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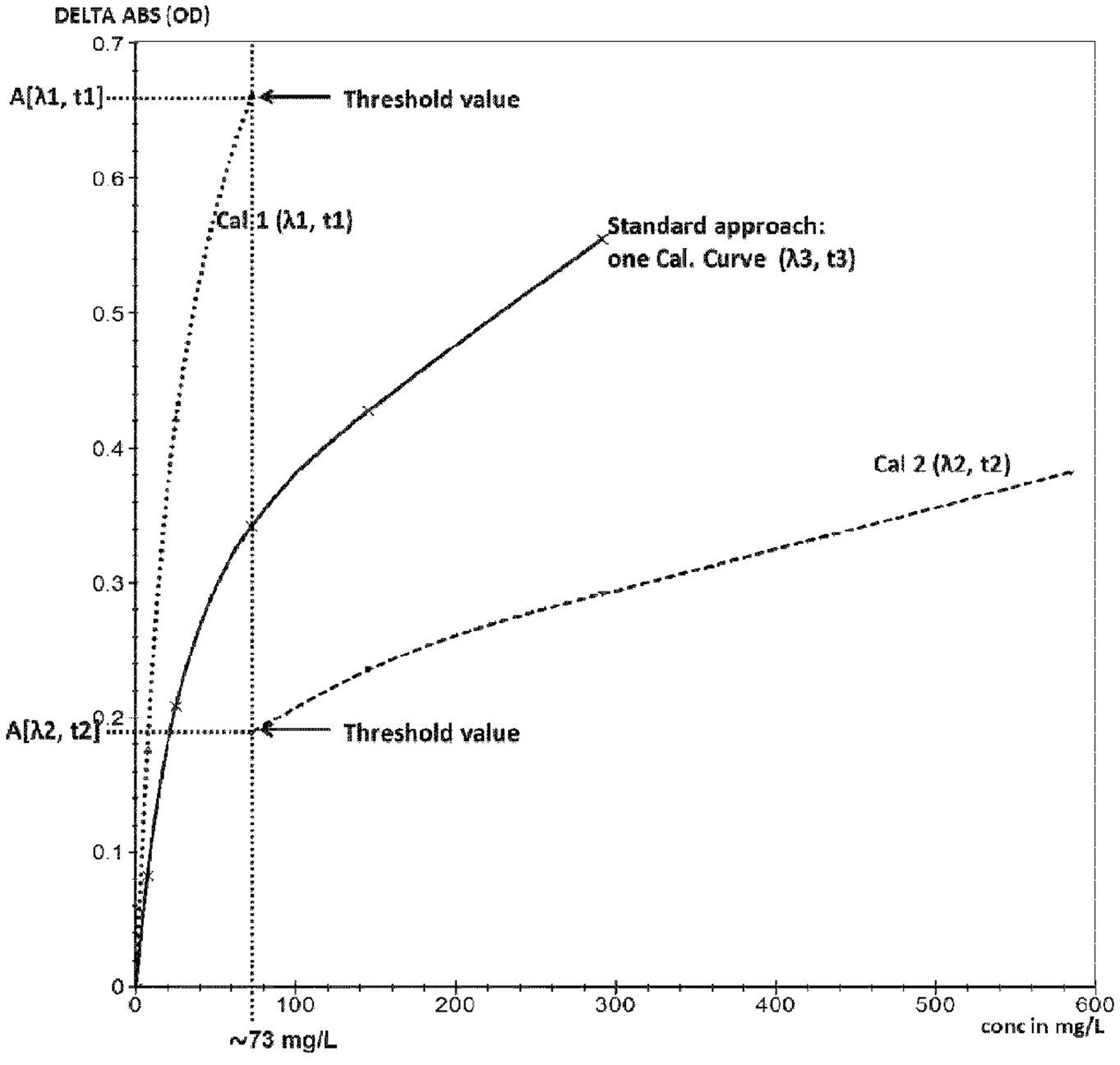
(54) **Titre : AMELIORATION DE LA SENSIBILITE ET DE LA PLAGE DYNAMIQUE D'ESSAIS PHOTOMETRIQUES PAR GENERATION DE MULTIPLES COURBES DE CALIBRAGE**

(54) **Title: IMPROVEMENT OF THE SENSITIVITY AND THE DYNAMIC RANGE OF PHOTOMETRIC ASSAYS BY GENERATING MULTIPLE CALIBRATION CURVES**



(57) **Abrégé/Abstract:**
The present invention relates to a method for determining the amount of a specific analyte by photometric assays, wherein the specific analyte in a sample reacts with an analyte specific reaction partner in a reaction mixture. At least two calibration curves are

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(57) **Abrégé(suite)/Abstract(continued):**
generating, the first calibration curve recorded at a first wavelength is optimized for low concentrations of the specific analyte thereby maximizing the lower detection limit and, the second calibration curve recorded at a second wavelength is optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit. The optimized lower detection limit and the optimized upper detection limit results in an extended dynamic range.

