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Theoretical assessments of errors in rapid immunoassays—how critical is the exact timing and reagent concentrations?

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Abstract

The reliability of rapid immunoassay is a concern due to an incomplete incubation to a non-equilibrium state and is susceptible to different error factors causing variance. The most critical point in the process should be found in order to improve the accuracy, and reproducibility of immunoassays, and enhance the system robustness.

In this paper, the behavior of rapid assays is predicted by simulations using mechanistic assay model, based on antibody–analyte binding reaction kinetics. This antibody–analyte binding reaction kinetics model was constructed for a generic three-component (immunometric) assay and the parameters were chosen to be those of a known surface binding assay. The effects of the exact incubation timing and the initial reagent concentrations were studied focusing on the early phase of incubation, the non-equilibrium state. The magnitudes of errors in the input parameters were estimated using knowledge from practical immunoassays. According to simulations, inaccurate incubation timing adds error in the results at very short incubation times, especially in low analyte concentrations. The inaccurate reagent concentrations increase variance at short incubation times, as well. The error decreases rapidly after the first few minutes of incubation.

Keywords: Reaction kinetics; Immunoassay; Simulation; Mechanistic reaction model

1. Introduction

Rapid immunoassays rely on interrupted incubation. This yields a non-equilibrium reaction state within the assay system. Another approach is to use a system that enables on-line monitoring of binding, thus omitting the potential error sources from the washing step [1]. However, providing a quantitative result by any rapid system with good quality after a short incubation time is a challenge. An immunoassay, like all analytical procedures, has inherent errors and these errors can be classified into three kinds: systematic, statistical and catastrophic errors [2]. It is proposed that incubation to a non-equilibrium state may be more susceptible to different error factors causing variance to assay results [3]. Timing, the immunoassay preparations and other complex phenomena are influencing the entire process of analysis. The most critical point in the assay process should be found in order to minimize the variation, increase the reliability, and attain the system robustness.

In this paper the error factors are studied theoretically by simulation methods. Non-equilibrium assays are compared to equilibrium assays in order to study how much the interrupted incubation causes inaccuracy, imprecision and variance to the response, and consequently, affects the quality of the assay process. The measurement technique and detection method will also add up errors, but in these theoretical studies the detection technology dependent errors are not considered.

1.1. The assessments of errors in reaction kinetics

The reaction kinetics in immunometric assays [4–6] are affected by many different factors and some of those are more easily controlled than the others. The variations within initial materials, in assay preparation errors, and interactions in immunoassays, such as varying pH conditions, nonspecific binding, diffusion, and matrix effects of the sample, must be considered as a probable cause of deviation in the immunoassay

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response. The major external error source affecting the reaction kinetics is the temperature variation and mixing. Changes in the thermodynamics of immunoassays affect the antibody–antigen interaction. Incubation temperature variations contribute to assay drift, because the reaction rate approximately doubles for each 10 °C rise in temperature [7,8].

1.2. Assay system kinetic model

Different modeling approaches have been used to study the kinetic behavior of assay systems [5,6,9,10]. Pure mathematical models (such as descriptive and empirical) may produce a large number of solutions. A good model is valid for any occasion with varying input parameters and should provide an unambiguous answer, with a single set of input parameters. A mechanistic assay model considers the antigen–antibody reaction, laws of chemical reaction kinetics [2,11] and it offers identifiable, physical and chemical parameters such as time and concentration.

In a mechanistic model approach three-component immunometric assay reaction can be written as Eq. (1):

$$\begin{aligned}
&\mathrm{Ag} + \mathrm{Ab} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \mathrm{AgAb}\\
&\mathrm{Ag} + \mathrm{Ab}' \underset{k_4}{\overset{k_3}{\rightleftarrows}} \mathrm{AgAb}'\\
&\mathrm{Ab} + \mathrm{AgAb}' \underset{k_6}{\overset{k_5}{\rightleftarrows}} \mathrm{AgAbAb}'\\
&\mathrm{Ab}' + \mathrm{AgAb} \underset{k_8}{\overset{k_7}{\rightleftarrows}} \mathrm{AbAgAb}', \qquad (1)
\end{aligned}$$

where k_{2n-1} stands for association and k_{2n} for dissociation rate constants. Ab denotes the primary, solid phase binding reagent, Ag the analyte (sample) and Ab′ the secondary binding reagent, i.e. the labeled antibody. By determining the association and dissociation constants, the behavior of the immunoassay can be described.

