APPLICATION NOTE

DELFIA Immunoassays

Key Features:

- Managing the effects of temperature, component concentration, dynamics and immunocomponent quality
- How to optimize these parameters for faster and simpler immunoassays
- DELFIA assays read using the VICTOR Multilabel Plate Reader enable multi-lanthanide measurements for savings in time, labour and reagents

How to Optimize Rapid and Simple Immunoassays

Introduction

The availability of monoclonal antibodies and the almost simultaneous progress in several non-isotopic labeling techniques have contributed to 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). 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.

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 investigated. In this study, the parameters temperature, concentration of antibodies, dynamics (including diffusion), and the quality of antibodies were optimized. 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. In all assays polystyrene microtitration wells were used, while time-resolved fluorometry and europium chelates were chosen as the appropriate label technology (Soini 1989).

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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 has been to substitute nonisotopic techniques for radioisotopes.

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 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-aceticacid, the Enhancement solution used for europium determination by time-resolved fluorescence, and the monoclonal antibodies against hTSH, hLH, and estradiol were obtained from PerkinElmer.

The recombinant protein (Rp24fA5) for the HIV-1 assay was kindly provided by Pharmacia Genetic Engineering (La Jolla, CA). The europium-labeled estradiol was used, and in all assays polystyrene microtiter strips were used (Labsystems Oy, Helsinki, Finland or Nunc, Roskilde, Denmark).

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

Labeling

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-labeled protein was separated from excess label by gel filtration on Sepharose-6B column (1.5 x 70 cm) and eluted with 0.05 M Tris-HCI 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 labeled 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, and 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 as 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, $k = AE^{-Ea/RT}$, where A is called the pre-exponential factor, Ea the activation energy and R is 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 the number of energetic 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 noncompetitive assay with two monoclonal antibodies; the temperature coefficient is about three. After 2 min of reaction, 67 and 25% of the maximal signal was reached at 35 and 25 °C, respectively. The Ea is about 84 $kJmol^{-1}$, assuming that the Arrhenius plot is linear. Accordingly, the higher the activation energy, the steeper the temperature dependence of the rate constant.

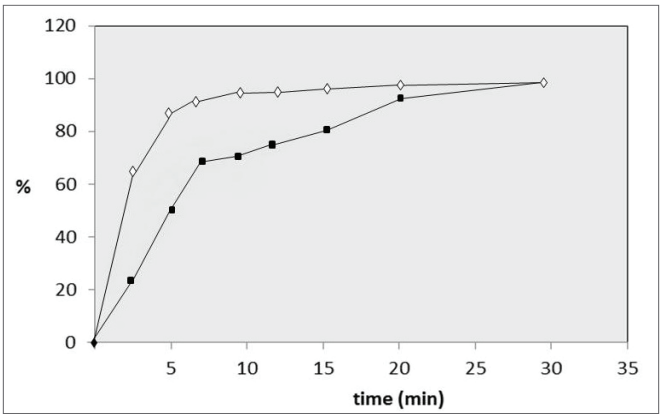

Figure 1. The effect of temperature on the second incubation step (labeled antibody) of a two-step non-competitive assay of hTSH. The total assay volume was 100 µl and the tracer concentration 200 ng/well. The standard concentration was 9 µIU/ml. The assays were done at 25 °C (■) and 35 °C (◊), respectively.

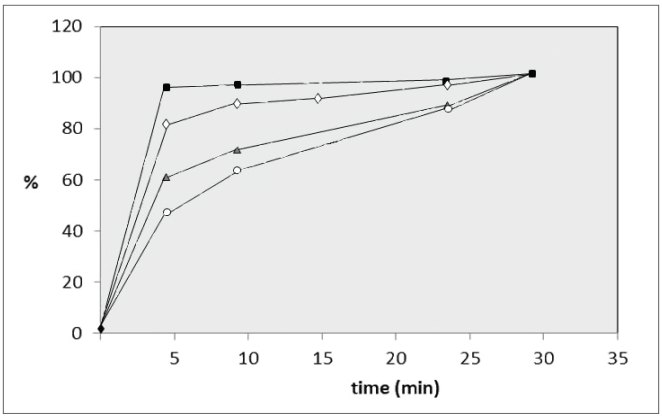

Figure 2. The effect of the reaction volume on the kinetics of the first incubation of a noncompetitive assay of hLH. 25 µl of an hLH standard (75 IU/l) was incubated with continuous shaking in the presence of various volumes (0-200 µl) of assay buffer. The subsequent incubation with labelled antibody was identical for all alternatives: (■) 0, (◊) 100, and (O) 200 µl assay buffer.