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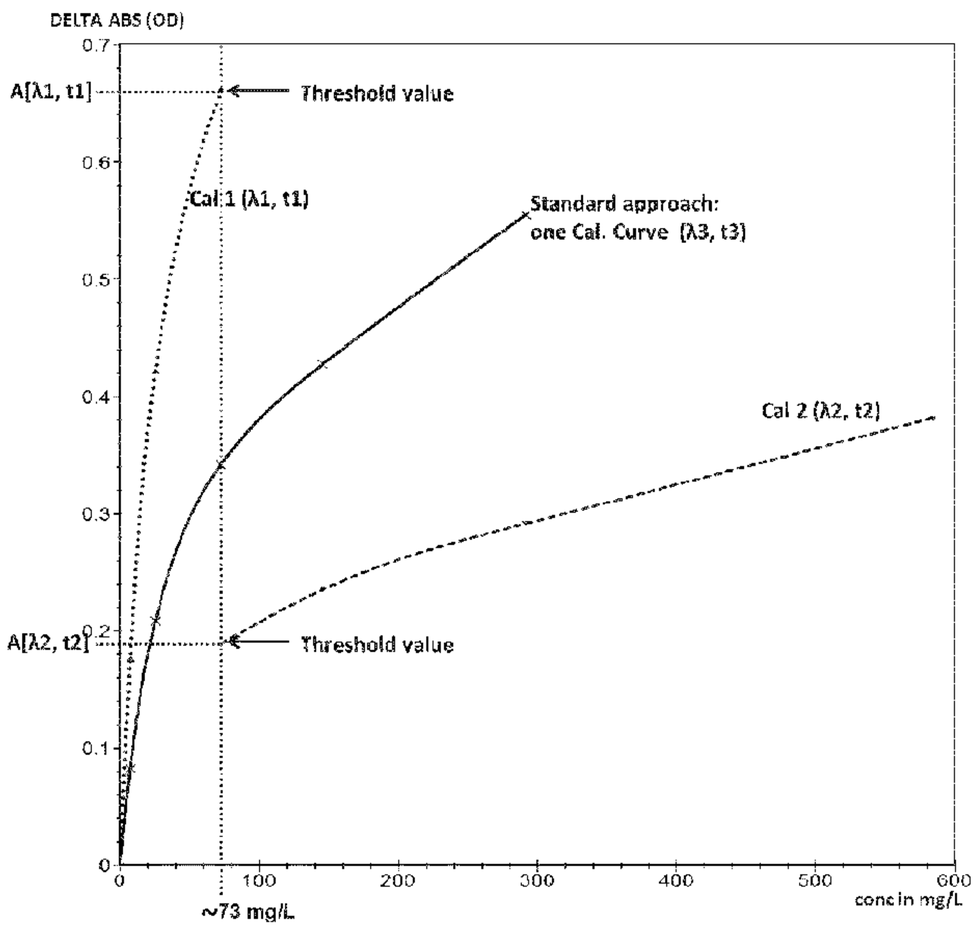
[Continued on next page]

(54) Title: IMPROVEMENT OF THE SENSITIVITY AND THE DYNAMIC RANGE OF PHOTOMETRIC ASSAYS BY GENERATING MULTIPLE CALIBRATION CURVES

Figure 3:



(57) Abstract: The present invention relates to a method for determining the amount of a specific analyte by photometric assays, wherein the specific analyte in a sample reacts with an analyte specific reaction partner in a reaction mixture. At least two calibration curves are generating, the first calibration curve recorded at a first wavelength is optimized for low concentrations of the specific analyte thereby maximizing the lower detection limit and, the second calibration curve recorded at a second wavelength is optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit. The optimized lower detection limit and the optimized upper detection limit results in an extended dynamic range.

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Improvement of the sensitivity and the dynamic range of photometric assays by generating multiple calibration curves

FIELD OF THE INVENTION

The present invention relates to a method for determining the amount of a specific analyte by photometric assays, wherein the specific analyte in a sample reacts with an analyte specific reaction partner in a reaction mixture. At least two calibration curves are generating, the first calibration curve recorded at a first wavelength is optimized for low concentrations of the specific analyte thereby maximizing the lower detection limit and, the second calibration curve recorded at a second wavelength is optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit.

In case of increasing calibration curves: The optical signal for the specific analyte of a sample to be determined in the reaction mixture is simultaneously measured at least at the first and second wavelength, thereby either the first or the second calibration curve is selected for quantification of the specific analyte. If the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength. If the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength.

In case of decreasing calibration curves: If the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength. If the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength. Finally, the amount of the specific analyte is quantifed by comparison with the selected calibration curve.

The method especially relates for increasing the sensitivity and / or the dynamic range of photometric assays by using different main wavelengths for determining the amount of a specific analyte by generating at least two calibration curves. Thereby, the first calibration curve records

the sensitive detection of the specific analyte, whereas the second calibration curve records the upper measuring range of the specific analyte.

RELATED ART

Agglutination is primarily a chemical phenomenon in which surface interaction between macromolecules leads to crosslinking and the formation of a large complex, resulting in an increase in light scattering. The formation of this large macromolecular complex can be observed using turbidimetric, nephelometric or colorimetric detection.