The mechanistic assay reaction model has been shown to predict the behavior of the assay kinetics over a wide range of analyte concentrations [9,11]. In practice, the assay related association and dissociation kinetic constants k_n can be determined by setting differential equation for each component as a function of time, constructing a Jacobian matrix and solving it, Eq. (2). The kinetic constants in the model are derived for a practical surface binding assay described in our earlier publications for human thyroid stimulating hormone assay (hTSH) utilizing TPX technology [9].

$$\begin{aligned}
\frac{\mathrm{d}[\mathrm{Ag}]}{\mathrm{d}t} &= k_2\cdot[\mathrm{AgAb}] - k_1\cdot[\mathrm{Ag}][\mathrm{Ab}] + k_4\cdot[\mathrm{AgAb}']\\
&\quad - k_3\cdot[\mathrm{Ag}][\mathrm{Ab}]\\
\frac{\mathrm{d}[\mathrm{Ab}]}{\mathrm{d}t} &= k_2\cdot[\mathrm{AgAb}] - k_1\cdot[\mathrm{Ag}][\mathrm{Ab}] + k_6\cdot[\mathrm{AbAgAb}']\\
&\quad - k_5\cdot[\mathrm{Ab}][\mathrm{AgAb}']\\
\frac{\mathrm{d}[\mathrm{Ab}']}{\mathrm{d}t} &= k_4\cdot[\mathrm{AgAb}'] - k_3\cdot[\mathrm{Ag}][\mathrm{Ab}'] + k_8\cdot[\mathrm{AbAgAb}']\\
&\quad - k_7\cdot[\mathrm{Ab}'][\mathrm{AgAb}]\\
\frac{\mathrm{d}[\mathrm{AgAb}]}{\mathrm{d}t} &= k_1\cdot[\mathrm{Ag}][\mathrm{Ab}] - k_2\cdot[\mathrm{AgAb}] + k_8\cdot[\mathrm{AbAgAb}']\\
&\quad - k_7\cdot[\mathrm{Ab}'][\mathrm{AgAb}]\\
\frac{\mathrm{d}[\mathrm{AgAb}']}{\mathrm{d}t} &= k_3\cdot[\mathrm{Ag}][\mathrm{Ab}'] - k_4\cdot[\mathrm{AgAb}'] + k_6\cdot[\mathrm{AbAgAb}']\\
&\quad - k_5\cdot[\mathrm{AgAb}'][\mathrm{Ab}]\\
\frac{\mathrm{d}[\mathrm{AbAgAb}']}{\mathrm{d}t} &= k_7\cdot[\mathrm{Ab}'][\mathrm{AgAb}] - k_8\cdot[\mathrm{AbAgAb}']\\
&\quad + k_5\cdot[\mathrm{AgAb}'][\mathrm{Ab}] - k_6[\mathrm{AbAgAb}'] \qquad (2)
\end{aligned}$$

2. Simulation methods

Modeling increases understanding of complicated processes and enables optimization without performing the actual measurements. Carefully built models provide simulation responses to changing parameters and gradually help in understanding and describing the details of the kinetics of the reaction. The magnitudes of errors in the input parameters were estimated by a priori knowledge from practical immunoassays.

2.1. Kinetic model simulation

Mechanistic kinetic assay model simulations were constructed to simulate the surface bound reaction fraction concentration [AbAgAb′], the kinetic curves, as a function of reagent concentration [Ag] or as a function of incubation time with open source R language and packages, which are collections of R subroutines and function collections for solving specific tasks [12]. This open source R language and environment provides statistical techniques and can be extended via such packages. In this work the R odesolve-package was used as a solver for ordinary differential equations [13].

