



Application Notes

How to optimize rapid and simple immunoassays

INTRODUCTION

The availability of monoclonal antibodies and the almost simultaneous progress in several non-isotopic labelling techniques have contributed to recent improvements in immunochemical assays. Especially the use of non-competitive assays has increased as they provide several advantages in performance and sensitivity when compared to competitive immunoassays (Ekins et al. 1985). The performance of an immunoassay is determined by parameters such as specificity, sensitivity, and reproducibility. When considering rapid and simple assays, particularly the factors that influence the reaction rate need to be optimized. Thus, in this study the effect of temperature, component concentration, dynamics, and quality of immunocomponents have been investigated.

When solid-phase alternatives were selected, membranes were not accepted because of the above requirements. The two most useful solid-phase supports, microparticles and microtitration wells, have their advantages and limitations. Particles provide a large surface, they are coated in batch form, and can give more rapid assay kinetics. On the other hand, the handling of particles is cumbersome and severe background problems may often occur. Microtitration wells are easily handled with available equipment and the background problem is insignificant. The capacity of the well surface is limited but adequate for most applications.

The detection principles and label technologies commonly used in immunochemical assays can be divided into four main categories: radio-activity, enzyme

activity, chemiluminescence, and fluorescence. The trend for some years has been to substitute radioisotopes for the nonisotopic techniques.

To exploit the full potential of a non-competitive assay design with regard to sensitivity, labels with very high specific activity, giving high signal-to-noise ratios are required. In addition, the technical aspects should be simple and useful for in-house research and routine applications. This requirement is not fulfilled by some of the new alternatives.

In the present contribution, parameters affecting the performance characteristics of rapid and simple immunoassays have been optimized. These parameters were temperature, concentration of immunocomponents, dynamics (including diffusion), and the quality of immunocomponents. In the final immunoassay, incubation was performed to equilibrium because kinetic assays are very difficult to control in a routine laboratory. Time-course studies were run to provide the necessary backup information. The work was carried out employing components that are generally available. In all assays polystyrene microtitration wells were used as the solid phase. Time-resolved fluorometry and europium chelates were chosen as the appropriate label technology (Soini 1989).



EXPERIMENTAL MATERIALS

The europium chelate of N^1 -(p-isothiocyanatobenzyl)-diethylenetriamine- N^1 , N^2 , N^3 , N^3 -tetra-acetic acid, the Enhancement solution used for europium determination by time-resolved fluorescence, and the monoclonal antibodies against hTSH, hLH, and estradiol were obtained from PerkinElmer Life Sciences.

The recombinant protein (Rp24fA5) for the HIV-1 assay was kindly provided by Pharmacia Genetic Engineering (La Jolla, CA). The europium-labelled estradiol was from Wallac Organic Chemistry Laboratory. In all assays polystyrene microtitre strips were used (Labsystems Oy, Helsinki, Finland or Nunc, Roskilde, Denmark).

The europium fluorescence was measured with a Wallac time-resolved fluorometer.

LABELLING

Depending on the conjugation conditions and the protein preparations, a 50 to 300-fold molar excess of europium chelate was used in conjugations performed overnight in carbonate buffer at pH 9.0-9.3 and 4° C. The europium-labelled protein was separated from excess of label by gel filtration on Sepharose-6B column (1.5 x 70 cm) and eluted with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.9% sodium chloride and 0.05% sodium azide. The number of europium ions incorporated per protein molecule was determined by measuring the fluorescence in comparison to that of known EuCl_3 standards. On the average, 5-15 europium ions were incorporated per protein molecule. Bovine serum albumin (1 mg/ml) was added as carrier to labelled protein solutions.

COATING OF MICROTITRATION STRIPS

Coating with antibody was performed by physical adsorption. Individual wells were treated with 0.2 ml of antibody preparation (5 µg/ml) in 0.1 M phosphate buffer, pH 4.9, for 20 h at room temperature, washed three times with saline, then saturated by treatment for 2 h with 0.3 ml of 0.05 M

Tris-HCl buffer, pH 7.4, containing 0.9% sodium chloride, 0.05% sodium azide, 0.5% bovine serum albumin. The saturated solution was aspirated and strips stored humid at 4° C until used.

IMMUNOASSAYS

All immunoassays were carried out in microtitration strip wells. The component concentrations and incubation conditions are provided separately in the legend of each figure. When the immunoreaction is complete, the europium ion is dissociated from the labelled immunocomponent (antibody, hapten, or recombinant protein) bound to the solid phase by adding the enhancement solution and then the europium fluorescence is measured by time-resolved fluorometry.