Turbidimetric, nephelometric and colorimetric assays have the benefits of being quasi-homogeneous assays which do not require any separation or wash step. Due to their easy one-step procedure and their short turn-around times homogeneous immunoassays (HIA) are ideal candidates for the application in automated analyzers. Homogeneous immunoassays are routinely used in the clinical diagnostics for the quantitation of serum proteins, therapeutic drugs and drugs of abuse on clinical chemistry analyzers. So far, the standard method is the use of one main wavelength generating one calibration curve for the determination of analytes in turbidimetric, nephelometric and colorimetric assays.

Although an excellent assay performance can be achieved with HIA on the clinical chemistry analyzers, there are still patient samples with concentrations which are outside of the measuring range. As consequence the samples have to be re-measured: after dilution in cases where the sample concentration exceeds the upper detection limit, or using higher sample volumes for samples with concentrations below the limit of detection. Re-measurements cause additional expenses and loss of time, both factors being critical for laboratories performing those assays. Therefore, an increase of the dynamic range would reduce the number of reruns. There are several approaches for solving the issues from the state-of the-art method.

EP 1845373 provides a method for simplifying the examination of samples with high concentrations of an analyte. Different calibration curves or different portions of a single calibration curve are used to calculate the analyte concentration depending on whether or not the sample contains high concentrations of analyte (prozone-positive). The specimen was mixed with an antibody and turbidimetric measurement of the samples was performed at a primary wavelength of 340 nm. A secondary wavelength of 800 nm is used for correcting interferences.

US 6,284,472 describes a calibration method that consists of generating two reaction rate measuring curves, the first curve generated using a sample of higher volume and the second curve using the same sample having a smaller volume. The turbidity was monitored at 340 nm and blanked by 700 nm. For antigen concentrations higher than the assay range, a high antigen signal utilizing the two rates avoids reporting false results.

JP 06109740 relates to an immunity nephelometry analyzing method by implementing optical measurements on plural kinds of standard specimens having different concentrations. A calibration curve is selected in response to the degree of the difference between the optical measured values twice at the initial stage of a reaction and after the progress and the virtual values calculated from the regression equation.

JP 06094717 describes a method for judging whether a sample is measured in proper antigen-antibody conditions or in prozone conditions in antigen antibody reactions. Two types of wavelengths are irradiated with the antigen-antibody reaction solutions to obtain a ratio of absorbances. When judged as the prozone condition, a shift factor is calculated, and a retest is performed in an analytical condition depending upon the shift.

JP 08075740 relates to a method of quantifying an antigen-antibody reaction by a nephelometry analyzing method by obtaining calibration curves for a plurality of wavelengths. The upper limit of the absorbance is pre-determined that satisfies the linearity for each wavelength as the quantitative determination limit. Thereby, the concentration of the subject analysis component is obtained by the calibration curve for the shortest wavelength that is equal or below the quantitative determination.

However, the above mentioned tools are far from optimal because the described methods do not solve the problem how to proceed with patient samples which are outside of the measuring range especially for automated systems. As a consequence the samples still have to be re-measured: After dilution in cases where the sample concentration exceeds the upper detection limit, or using higher sample volumes for samples with concentrations below the limit of detection. Remeasurements, so-called re-runs, cause additional expenses and loss of time, both factors being critical for laboratories performing those assays.

Furthermore, for several analytes two test systems are available for the measurement of high analyte concentrations and high sensitive test systems for the determination of a low analyte concentration. For example, for the analyte CRP (C-reactive protein) an additional high

sensitivity C-reactive (hs-CRP) immunoassay for cardiovascular risk assessment exists (Tina-quant Cardiac C-reactive protein (Latex) high sensitive) for the determination of low analyte concentration.

As a result, there is a high need for increasing of the sensitivity and extending of the dynamic range in photometric assays. The new approach should afford a minimal implementation and retention of an excellent precision without any changes of reagents and their formulations.

PROBLEM TO BE SOLVED

The problem addressed by the invention is therefore to provide an improved turbidimetric, nephelometric and colorimetric assay that offers an increased dynamic range and sensitivity. It has now surprisingly be found that generating of at least two calibration curves, a first calibration curve recorded at a first wavelength optimized for low concentrations of the specific analyte thereby maximizing the lower detection limit, and a second calibration curve recorded at a second wavelength optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit, lead to an improvement of the dynamic range and sensitivity of photometric assays. The present invention is expected to (at least partially overcome) the problem of remeasurements of samples.

SUMMARY OF THE INVENTION

At least one of these objectives is accomplished by the provision of the subject matter defined in the claims and herein below. In a first aspect, the present invention is directed to a method for determining the amount of a specific analyte by photometric assays, wherein the specific analyte in a sample reacts with an analyte specific reaction partner in a reaction mixture.

At least two calibration curves are generating, the first calibration curve recorded at a first wavelength is optimized for low concentrations of the specific analyte thereby maximizing the lower detection limit and, the second calibration curve recorded at a second wavelength is optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit.