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, $V_{ass} = k_{+}$l[Ab] [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 µl) while the total assay volume was decreased (from 225 to 25µl) 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 labeled 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) equilibruim was reached in 10-15 min

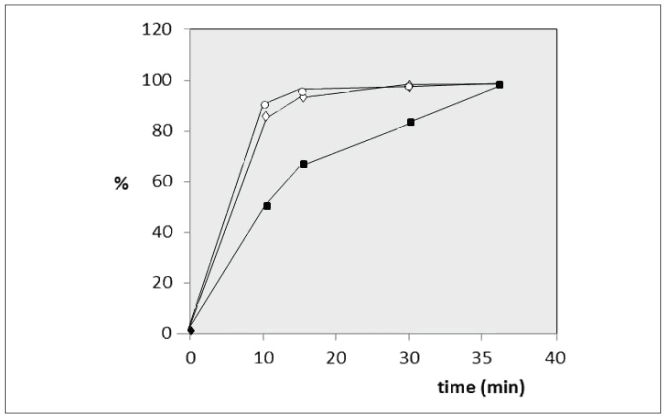

Figure 3. Effect of various concentrations of labeled antibody on the kinetics of a one-step non-competitive assay of human TSH. 50 µl hTSH standard (9 µIU/ml) was incubated with 50 µl of different concentrations of tracer dilutions. The assays were run with continuous shaking at room temperature. The amount of tracer in the well was 50 ng (■), 200 ng (◊), and 600 ng (O), 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 $M^{-1}second^{-1}$ or greater.

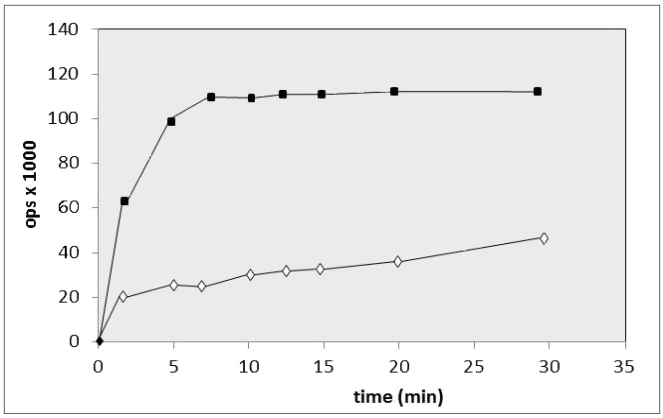

Figure 4. The effect of mixing on the first incubation in a two-step non-competitive assay of hTSH. 50 µl hTSH standard (9 µIU/ml) and 50 µl of assay buffer was incubated with continuous mixing (■) or without mixing (◊) at room temperature. After the first incubation was completed, the wells were washed and 100 µl 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 antibodies

The quality of the antibodies 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 etal.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 antibodies 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 labeled 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.

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} M^{-1}	Labelled
A	5×10^9	2×10^{10}
B	2×10^{10}	1.8×10^{10}

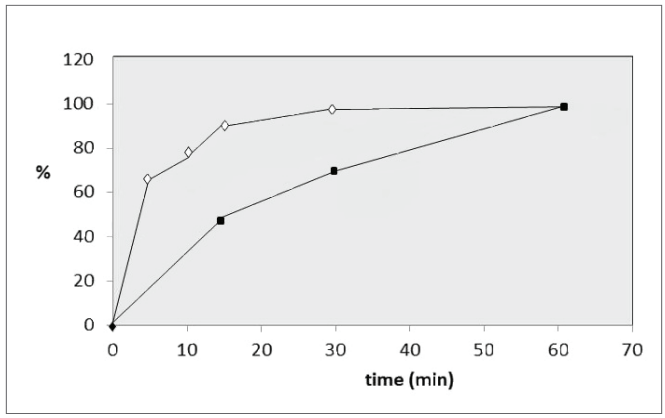

Figure 5. Time course of reactions of two combinations of monoclonal antibodies (A and B as described in table 2) in one-step noncompetitive assay of hTSH. 50 µl hTSH standard (1.5 µIU/ml) was incubated under continuous shaking with 200 µl assay buffer containing 50 ng tracer antibody at room temperature.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

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 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 labeling of hapten molecules is likely to affect both the thermodynamic and kinetic parameters of the binding reaction.

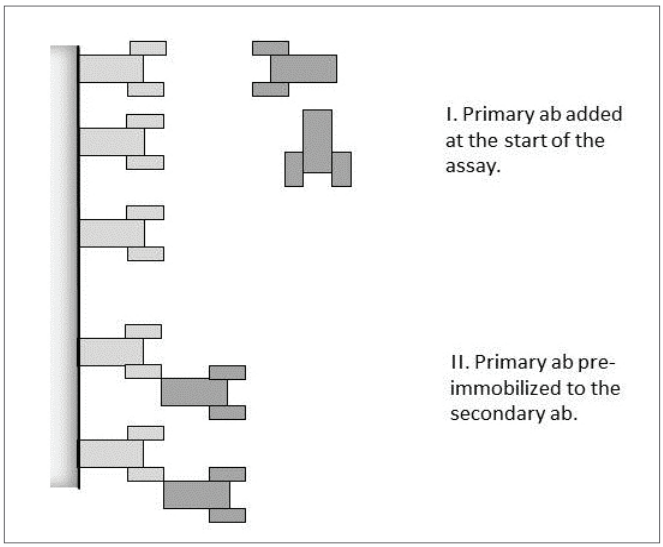

Figure 6. Schematic representation of two principles (I and II) of competitive solid-phase assays. Secondary antibody is indicated with open and primary antibody with shaded symbols.

