) The detection of *Salmonella* spp. The use of acridinium ester labels allows the detection of fewer than 1000 c.f.u. ml^{-1}, at least a 100-fold improvement insensitivity over conventional enzyme labelled assays using the same antibodies. The blank reading corresponds to 10^6 *Escherichia coli*. (b) Detection of human AFP using acridinium ester labelled antibodies. Replacement of the radio label currently used in these assays with acridinium ester label allows the sensitive detection covering the same dynamic range without any of the drawbacks of radioisotopes.

luminescent immunoassays (Smith *et al*. 1995; Laios, Loannou & Christopolous, 1998). Phosphors are another group of compounds which have seen some use in immunoassay, and sensitivities down to <10 fg can be obtained using these compounds (Beverloo *et al*. 1992).

Luminescent compounds have seen extensive usage in various immunoassay formats. These labels are generally safe, stable, easy to prepare and can be measured readily without the need for expensive equipment. Luminol, isoluminol and their derivatives have been widely used, and one advantage they have is that peroxide labels (which are very commonly used and available) can be used in a luminol system (Arakawa, Maeda & Tsuji, 1979). These labels can provide very sensitive and easy to use assays. However one drawback is the time required to develop optimal light production (sometimes >1 h).

A number of alternatives to luminol have been developed, aryl oxalates and oxamines, aryl hydrazides (Nakashima *et al*. 1989; Jonsson, Emteborg & Irgum, 1998), and acridinium esters (Weeks *et al*. 1983). The low aqueous solubility of the aryl oxalates, oxamines and hydrazides coupled with their high loss of activity on conjugation have limited their use. However, acridinium esters provide highly active compounds which can be easily conjugated to proteins and antibodies to give very sensitive/reliable, accurate and simple immunoassays.

In work carried out in our laboratory, acridinium esters have been used in developing a number of highly sensitive, easy to use assays. This involved an extremely simple and rapid conjugation step to produce stable acridinium ester conjugates (Weeks *et al*. 1983). The light emission of these conjugates is readily triggered by the addition of an acidic hydrogen peroxide solution (H_2O_2 in HNO_3) followed by the addition of a sodium hydroxide solution containing a surfactant. The light generated is then counted over 2–5 sec in a luminometer. This can be done on a wide variety of commercially available plate reading luminometers. However, there is no need for such expensive equipment and inexpensive portable luminometers which can carry out measurements with sensitivity comparable to more expensive devices are available for under £1000.

Using this system we have developed a number of assays which show greatly improved sensitivity over those using conventional enzyme labels (see Fig. 13a) with levels of detection less than 1000 c.f.u. ml^{-1} compared to levels of 2×10^5 ml^{-1} using enzyme labels, allowing rapid detection of bacterial contamination in under 24 h, compared with over 48 h. Assays have been developed in which the previously used radioactive label (I^{125}) is replaced with acridinium ester without any loss of sensitivity or dynamic range (Fig. 13b) and avoiding the problems associated with radioactivity (short half life and waste disposal problems).

The ease of use of acridinium esters as an immunoassay label, both in terms of conjugation procedure and triggering of the light-producing

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reaction, coupled with the availability of inexpensive, reliable detection equipment allows this technique to be applied to areas such as near patient testing or screening in less developed countries where access to expensive equipment and highly trained staff is not applicable. However, the high sensitivity achievable with these labels and their ease of use and compatibility with automation also makes them ideal for use in centralized testing laboratories.

Immuno-polymerase chain reaction (*PCR*)

An extremely sensitive label for use in immunoassay can be provided by labelling antibodies with DNA or RNA fragments which can be detected after amplification by PCR. These techniques can provide amplification factors in the 10^6 range (25 cycles of PCR produce a 34 million-fold amplification for DNA and for RNA 4 cycles provides a 2–5 million-fold amplification) (Kricka, 1994), and a number of extremely sensitive assays have been developed based on this principle (Kakizaki *et al*. 1996; Case *et al*. 1997; Mahbubani *et al*. 1998). An immuno PCR assay detected 15 fg (10^{-19} M) IgG representing a 1000-fold increase in sensitivity over the same enzyme immunoassay (Niemeyer, Adler & Blohm, 1997), and an assay utilizing immuno-PCR detected the presence of 3·4 c.f.u. ml^{-1} of bacteria compared to $3{\cdot}4 \times 10^4$ ml^{-1} by enzyme immunoassay (Kakizaki *et al*. 1996).