The optical signal for the specific analyte of a sample to be determined is measured simultaneously in the reaction mixture at least at the first and second wavelengths. Either the first or the second calibration curve is selected for quantification of the specific analyte, by the following criteria:

For increasing calibration curves:

- if the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength;

- if the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength.

For decreasing calibration curves:

- if the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength;

- if the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength. The amount of the specific analyte is quantified by comparison with the selected calibration curve.

In turbidimetric and nephelometric immunoassays the specific analyte in a sample is quantified from the change in the turbidity of the reaction mixture based on the aggregation of the specific analyte and an analyte specific binding partner, wherein in colorimetric immunoassays the specific analyte in a sample is quantified with the aid of a color reagent.

In a second aspect the invention relates to a method for increasing the sensitivity and/or the dynamic range of photometric assays, wherein a first calibration curve recorded at a first wavelength is optimized for low concentrations of the specific analyte thereby maximizing the lower detection limit and, a second calibration curve recorded at a second wavelength is optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit.

In a third aspect, the invention relates to the use of at least two calibration curves for quantifying the amount of a specific analyte of photometric assays, wherein a first calibration curve recorded at a first wavelength is optimized for low concentrations of the specific analyte thereby maximizing the lower detection limit and a second calibration curve recorded at a second wavelength is optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention is directed to a method for determining the amount of a specific analyte by photometric assays, wherein the specific analyte in a sample reacts with an analyte specific reaction partner in a reaction mixture.

At least two calibration curves are generating, the first calibration curve recorded at a first wavelength is optimized for low concentrations of the specific analyte thereby maximizing the lower detection limit and, the second calibration curve recorded at a second wavelength is optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit.

The optical signal for the specific analyte of a sample to be determined is measured simultaneously in the reaction mixture at least at the first and second wavelengths. Either the first or the second calibration curve is selected for quantification of the specific analyte, by the following criteria for increasing calibration curves:

For increasing calibration curves:

- if the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength;

- if the at least one optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength.

For decreasing calibration curves:

- if the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength;

- if the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength. The amount of the specific analyte is quantified by comparison with the selected calibration curve.

The inventors of the present invention surprisingly found that the simultaneous measurement of a specific analyte at two different wavelengths and different reaction times generating at least two calibration curves from one run (one measurement) increases the dynamic range of photometric assays.

Photometric assays encompass turbidimetric and nephelometric immunoassays as well as colorimetric assays. In turbidimetric and nephelometric immunoassays the specific analyte is quantified from the change in the turbidity of the reaction mixture based on the agglutination of the specific analyte and an analyte specific binding partner, while in colorimetric assays the specific analyte is quantified with the aid of a color reagent.

The measurement at a first in most instances short wavelength at a first reaction time allows an optimal detection with respect to sensitivity, and the parallel measurement at a second in most instances larger wavelength at a second reaction time ensures a high upper detection limit, both resulting in an extended dynamic range compared to the measurement at only one wavelength at one reaction time. As a consequence, an extension of the dynamic range is achieved without the need to modify the assay reagents and their formulations, thus ensuring a fast and cost-effective realization. Subsequently, depending upon the signal value obtained for a sample, a suitable calibration curve is used for the quantification of the specific analyte. It is now possible to increase the dynamic range of the assay in the comparison with standard techniques.

It is a further embodiment of the present invention, that there are cases where a large wavelength at a first reaction time allows an optimal detection with respect to sensitivity, and the parallel measurement at a second smaller wavelength at a second reaction time ensures a high upper detection limit, both resulting in an extended dynamic range compared to the measurement at only one wavelength at one reaction time.

When performing an agglutination assay in the competitive inhibition format the measurement at a first in most instances larger wavelength at a first reaction time allows an optimal detection with respect to sensitivity, and the parallel measurement at a second in most instances shorter wavelength at a second reaction time ensures a high upper detection limit, both resulting in an extended dynamic range compared to the measurement at only one wavelength at one reaction time.

It is a further embodiment of the present invention,, there are cases where a small wavelength at a first reaction time allows an optimal detection with respect to sensitivity, and the parallel measurement at a second larger wavelength at a second reaction time ensures a high upper detection limit, both resulting in an extended dynamic range compared to the measurement at only one wavelength at one reaction time.

Definitions:

The term “determining” as used herein means assessing, diagnosing, deciding, identifying, evaluating, or classifying whether a specific analyte in a sample is quantified from the change in the turbidity of the reaction mixture based on the agglutination of the specific analyte and an analyte specific binding partner by turbidimetric and nephelometric immunoassays or whether the specific analyte is quantified with the aid of a color reagent in colorimetric assays.

The term “amount” as used herein is encompasses the absolute amount of an analyte or the relative amount and/or concentration of said analyte and/or any value and/or parameter which may correlate thereto and/or may be derived therefore.