RESULTS AND DISCUSSION

In the optimization of rapid and simple immunoassays the influence of the following four parameters were separately studied: (1) temperature; (2) concentration; (3) dynamics; and (4) quality of immunocomponents. Influence of the assay design on the specificity and sensitivity is discussed in more detail by Dr. Karsilayan in *Commun. Lab. Med.* (1992).

(1) Temperature

In the collision theory of reactions it is assumed that the reaction rate is proportional to the frequency with which the reactants collide with each other and also to the probability that the collision is sufficiently energetic for a reaction to occur. Many reactions have rate constants that follow the Arrhenius equation, $k = A e^{-E_a/RT}$, where A is called the pre-exponential factor, E_a the activation energy and R the molar gas constant. Thus, the temperature dependence of the rate constant reflects the number of encounters that are sufficiently energetic to lead to reaction. The higher the temperature, the greater is the number of vigorous encounters assuming that no transition temperature exists which indicates e.g. protein denaturation. The temperature dependence of the reaction rate is frequently expressed in terms of a

temperature coefficient Q_{10} which is the factor by which the rate increases by raising the temperature 10°C .

A general assumption is that the reaction rate increases at least twofold when the temperature increases by 10°C . Obviously, the selected assay temperature will have a considerable effect on the reaction time for the immunoassay to reach equilibrium. The temperature effect on the reaction rate is shown in Fig.1 for the second incubation step of a two-step assay of human thyrotropin (hTSH) employing a non-competitive assay with two monoclonal

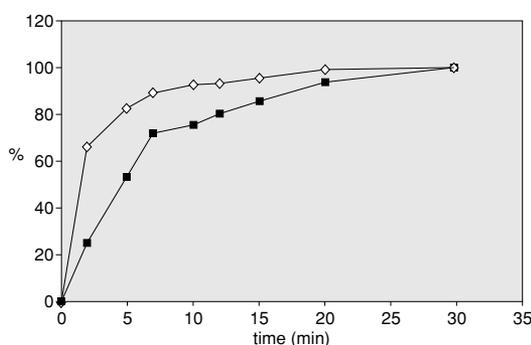


Fig.1. The effect of temperature on the second incubation step (labelled antibody) of a two-step non-competitive assay of hTSH. The total assay volume was $100\ \mu\text{l}$ and the tracer concentration $200\ \text{ng/well}$. The standard concentration was $9\ \mu\text{IU/ml}$. The assays were done at 25°C (■) and 35°C (◇), respectively.

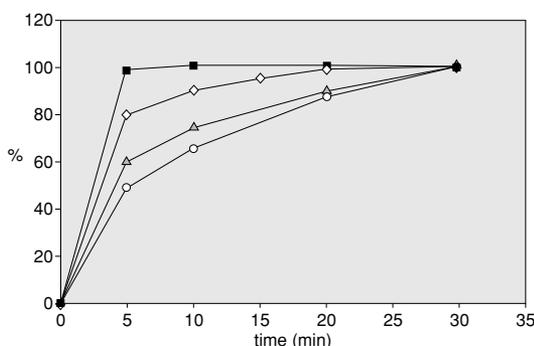


Fig. 2. The effect of the reaction volume on the kinetics of the first incubation of a non-competitive assay of hLH. $25\ \mu\text{l}$ of an hLH standard ($75\ \text{IU/l}$) was incubated with continuous shaking in the presence of various volumes (0 - $200\ \mu\text{l}$) of assay buffer. The subsequent incubation with labelled antibody was identical for all alternatives: (■) 0 , (◇) 100 , and (○) $200\ \mu\text{l}$ assay buffer.

antibodies. The temperature coefficient is about 3. After 2 min. of reaction, 67 and 25% of the maximal signal was reached at 35 and 25°C , respectively. The E_a is about $84\ \text{kJmol}^{-1}$ assuming that the Arrhenius plot is linear. Accordingly, the higher the activation energy, the steeper the temperature dependence of the rate constant.

(2) Concentration

The reaction between a single antibody binding site and antigen, via one antigenic determinant, can be described as a single equilibrium reaction in which the association rate follows second-order kinetics. Thus, the association rate of the reaction is proportional to the product of the concentrations of the two species of reactants, $V_{\text{ass}} = k_{+1}[\text{Ab}][\text{Ag}]$; the association rate constant (k_{+1}) being independent of the concentrations but dependent on temperature.