Immunometric human thyroid stimulating hormone (hTSH) assay was used as an assay example. The association and the dissociation rate constants for hTSH kinetic reaction had been measured and determined in our earlier work, Eq. (3) [9], where the measurements were carried out by observing the reactions at the surface of a microparticle in the TPX-system [14].

$$\begin{aligned}
&k_1 = k_5 = k_7 = 9{*}10^5\ \frac{\text{liter}}{\text{mole}{*}\text{s}}\\
&k_2 = k_6 = k_8 = 4.5{*}10^{-5}\ \mathrm{s}^{-1}\ (K_\mathrm{a} = 2{*}10^{10}\ \mathrm{M}^{-1})\\
&k_3 = 8{*}10^6\ \frac{\text{liter}}{\text{mole}{*}\text{s}}\\
&k_4 = 1.33{*}10^{-3}\ \mathrm{s}^{-1}\ (K_\mathrm{a} = 6{*}10^9\ \mathrm{M}^{-1})
\end{aligned} \qquad (3)$$

The simulation response as a function of the incubation time is ‘analogous’ to monitoring a real signal measurement response continuously. In the simulations, the ideal output values were compared with those created by adding random effects, or a constant error effect to the input parameters (i.e. time, concentrations). The random effects in our simulations followed Gaussian distribution statistics.

2.2. Simulation input set-up

The incubation time, the dynamic range, and, the magnitudes of error for simulations are estimated from previous practical assay experiences.

2.2.1. Concentrations

The concentration range for analyte [Ag] was chosen to cover the dynamic range of hTSH: 1.4 mIU/l–700.0 mIU/l, corresponding approximately to 10^{-11} mol/l–$5*10^{-9}$ mol/l. This concentration range was studied in 10 steps and special attention was paid to four different analyte concentrations: 4 mIU/l, 50 mIU/l, 150 mIU/l and 1200 mIU/l. These specific concentration values were selected for further studies also because real hTSH kinetic data had already been measured earlier for these concentrations [9]. A concentration of 1200 mIU/l was introduced as an example of analyte concentration exceeding the reagent capacity leading to the well-known "hook effect" and resulting in a false quantity in the read-out with an end-point analysis [6].

The value for the solid phase antibody concentration [Ab] was 0.9 nmol/l and for the labeled antibody concentration [Ab′] 1.0 nmol/l. A +10% and −10% error was induced into these concentrations in order to study the effect of extra and reduced antibody concentrations, [Ab] and [Ab′]. The same experimental conditions and set-up were assumed as in the article determining the association and the dissociation kinetic constants [9]. An error in both [Ab] and [Ab′] concentrations were introduced and the deviations in response were evaluated by comparing the simulated kinetic curve with the ideal kinetic curve as a function of the incubation time.

2.2.2. Incubation times

Kinetic curves as a function of 2.5 h incubation time were constructed by simulations, from the start of the incubation to the equilibrium state. The sampling rate was at 1 s intervals (measurement/s). The incubation times were studied more closely in a non-equilibrium state (60 s, 120 s, 300 s and 600 s), because the rapid assays are of interest.

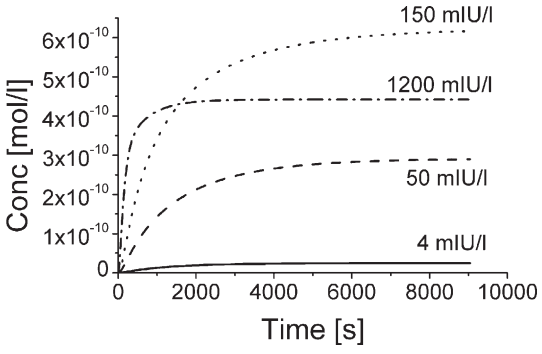

Fig. 1. Kinetic curves for analyte concentrations of 4 mIU/l, 50 mIU/l, 150 mIU/l, and 1200 mIU/l. The last concentration is an example of antigen concentration exceeding the antibody concentration. This phenomenon called "hook effect" saturates the signal curve to a lower level and will result to a false quantity in end-point analysis.

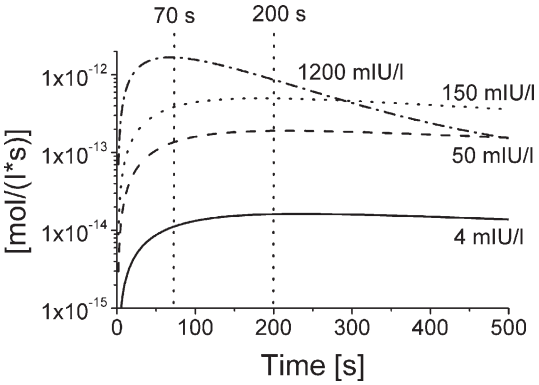

Fig. 2. The first derivative of ideal modeled kinetic assay curves for analyte concentrations of 4 mIU/l, 50 mIU/l, 150 mIU/l and 1200 mIU/l. The deflection point for 1200 mIU/l out-of-range concentration suffering from hook effect is reached already in 70 s, whereas the deflection point for concentrations on range is reached after 200 s.