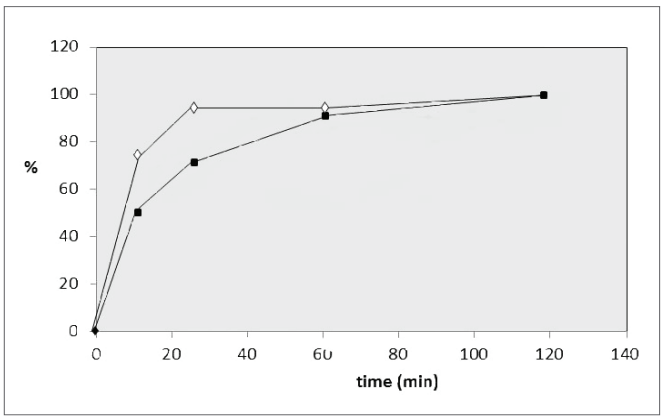

Figure 7. Effect of two assay principles (I and II as described in Fig. 6) on the time-course reaction of and estradiol assay. 25 µl estradiol standard (0.5 nmol/l) was incubated in a total volume of 100 µl under continuous shaking at 35°C. (Effect of two assay principles (I and II as), principle I, 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.

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

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 fulfill 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 VICTOR™ Multilabel Plate Reader is equipped with filters for Eu, Sm, Tb and Dy. The instrument program will correct for the lanthanide spillover. Labeling reagents are available for Eu, Tb, and Sm labeling. For labeling with Dy, contact your local representative for labeling 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 3. Examples of multilabeled detection using DELFIA immunoassays.

Analytes	Labels	Ref. 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
TNFα 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 antibodies and optimization of reaction conditions are required.
3. The design of VICTOR equipment is based on an understanding of assay optimization needs.

References and Additional Reading

1. Hemmilä et al, Double-label time-resolved immunofluorometry of lutropin and follitropin in serum, Clin. Chem. 33; 2281-83 (1987)
2. Holttinen S., et al, Double-label time-resolved immunofluorometric assay for simultaneous determination of cancer associated antigens CA-50 + CEA. Manuscript (1993)
3. Saarma M., et al, Simultaneous quantifications of two plant viruses by double-label time-resolved immunofluorometric assay. J.Virol.Methods 23; 47-54 (1989)
4. Siitari H., Dual-label time-resolved fluoroimmunoassay for the simultaneous detection of adeno-virus and rotavirus in faecas. J.Virol.Methods 28; 179-88 (1990)
5. Xu, Y-Y., et al, Triple-label time-resolved fluroimmunoassay of thyrotropin, 17a-hydroxyprogesterone and immunoreactive trypsin from dried blood spots. Abstract of paper presented in 8th Int. Neonatal Screening Symposium and Inaugural Meeting of the Int. Soc. for Neonatal Screening. Fairmont NSW. Australia (Nov. 1991)
6. Xu, Y-Y., et al, Simultaneous quadruple-label flurometric immunoassay of thyroid stimulating hormone, 17a-hydroxyprogesterone, immunoreactive trypsin and creatine kinase MM isoenzyme in dried blood spots. Clin. Chem. 38; 2038-43 (1992)
7. Lövgren, T., Leivo, P., Siitari, H., Pettersson, K., (1991) How to optimize rapid and simple immunoassays, In: A. Vaheri, R.C. Tilton, A. Bulows (eds), Rapid methods and automation in microbiology and immunology, Springer Verlag, Berlin 84 - 95.
8. Hoylaerts MF, Bollen A, De Broe ME (1990) The application of enzyme kinetics to the deter-mination of dissociation constants for antigen-antibody interactions in solution. J Immunol Methods 126: 253-261
9. Lövgren, T., Hemmilä, I., Pettersson, K., Eskola, J.U., Bertolt, E. (1984) Determination of hormones by time-resolved fluoroimmunoassay. Talanta 31:909-916
10. Mason DW, Williams RF (1986) Kinetics of antibody reactions and the analysis of cell surface antigens. In: Weir DM et al. (eds) Handbook of experimental immunology, vol 1. Immunochemistry. Blackwell Scientific Publications, Oxford pp 38: 1-34.
11. McAllan et el: Time Resolved Fluorescent and Resonance Energy Transfer-Based High-Throughput Protein Interaction Assays for Drug Discovery NMHCC's Second International Conference, Jan 1998, San Diego
12. Ilonen et al: Rapid HLA-DQB1 Genotyping for forur Alleles in the Assasment of Risk for IDDM in the Finnish Population. Diabetes Care, Vol 19, No 8, August 1996
13. Blomberg, Kaj: Simoultaneous measurement of NK cell cytotoxicity against each of three different target cells (J Imm Methods, 1993)

Acknowledgements

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