The rate of an immunochemical reaction can obviously be affected by changing the concentrations of the reactants. For a non-competitive immunoassay this can be accomplished without any major effect on the standard curve of the test, although some change in the working range can be expected. In a competitive assay the situation is completely different. A change in the concentration of the reactants, especially the antibody concentration, has an influence on the shape of the standard curve and consequently the sensitivity and working range of the test will change.

In a non-competitive assay the concentration of reactants can be increased in three ways, by decreasing the total reaction volume, by increasing the concentration of the labelled antibody, and by increasing the concentration of the solid-phase antibody (surface capacity). In each case an increase in reaction rate should be observed. The effect of a decrease in the total reaction volume is shown in Fig.2 when measuring human lutropin (hLH) with a two-step assay. The serum sample volume was constant ($25\ \mu\text{l}$) while the total assay volume was decreased (from 225 to $25\ \mu\text{l}$) in the first incubation. According to the time course of the reaction, the maximal signal (equilibrium) was reached in 10 min. when the smallest assay volume was applied. When the largest assay volume was used only 65% of the maximal

signal was obtained in 10 min. and it took 40 min. for the reaction to reach equilibrium.

The concentration of the labelled antibody was increased in a one-step non-competitive assay of hTSH (Fig.3). When a relatively low antibody concentration (50 ng/well) was employed, the reaction required 45 min. to reach equilibrium. With a high antibody concentration (600 ng/well) equilibrium was reached in 10-15 min.

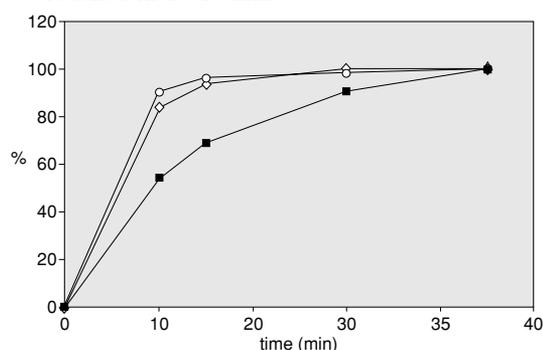


Fig. 3. Effect of various concentrations of labelled antibody on the kinetics of a one-step non-competitive assay of human TSH. 50 μ l hTSH standard (9 μ U/ml) was incubated with 50 μ l of different concentrations of tracer dilution. The assays were run with continuous shaking at room temperature. The amount of tracer in the well was 50 ng (■), 200 ng (◇), and 600 ng (○), respectively.

The concentration effect can thus be efficiently applied to increase the rate of non-competitive immunoreactions. Other assay parameters such as non-specific binding and specificity must, however, be carefully followed simultaneously in order to maintain or preferably improve the performance characteristics of the assay.

(3) Dynamics

The reaction rate is diffusion controlled when it is governed by the rate at which the reactant particles diffuse through the medium. A chemical reaction cannot proceed faster than the speed with which molecules meet by diffusion. This implies that every collision between the reactant particles is supposed to lead to a reaction. In a diffusion-controlled reaction the rate constant is in the order of $10^9 \text{ M}^{-1}\text{second}^{-1}$ or greater.

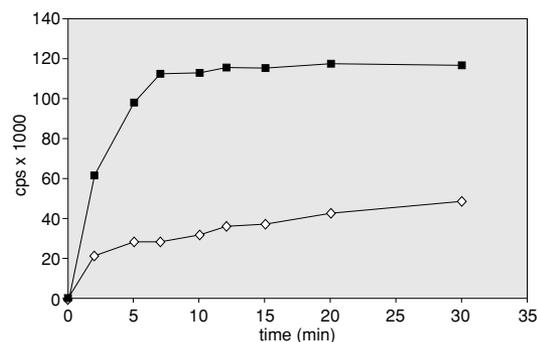


Fig. 4. The effect of mixing on the first incubation in a two-step non-competitive assay of hTSH. 50 μ l hTSH standard (9 μ U/ml) and 50 μ l of assay buffer was incubated with continuous mixing (■) or without mixing (◇) at room temperature. After the first incubation was completed, the wells were washed and 100 μ l of labelled antibody solution (200 ng/well) were added to the wells and mixed continuously for a fixed time.

In the fast diffusion-controlled immunoreaction the rate of the reaction can be increased by efficient mixing when the assay is carried out in coated microtitration wells using a limited amount of antigen or antibody that reacts with the surface-bound component. The situation is different in the second incubation of a two-step non-competitive assay, if the labelled antibody is present in large excess.