The incubation timing errors induced into simulations were studied using both a randomized Gaussian error distribution and a constant absolute error. A relative error was not considered as an option, because timing errors are always absolute. The simulations were carried out by 100 replicates with this randomized error. Input variations were 3, 6 and 12 s as a standard deviation for the Gaussian distribution. Also a constant absolute error of 6 s was used as incubation timing error for all studied non-equilibrium reactions.

Ideal kinetic curves (without input errors and variance in response) were calculated for analyte concentrations of 4 mIU/l, 50 mIU/l, 150 mIU/l, and 1200 mIU/l (hTSH), Fig. 1.

The behavior of the reaction kinetics during the first 1000 s is studied by taking the first derivative of the ideal curves and finding out the inclination point of the growth curve [11]. The greater the derivative of the curve, the more sensitive it is to imprecisions. As seen in Fig. 2, the reaction kinetics is most sensitive to imprecisions in the early phase of the incubation, in a non-equilibrium state. However, the variance in response diminishes as the incubation time increases. The reaction with excess analyte concentration, leading to the hook effect, already reaches an inclination point after 70 s, however, the inclination point for analyte concentrations within the normal working range is reached in 200 s.

3. Results

3.1. The incubation timing variation simulations

The collection of simulation responses for Gaussian randomized variation in incubation timing simulations are shown in Fig. 3. In the response the coefficient of variation (% CV) for each analyte concentration and for four different interrupted incubation times (60 s, 120 s, 300 s, 600 s) are plotted as a function of the analyte concentration.

An overview of the studied concentration range (Fig. 3) shows that a variance in incubation timing leads to the largest effect in rapid assays with very short incubation times. The variance of incubation timing is even more critical when low

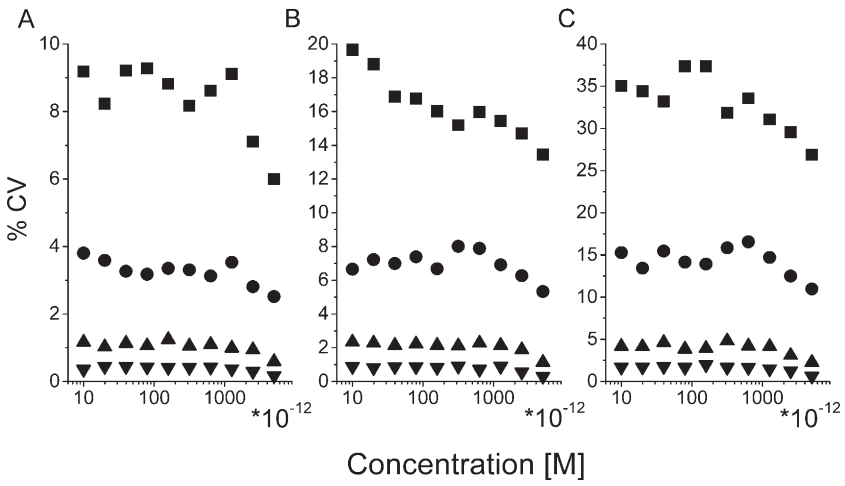

Fig. 3. Percentage of coefficient variation (% CV) of bound fraction (AbAgAb′) as a function of studied analyte concentration range and incubation time point with Gaussian randomized error defined by standard deviation of (A) 3 s, (B) 6 s and (C) 12 s. Incubation time points: 60 s (square), 120 s (circle), 300 s (triangle) and 600 s (upturned triangle). The % CV is calculated by using 100 replicates for each concentration point.

analyte concentrations are used. It is noteworthy that the response % CV drops significantly between 60 s and 120 s incubation times. An incubation time of 5 min (300 s) with a random Gaussian input error, $\sigma = 6$ s, results under 3% CV for all analyte concentrations. The relative response error caused by absolute timing error of 6 s, introduced in Fig. 4, is calculated by subtracting the kinetic curve with the error from the ideal curve. This points out the error effect in the early phase of the incubation and the drop of error due to incubation timing during the first hundreds of seconds. There was no major difference in the percentage of error in response detected in concentrations 4 mIU/l, 50 mIU/l and 150 mIU/l. However, the concentration beyond the assay capacity (1200 mIU/l), due to quick saturation of binding surface, shows a much lower deviation from the original than the analyte concentration in the working range.