The term “sample” as used herein is refers to a sample of a body fluid selected from blood, i.e. whole blood, plasma, or serum, or urine, CSF, sputum or to a sample of separated cells or to a sample from a tissue or an organ of a respective individual. Samples of body fluids can be isolated by well-known techniques. Tissue or organ samples may be isolated from any tissue or organ by, e.g., biopsy. Separated cells may be isolated from the body fluids or the tissues or organs by separating techniques such as centrifugation or cell sorting. Preferably, lysates from cell-, tissue- or organ samples are isolated from those cells, tissues or organs which express or produce the peptides referred to herein.

The term “agglutination” as used herein is primarily a chemical phenomenon in which surface interaction between macromolecule leads to crosslinking and to the formation of a large complex.

The formation of this large complex leads to an increase in light-scattering properties that, depending on the size of the complex, can be observed with the naked eye or monitored photometrically using turbidimetric and nephelometric detection (see Christopher P. Price, Encyclopedia of Life Sciences 2001, p. 1-7).

The assay method of the present invention can be any type of light scattering assay, in particular turbidimetric, nephelometric and colorimetric assays. The assay can be used for determining the amount of any specific analyte suitable to be determined by turbidimetric, nephelometric and colorimetric assays, i.e. any analyte for which there are reaction partners apt to be bound which specifically recognize the analyte.

The term "turbidimetry and nephelometry" are methods known in the art for determining the amount of cloudiness, or turbidity, in a solution based upon measurement of the effect of this turbidity upon the transmission and scattering of light. Turbidity in a liquid is caused by the presence of finely divided suspended particles. If a beam of light is passed through a turbid sample, its intensity is reduced by scattering, and the quantity of light scattered is dependent upon the concentration and size distribution of the particles. The spectrophometer measures the increased turbidity (i.e. the reduction of light in the intensity transmitted light), which is due to the increasing particle size resulting from the immunoagglutination reaction. This increased turbidity is a direct measure of the immunagglutination caused by the analyte or an indirect measure of the immunagglutination inhibition caused by the analyte. In nephelometry the intensity of the scattered light is measured, while in turbidimetry, the intensity of light transmitted through the sample is measured.

Turbidimetric assays involve measurement of the intensity of the incident beam as it passes through the sample. The light beam may pass through a suspension or be absorbed, reflected, or scattered by the particles. As a consequence, the intensity of light decreases as it is transmitted through the suspension. For nonabsorbing particles the decrease in light intensity due to scattering is expressed as turbidity.

Nephelometric assays refer to the measurement of the light scattered at an defined angle of θ from the incident beam when the incident beam is passed through the sample. In nephelometry the change in the intensity of the scattered light after a time is measured because the scattering

species rapidly increase size. The scattered light is proportional to the initial antigen concentrations when measured in the presence of a fixed antibody-latex complex. Further explanations are described by J.A. Molina-Bolivar et al., Journal of Macromolecular Science, Part C-Polymer Review, 45:59-98, 2005.

The immunoassay method of the present invention works with all known agglutination tests with and without microparticles enhancement.

Preferably used within the present invention is a “microparticle-enhanced light scattering agglutionation tests” which is also called “particle-enhanced turbidimetric immunoassays” (PETIA). Particle-enhanced immunoassays are routinely used in clinical diagnostics for the quantitation of serum proteins, therapeutic drugs and drugs of abuse on clinical chemistry analyzers, because they have the benefits of being quasi-homogeneous assays which do not require any separation or wash step. To enhance the optical detection between the specific analyte and an analyte specific binding partner in the reaction mixture, the analyte or the analyte specific binding partner is linked to suitable particles. Thereby, the analyte reacts and agglutinates with the particles which are coated with analyte specific binding partners. With increasing amount of analyte, the agglutination and the size of the complexes are increasing, leading further to a change of light scattering. The agglutinated particles are determined by turbidimetric and nephelometric measurements.

The material of the microparticles may be any inorganic, organic, or polymer material suitable for microparticle enhanced light scattering assays. The material of the microparticles may be any inorganic, organic, or polymer material suitable for microparticle enhanced light scattering assays. Such materials include for example selenium, carbon, gold; nitrides of carbon, silicium or germanium, e.g. Si3N4; oxides of iron, titanium or silicium, e.g. TiO2 or SiO2; and polymeric materials such as for example polystyrene, poly(vinyl chloride), epoxy resins, poly(vinylidene chloride), poly(alphanaphtyl methacrylate), poly(vinylnaphtalene), or copolymers thereof, in particular copolymers of styrene and a copolymerizable ethylenically unsaturated compound, e.g. styrene-(meth)acrylate co-polymers. Microparticles made of polymeric materials, as well as core-shell particles consisting of an inner core polymerized from styrene and an outer shell formed by copolymerization from styrene with a copolymerizable ethylenically unsaturated compound, as described e.g. in US Patent No. 4,210,723, are particularly suitable. The majority of particle based assays employ latex particles, with the predominant type of being polystyrene.

A review of latex agglutination test is described in (J. L. Ortega-Vinuesa, J. Biomater. Sci. Polymer Edn, Vol. 12, No. 4 pp.379-408 (2001).

There are different test formats for particle-enhanced turbidimetric immunoassays (PETIA), the competitive format and the direct format.