The importance of dynamics is demonstrated in the first step (analyte incubation) of a two-step non-competitive assay of hTSH (Fig. 4). With mixing, equilibrium is reached in less than 10 min, while it takes hours to reach equilibrium without mixing. As the second-order rate constants are identical, the diffusion rate has to limit the rate of the reaction when no mixing is used.

(4) Quality of immunocomponents

The quality of the immunocomponents that affect the rate of the immunoreaction can be deduced from the fact that the reaction between a single antibody binding site and antigen can be described by a simple equilibrium reaction in which association is a second-order reaction and dissociation follows first-order kinetics. The relevant kinetic and thermodynamic parameters are shown in Table 1. When rapid immunoreactions are set as the objective, the

theory based on interactions at equilibrium has to be supported by kinetics of association and dissociation. The range of the kinetic constants is also given in Table 1, although the available experimental data is quite limited (Mason et al. 1986; Hoylaerts et al. 1990).

Table 1. Thermodynamic and kinetic constants of immunochemical reactions

Constant	Range
Affinity constant, $K = k_{+1}/k_{-1}$	10^7 - 10^{11} M^{-1}
Association rate constant, k_{+1}	10^4 - 10^7 $M^{-1}s^{-1}$
Dissociation rate constant, k_{-1}	10^{-3} - 10^{-5} s^{-1}
(Half-life of the dissociation reaction, $t_{1/2}$)	700-70 000 s)

The affinity constants for antibodies are usually in the range of 10^7 - $10^{11}M^{-1}$. Monoclonal anti-bodies are found both against haptens and protein antigens with affinities $> 10^9 M^{-1}$. The difference in antibody affinity can be due to differences in association or dissociation rate.

In a model study four different monoclonal antibodies were compared in a one-step, noncompetitive assay of hTSH. Table 2 shows the affinity constant for four different monoclonal antibodies and the combination in which they were used in a one-step non-competitive assay. The labelled antibodies have an equal affinity for hTSH, while the solid-phase antibody of assay version B has a fourfold higher affinity compared to the solid-phase antibody in assay version A. When the time-course of the two assay versions is analysed, the reaction rate of assay B is sixfold when compared to assay A and equilibrium is reached in about 15 min (Fig. 5). It is important to realize that in addition to the affinity constants, the rate constants also affect the usefulness of an antibody for a rapid assay.

The time course of competitive assays is also influenced by the association rate constants of the utilized immunocomponents. Figure 6 shows the principle of two solid-phase competitive assays. In assay version I the hapten-specific monoclonal antibody is in solution and it is bound to the anti-mouse

Table 2. Affinity constants of monoclonal antibodies to hTSH and the combination in which they were used in a one-step non-competitive assay

Assay	Monoclonal antibody affinity	
	Solid phase M^{-1}	Labelled M^{-1}
A	5×10^9	2×10^{10}
B	2×10^{10}	1.8×10^{10}

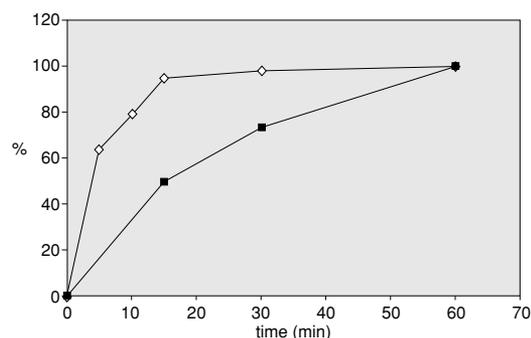


Fig. 5 Time course of reactions of two combinations of monoclonal antibodies (A and B as described in table 2) in one-step non-competitive assay of hTSH. 50 μ l hTSH standard (1.5 μ IU/ml) was incubated under continuous shaking with 200 μ l assay buffer containing 50 ng tracer antibody at room temperature. (■) Mab combination A; (◇) Mab combination B.

antibody on the solid phase during the analyte incubation. In assay version II the hapten-specific monoclonal antibody is preimmobilized before the analyte incubation. A considerable difference in the overall reaction rate of an estradiol assay is observed employing these principles (Fig. 7).

Assay version II reaches equilibrium in 30 min, while more than 1 h is required for assay version I. Obviously, the association rate for the hapten is quite rapid in both assay versions, but the association rate of the antibody to the solid phase is rate-limiting in assay version I. The selection of the hapten-specific monoclonal with a high association rate constant will naturally be of importance for the assay version. Moreover, the labelling of hapten molecules is likely to affect both the thermodynamic and kinetic parameters of the binding reaction.