3.2. Antibody concentration simulations

Kinetic curves for an ideal reaction and reactions with an extra and a reduced antibody concentrations for solid phase and labeled antibody were modeled. The response deviation caused by these inaccuracies in reagent concentrations is given as a relative error.

$$R_{\text{rel}} = |R_{\text{error}} - R_{\text{ideal}}|/R_{\text{ideal}}$$

The reduced antibody concentration (−10%) resulted in a greater response deviation than (10%) the extra concentration. This trend is seen in Fig. 5 for the inaccuracy in labeled antibody concentration and in Fig. 6 for the inaccuracy of solid phase antibody concentration, where the response error % is given as a function of the incubation time for studied analyte concentrations.

The behavior of relative error curves as a function of incubation time were different for solid phase and labeled antibody inaccuracies, see Figs. 5 and 6. It was also seen that as the analyte concentration increased, the error in the response increased as well.

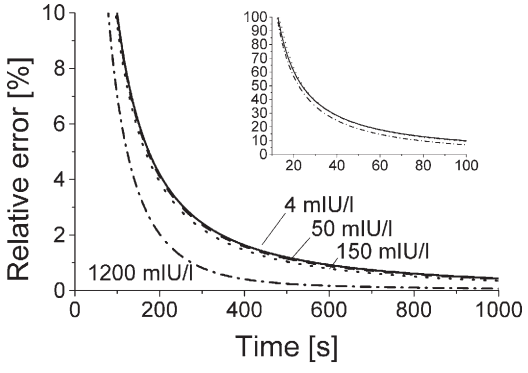

Fig. 4. Relative error in bound fraction, when absolute timing error is 6 s for 4 mIU/l, 50 mIU/l, 150 mIU/l and 1200 mIU/l. The insert points out the decrease of error in first hundred seconds.

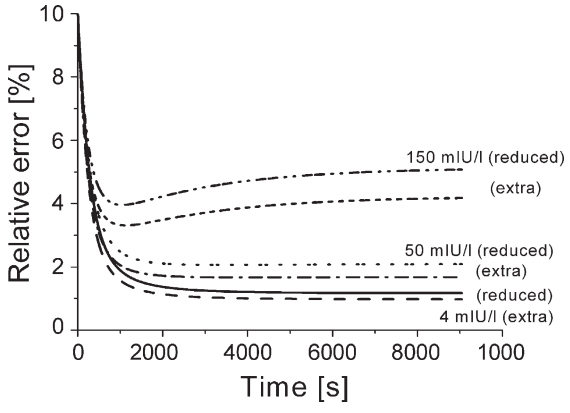

Fig. 5. Relative error in percentage caused by inaccuracy in labeled antibody concentration [Ab′] as a function of incubation time for analyte [Ag] concentrations of 4 mIU/l, 50 mIU/l and 150 mIU/l. The initial error induced was −10% for reduced and +10% for extra in [Ab′]. A local minimum of response error is detected for 150 mIU/l analyte concentration, after 1000 s of incubation.

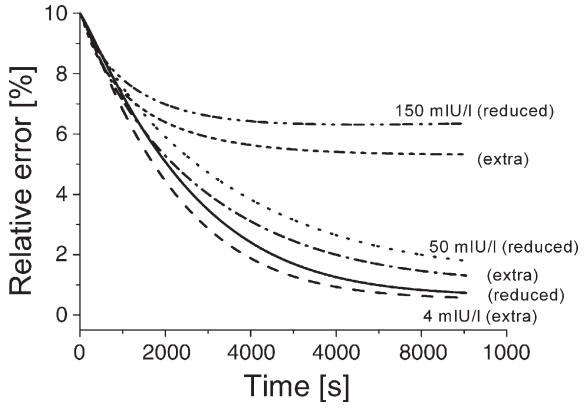

Fig. 6. Relative error in percentage caused by inaccuracy in solid phase antibody concentration [Ab] as a function of incubation time for analyte [Ag] concentrations of 4 mIU/l, 50 mIU/l and 150 mIU/l. The initial error induced was −10% for reduced and +10% for extra in [Ab].

The inaccuracy in [Ab] produced greater error in non-equilibrium state than [Ab′], even though, the deviation decreases as the reaction reaches the equilibrium for low concentrations.