The direct format is preferably applied for analytes having a large size. These analytes are polyvalent antigens with multiple epitopes, e.g. proteins and microorganisms. For the direct format the particles are coated with antibodies which agglutinate with the analyte.

Turbidimetric and nephelometric assays may also be performed in a competitive inhibition format. This format is used most often to measure small molecules, such as haptens and is usually applied in diagnostics for the drugs of abuse testing and therapeutic drug monitoring. In this format the assay reagent not only contains an analyte specific binding partner, but also a chemically modified analyte obtained by attaching it to a microsphere surface or to another carrier molecule, such as a protein (e.g. bovine serum albumin) or a soluble polymer or oligomer. In contrast to the unmodified analyte, this reagent is able to agglutinate in the presence of an analyte specific binding partner due to the multiple copies of analyte present in the molecule. The analyte in a sample is quantified from the change in the turbidity of the reaction mixture based on the aggregation of the specific analyte and an analyte specific binding partner in the presence of the modified analyte.

The antigens are linked to a cross-linking agent e.g. polyhaptens, which competes against the antigen of the sample for the binding site of the antibody as shown in EP 545350. Here a soluble polymer, a protein or a microparticle acts as carrier molecule for multiple copies of the antigen. The amount of unlabeled antigen in the test sample is measured by its ability to compete with labeled antigen in the immunoassay. The unlabeled antigen blocks the ability of the labeled antigen to bind because that binding site on the antibody is already occupied. Thus, in a competitive immunoassay, less label measured in the assay means more of the unlabeled (test sample) antigens is present.

Preferably used is a microparticle enhanced light scattering agglutination assay for determining the amount of an analyte which comprises using a mixture of particles of strong light scattering properties carrying at least one binding partner of high reactivity for the analyte and particles of weak light scattering properties carrying at least one binding partner of low reactivity for the

analyte as described in EP 0898169. The particles of strong light scattering properties have a larger size and/or a higher refractive index than the particles of weak light scattering properties. The microparticle reagent for a microparticle enhanced light scattering immunoassay for determining the amount of an analyte, which comprises a mixture of microparticles of 30 to 600 nm in diameter, including particles of strong light scattering properties carrying at least one binding partner of high reactivity partner for the analyte and particles of weak light scattering properties carrying at least one binding partner of low reactivity for the analyte. The microparticles are usually approximately spherical with a narrow size distribution, a good representation of their size being their mean diameter. According to the law of light scattering (D: J. Newman et al., 1992, Ann. Clin. Biochem. 29, 22-42) strong light scattering properties result from a large particle size and/or a high ratio of the refractive index of the particle to that of the medium, whereas weak light scattering result from a small particle size and/or a low ratio of the refractive index of the particle to that of the medium.

The term “spectrophotometric assay”, also called “photometric assay”, is well known in the art. Photometric assays encompass turbidimetric and nephelometric immunoassays as well as colorimetric assays. In turbidimetric and nephelometric immunoassays the specific analyte is quantified from the change in the turbidity of the reaction mixture based on the agglutination of the specific analyte and an analyte specific binding partner, while in colorimetric assays the specific analyte is quantified with the aid of a color reagent. In spectrophotometric assays, the course of the reaction is followed by measuring a change in how much light the assay solution absorbs. If this light is in the visible region you can actually see a change in the color of the assay, these are called colorimetric assays. The MTT assay, a redox assay using a tetrazolium dye as substrate is an example of a colorimetric assay. UV light is often used, since the common coenzymes NADH and NADPH absorb UV light in their reduced forms, but do not in their oxidized forms. An oxidoreductase using NADH as a substrate could therefore be assayed by following the decrease in UV absorbance at a wavelength of 340 nm as it consumes the coenzyme.

Even when the enzyme reaction does not result in a change in the absorbance of light, it can still be possible to use a spectrophotometric assay for the enzyme by using a coupled assay. Here, the product of one reaction is used as the substrate of another, easily detectable reaction. An example for a coupled assay is the enzyme hexokinase, which can be assayed by coupling its production of glucose-6-phosphate to NADPH production, using glucose-6-phosphate dehydrogenase.

The term "colorimetric assays" according to the present invention are routinely used in the clinical diagnostics on highly automated clinical chemistry analyzers. Due to their easy one-step procedure and their short turn-around times homogeneous colorimetric assays are ideal candidates for the application in automated analyzers. A broad test menu for the clinical chemistry analyzers are actually offered e.g. Roche Diagnostics cobas® c. Colorimetric assays are characterized by formation or change or the depletion of the color in the presence of the analyte to be quantified. Typical colorimetric tests running on lab analyzers are the clinical chemistry tests and enzyme-immuno tests (CEDIA, EMIT). Such assays are detected by spectrophotometry in spectrometers. The detection of these assays on the cobas® c instruments is based on a photometer with a tungsten halogen lamp as irradiation source and photodiode array (12 diodes yielding 12 wavelengths between 340 and 800 nm) as detector. The O.D. is directly proportional to the concentration of the colored compound. If the development of color is linked to the concentration of a substance in solution, the concentration can be measured by determining the extent of absorption of light at the appropriate wavelength. An embodiment of the present invention is the method of the present invention, wherein in colorimetric immunoassays the specific analyte is quantified with the aid of a color reagent.