MULTILABEL IMMUNOASSAYS

In clinical routine work there is frequently the need to measure two or more analytes from a single sample. In addition to the clinical needs there are other reasons why

double, triple or quadruple-label technologies would be especially valuable;

- savings in time, labour, reagents
- increased test throughput
- reduced overall costs
- internal control
- limited sample volumes

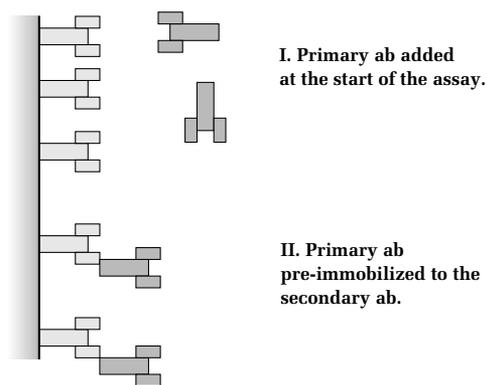


Fig. 6 Schematic representation of two principles (I and II) of competitive solid-phase assays. Secondary antibody is indicated with open and primary antibody with shaded symbols.

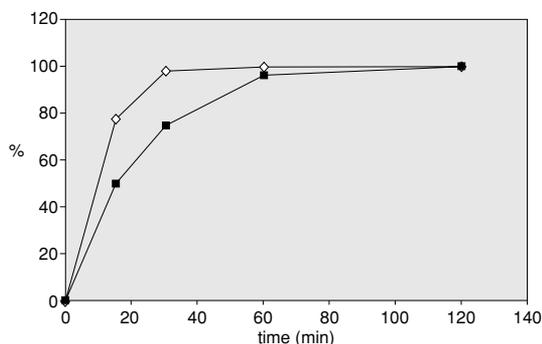


Fig. 7 Effect of two assay principles (I and II) as described in Fig. 6) on the time-course reaction of an estradiol assay. 25 μ l estradiol standard (0.5 nmol/l) was incubated in a total volume of 100 μ l under continuous shaking at 35°C. (■), principle I, where the estradiol monoclonal antibody was added in the assay buffer (◇), principle II, where the estradiol monoclonal antibody was preimmobilized to the solid-phase bound secondary antibody.

The development of two-site assay technologies based on monoclonal antibodies have created assays that have extremely high sensitivity and wide dynamic range, and consequently, the requirements placed on immunoassays in general and multilabel assays in particular are increased. Other label technologies do not fulfil these requirements due to the relatively narrow assay dynamics achieved – limited by signal cross-talk between the labels. The limitations of most of the label technologies are partly the reason for the relatively uncommon use of double-label technologies today. Time-resolved fluorometry when combined with lanthanide chelate labels offers, however, an ideal technology for multilabel assays. The function of the DELFIA reader in the assay optimization process is quite critical for multi-lanthanide measurements especially to correct for inter-lanthanide signal spillover. The Wallac 1420 VICTOR²™ multilabel counter is equipped with filters for Eu, Sm, Tb and Dy. The instrument program will correct for the lanthanide spillover. Labelling reagents are available for Eu and Sm labelling. For labelling with Tb and Dy, contact your local representative for labelling service details.

The ultimate goal for multilabel technologies is the measurement of whole panels of analytes simultaneously from a single sample. Even though it is possible to measure up to four labels simultaneously from a single well, assays have been described where prompt fluorescence labels and time-resolved labels have been combined allowing more than the original four determinations.

Table 2.

Analytes	Labels	Refs. No.
Double-label:		
LH and FSH	Eu + Tb	1
CA-50 and CEA	Eu + Tb	2
Potato viruses PVM and PVX	Eu + Sm	3
Human viruses Adeno and rota	Eu + Tb	4
TNF α receptor binding	Eu + Sm	14
Triple- and quadruple-label:		
TSH, IRT, T ₄	Eu + Sm + Tb	5
TSH, IRT, T ₄ , CKMB	Eu+Sm+Tb+Dy	6
HLA-DQB1 Genotyping	Eu, Sm, Tb	15
NK cell cytotoxicity	Eu, Sm, Tb	16

CONCLUSIONS

The outcome of the optimization of rapid and simple immunoassays can be summarized in the following general conclusions:

1. A generally available sensitive label technology, e. g., lanthanide chelates, has to be used.
2. Careful selection of immunocomponents and optimization of reaction conditions are required.
3. The design of Wallac equipment is based on an understanding of assay optimization needs.

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