4. Conclusions and discussion

4.1. Timing error

According to the simulation results, the inaccuracy in incubation timing is most critical for the assay response in the very early phase of the incubation, and, is even more critical when low reagent concentrations are measured. For the lowest analyte concentration (4 mIU/l) and for the shortest incubation time (60 s) with a $\sigma = 6$ s timing error, the % CV of the assay response approaches the limit of functional sensitivity (20% CV). However, the response % CV drops significantly between 60 s and 120 s incubation times.

A 5-min (300 s) incubation with an incubation timing error of $\sigma = 6$ s will result in a 3% CV response, for all studied analyte concentration values. Using an absolute timing error confirms this drop of response variance as the incubation time increases and the input timing error remains the same.

4.2. Reagent concentration error

The error in response, i.e. the error in the concentration of the bound fraction, due to reduced antibody concentration, was greater at the early phase of incubation. An inaccuracy of the initial concentration of solid phase antibody was more detrimental to the assay response than that of the labeled antibody in the early phase of incubation in rapid assays. This points out the fact that using excess of labeled antibody concentration in order to speed up the reaction is advantageous also with respect to the concentration errors of this reagent component. Though, for obvious reasons, the use of labeled antibody excess may be a drawback in one-step assay formats, if the unbound labeled antibody binding reagent is present during the signal measurement and the detection method does not suppress the signal caused by this.

Due to the reaction rates, the optimal reaction conditions for non-equilibrium rapid immunoassays may be different from those for equilibrium immunoassays. The assay conditions can be adjusted to the most optimal using simulation methods. However, the use of simulations based on mechanistic models requires the determination of kinetic rates for each assay material combination.

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References

[1] E. Soini, N.J. Meltola, A.E. Soini, J. Soukka, J.T. Soini, P.E. Hänninen, Two-photon fluorescence excitation in detection of biomolecules, Biochemical Society Transactions 28 (part 2) (2000) 70–73.
[2] Peter Ragatt, Data Processing, Principles and Practice of Immunoassay, Stockton Press, New York, 1991.
[3] Tero Soukka, Immunoassay Designs and Potential of Particulate Photoluminescent Lanthanide Reporters, Annales Universitatis Turkuensis AI 304, Doctorate thesis, University of Turku (2003).
[4] R. Ekins, Immunoassay Design and Optimisation, Principles and Practice of Immunoassay, Stockton Press, 1991.
[5] D. Rodbard, Y. Feldman, Kinetics of two-site immunoradiometric ('sandwich') assays—I. Mathematical models for simulation optimization, and curve fitting, Immunochemistry 15 (1978) 71–76.
[6] D. Rodbard, Y. Feldman, M.L. Jaffe, L.E.M. Miles, Kinetics of two-site immunoradiometric ('sandwich') assays—II. Studies on the nature of the 'high-dose hook effect', Immunochemistry 15 (1978) 77–82.
[7] D. Wild, The Immunoassay Handbook, MacMillan Press Ltd., London, 1994.
[8] M. Van Regenmortel, Thermodynamic parameters in immunoassay, Clinical Chemistry and Laboratory Medicine 36 (6) (1998) 353–354.
[9] P. Hänninen, M. Varis, M. Kettunen, E. Soini, Reaction kinetics of a two-photon excitation microparticle based immunoassay—from modelling to practice, Biophysical Chemistry 105 (2003) 23–28.
[10] E. Zuber, L. Rosso, B. Darbouret, F. Socquet, G. Mathis, J.-P. Flandrois, A descriptive model for the kinetics of a homogenous fluorometric immunoassay, Journal of Immunoassay 18 (1997) 21–47.
[11] E. Zuber, G. Mathis, J.-P. Flandrois, Homogeneous two-site immunometric assay kinetics as a theoretical tool for data analysis, Analytical Biochemistry 251 (1997) 79–88.
[12] R Development Core Team (2005). R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.r-project.org.
[13] R. Woodrow Setzer (2004). Odesolve: Solvers for Ordinary Differential Equations. R package version 0.5-12.
[14] Pekka Hänninen, Aleksi Soini, Niko Meltola, Juhani Soini, Jori Soukka, Erkki Soini, A new microvolume technique for bioaffinity assays using two-photon excitation, Nature Biotechnology 18 (2000) 548–550.