The term "color reagent" encompasses any assay reagent or a mixture of assay reagents that lead to a color change, color formation or color depletion of the assay that can be measured on the photometer with typical wavelengths ranging from 340 to 800nm. Many colorimetric assays involve an enzyme and the corresponding substrate which lead to colored products in a one- or more-step-reaction; the color change may be induced by corresponding enzymatic co-factors like NAD/NADH rather than by the substrate itself. There are also colorimetric assays based on the specific reaction of the analyte with a chemical reagent which leads to a colored product in a one or more step-reaction. In colorimetric immunoassays like EMIT or CEDIA the color is typically formed by the reaction of a reporter enzyme, like ß-galactosidase or a dehydrogenase, with its corresponding substrate leading to a product with characteristic and detectable absorption properties. The reaction of the reporter enzyme with the substrate typically takes place after the immunoreaction between analyte and antibody which then triggers or inhibits the enzymatic reaction. In other colorimetric tests, like typical clinical chemistry tests for lab analyzers, the color is formed, changed or depleted by the reaction of the analyte with enzymes or any other specific chemical reagent or a combination here-off. In some cases the analyte itself acts as enzyme. Even when the enzyme reaction does not result in a change in the absorbance of light, it can still be possible to use a spectrophotometric assay for the enzyme by using a coupled assay.

Here, the product of one reaction is used as the substrate of another, easily detectable reaction. An example for a coupled assay is the enzyme hexokinase, which can be assayed by coupling its production of glucose-6-phosphate to NADPH production, using glucose-6-phosphate dehydrogenase.

The term “Rayleigh scattering” is the elastic scattering of light or other electromagnetic radiation by particles much smaller than the wavelength of the light. The particles may be individual atoms or molecules. It can occur when light travels through transparent solids and liquids, but is most prominently seen in gases. Rayleigh scattering is a function of the electric polarizability of the particles. Scattering by particles similar to or larger than the wavelength of light is typically treated by the Mie theory, the discrete dipole approximation and other computational techniques. Rayleigh scattering applies to particles that are small with respect to wavelengths of light, and that are optically "soft" (i.e. with a refractive index close to 1). The amount of Rayleigh scattering that occurs for a beam of light depends upon the size of the particles and the wavelength of the light.

The term “analyte” according to the present invention encompasses any “in vitro diagnostic compound” such as e.g. serum proteins, therapeutic drugs and drugs of abuse. Representative analytes include, but are not limited to antigens, haptens, antibodies, proteins, peptides, amino acids, hormones, steroids, cancer cell markers, tissue cells, viruses, vitamins, nucleic acids, and pesticides. As used herein, an “analyte” or “specific analyte” refers to the substance whose presence and/or concentration in a sample is to be determined. The term “analyte” includes any substance for which there exists a specific reaction partner (e.g., a binding molecule or substance which specifically binds the analyte), or for which a specific binding partner can be prepared.

Analytes that can be determined by the assay of the invention include antigenic analyte, the binding partner then suitably being immunological binding partners. The antigenic analytes may be monomeric or polymeric, with or without repetitive epitopes.

Suitable antigenic analytes include:

a) Specific proteins such e.g. e.g. alpha-1-acid glycoprotein (AAGP), alpha-1-antitrypsin (AAT), albumin in serum (ALBS), microalbumin (ALBU), apolipoprotein A-1 (APOA), apolipoprotein B (APOB), antistreptolysin O (ASO), antitrombin III, (AT III), complement

C3c (C3C), complement C4 (C4), C-reactive Protein (CRP), fibrinogen (FIBG), fibronectin (FIBR), haptoglobulin (HAPT), immunglobulin A, G, M (IgA, IgG, IgM), lipoprotein a (LPA), rheumatoid factors (RF), transferrin (TRSF), serum amyloid A (SAA);

b) Tumor markers such as e.g. for example alpha-fetoprotein (AFP), human chorionic gonadotropin beta-subunit (b-HCG), beta-2-microglobulin, carbohydrate antigens such as CA 125, CA 15-3, CA 19-9, CA 72-4, carcinoembryonic antigen (CEA), ferritin, mucin-like carcinoma associated antigen (MCA), neuron specific enolase (NSE), prostate specific antigen (PSA);

c) Cardiovascular or fibrinolysis markers such as e.g. fatty acid binding protein (FABP), fibrin and fibrinogen degradation products (FDP), FDP D-dimer, troponin, myoglobin, glycated hemoglobin A1c (HbA1c);

d) Virus markers such as e.g. influenza virus, Herpes simplex virus (HSV);

e) Immunoglobin E (IgE), Insulin, Cystatin C

f) Immunsupressiva such as e.g. Cyclosporine, Everolimus, MPA (Mycophenolic Acid), Sirolimus, Tacrolimus

g) Therapeutic drugs such as e.g. Acetaminophen, Aminoglycosides, Amphetamines, Amikacin, Barbiturate, Benzodiazepines, Buprenorphine, Carbamazepine, Cocaine, Digoxin, Digitoxin, Ecstasy, Gentamicin, HIV Protease Inhibitors, Lidcaine, LSD, MDEA, Methadone, Methamphetamine, Methaqualone, 6-Monoacetyl-morphine, Napa, Procainamide, Phenytoin, Phencyclidine, Phenobarbital, Propoxyphene, Quinidine, Salicylate, Theophylline, THC, Tricyclic Antidepressants, Tobramycin, Vancomycin, VPA