Miscellaneous immunoassays

As well as the traditional and emerging immunoassay techniques discussed, a number of alternative types of immunoassay continue to generate interest. Most of these represent areas of active development and are not yet available or even widely applicable but are worth mentioning. Immunoassays based on electrochemical detection are being developed which include the estimation of enzyme products and the direct detection of non-enzymatic labels (Sadik & Vanemon, 1996; Qu, Berghman & Vandesande, 1998). Liposome immunoassays are another group of assays that promise to be of great interest. Liposomes are used to entrap large amounts of traditional labels, fluorescent compounds or enzymes, which are then released from the liposomes upon antigen-antibody binding. The main advantage with this form of assay is that the interaction of a single antibody can lead to the release of a large amount of signal, therefore making extremely sensitive assays a real possibility (Kim & Lim, 1993; Lim & Kim, 1997; Rongen, 1997; Okabayashi & Ikeuchi, 1998; Wink *et al*. 1998). Other areas of interest have included flow injection analysis or liquid chromatography to perform immunoassays which have the advantage of both speed and ease of automation.

CONCLUSIONS

Immunoassay technology has contributed greatly to the advancement of scientific understanding by offering the possibility of detecting, often for the first time, a vast range of biological molecules with exceptional accuracy, sensitivity and specificity. Importantly, these technologies have also proven to be crucial in the development of certain aspects of modern clinical practice, especially in the area of therapeutic drug monitoring.

Since the early 1970s the scope and diversity of immunoassays has witnessed phenomenal growth, with a vast array of different labels and assay strategies being developed to meet the requirements for sensitivity, accuracy and convenience of immunoassay. The development of more sensitive labels and detection equipment, coupled with advances in molecular biology making possible the production of fusion protein conjugates, and improvements in methods of monoclonal antibody construction, has seen a drastic reduction in the detection limit of immunoassays – a whole new nomenclature for the sub-attomole concentrations has now become essential (attomole = 10^{-18} M; zeptomole = 10^{-21} M; yoctomole = 10^{-24} M). Several labels and assay systems have been utilized in these ultra sensitive assays such as enzyme amplification, luminescence and fluorescence, time-resolved fluorescence with lanthanide chelates and electro-chemiluminescence; and the use of immuno-PCR has allowed detection levels as low as 1000 molecules.

Many of the advances in immunoassay technology will have limited applicability, as the need for expensive equipment and highly trained personnel limit the use of these techniques to a few centralized research or testing facilities. However, immunoassay techniques have also been developed which offer the same qualities associated with more costly systems (rapid, accurate quantification of minute amounts of analyte) without the need for expensive equipment or highly trained personnel.

Some labels and techniques are readily applicable to such assays. The light signal generated by luminescent and fluorescent labels (or luminescent substrates for enzymes) can be easily detected without the need for complex equipment, and can readily provide sensitivities in the low attomole region. Homogeneous, or separation-free, assays are ideally suited to this purpose, the simplified methodologies of these assays often compensating for the reduced sensitivity these assays have compared to their heterogeneous counterparts. Another area of more recent interest is immunosensors, and such technology has been applied to many different areas with a number of inexpensive, simple to use systems becoming available for point-of-care testing.

Dissatisfaction with various aspects of existing immunoassay performance (sensitivity, reliability,

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cost, speed and ease of use) will continue to drive the development of new labels and assay systems. Many of these developments will only be of use in large centralized laboratories. Nevertheless, the use and applicability of immunoassay technology to new and more diverse areas (point-of-care testing, screening of parasitic diseases in poorer countries, home testing, etc.), where they may once have been considered too expensive or awkward to apply, will be expanded by the development of assay systems delivering the required sensitivity, accuracy and throughput, in an inexpensive, easy to use format.

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