Analytes that can be determined by the assay of the invention further include nucleic acids, the binding partners then suitably being oligonucleotide capture probes showing sufficient sequence complementarity for hybridization to take place. Suitable nucleic acid analytes include DNA, RNA and derivatives thereof, the determination of the amount of which is of interest in the diagnostic or pharmaceutical field. Examples of such nucleic acids that be quantitatively

determined using the assay of the invention are HIV1-RNA, HIV2-RNA, HCV-RNA, enterovirus RNA, HTLV-DNA, CMV-DNA and Mycobacterium tuberculosis DNA.

As an example, the analytes CRP (C-reactive protein), Ferritin, Myoglobin, D-Dimer, RF II (Rheuma-factor II), DIG (Digoxin) and Phenobarbital were determined in a sample according to the methods of the present invention.

The term "analyte specific reaction partner" as used herein is able to react with the specific analyte so as to form a reaction complex, like an antigen-antibody immunocomplex, or to form a new product, like the product resulting from an enzyme-substrate reaction. Typical analyte specific reaction partners include, but are not limited to, binding proteins, antigens, antigen fragments, antibodies, antibody fragments, nucleic acids, receptors and particle enhanced binding partners, enzymes, substrates (in cases where the analyte is an enzyme), specific chemical reagents leading to a color change in the presence of analyte. Such reaction partners specific for a given analyte may be obtained from commercial sources or may be prepared in accordance with standard procedures known to those skilled in the art. Examples of analyte specific reaction partner pairs include, but are not limited to, hapten:antibody, biotin:avidin, hormone:receptor, polypeptide:antibody, and oligonucleotide:complementary DNA or RNA, enzyme-substrate. For analyte specific reaction partners leading to the formation of a binding complex with the analyte, as it is the case with antibodies, the term "analyte specific bindin partner" can equally be used instead of "analyte specific reaction partner".

The term "antibody" as used herein refers to immunoglobulins that are produced in response to the detection of a foreign substance, and includes intact molecules as well as functional fragments thereof, such as Fab, F(ab′)2, and Fv. Antibodies that can be used as immunological binding partners in the assay of the present invention include polyclonal antibodies of any species, monoclonal antibodies of any species (including chimeric antibodies and/or recombinant antibodies). Because of their capacity of being produced identically in unlimited amounts, monoclonal antibodies or fragments thereof are generally preferred.

The term "antigen" as used herein is characterized by its ability to be bound at the antigen-binding site of an antibody. The region of an antigen that is recognized by an antibody, and to which the antibody binds, is referred to as an "epitope." An antigen is a substance which is

capable of inducing an immune response, i.e., antibody production, when introduced into an animal or human body. A hapten is a small molecule that can elicit an immune response only when attached to a large carrier such as a protein. The carrier may be one that also does not elicit an immune response by itself. Once the body has generated antibodies to a hapten-carrier adduct, the small-molecule hapten may also be able to bind to the antibody.

It is common practice to determine the concentration of an analyte by using a “calibration curve” (also commonly referred to as standard curve or working curve) which has been preliminarily drawn by plotting the interrelation between the known concentrations of the analyte in the standard samples and the analytical measured values such as optical densities of the standard samples. When the calibration curve has an adequate linearity over a wider range in the region of quantitative analysis, the calibration curve can be prepared with a relatively smaller number of standard samples, which are near the upper limit, lower limit and in the intermediate point in the determination range of the quantitative analysis.

In practice, however, there are many calibration curves which are not linear in general. The calibration curve of turbidimetric, nephelometric or colorimetric assays, prepared from the absorbance of a specific wavelength, typically is a nonlinear S-shape calibration curve where the sensitivity is deteriorated at the concentration near zero, and is saturated at a higher concentration side. The determination of the S-shape calibration requires a multipoint calibration where the use of the standard samples of the plurality of the concentrations is obliged. Furthermore, this S-shape calibration curve lacks of reportable measuring range at very low and at very high concentrations. Although several methods exist there are still patient samples with concentrations which are outside of the measuring range. As a consequence the samples have to be diluted in cases where the sample concentration exceeds the upper detection limit, or a higher volume of the samples have to be used for samples with concentrations below the limit of detection. These remeasurements, so-called re-runs, cause additional expenses and loss of time, both factors being critical for laboratories performing those assays. To avoid these disadvantages new methods are provided herein for increasing the sensitivity and extending the dynamic range of turbidimetric, nephelometric or colorimetric assays.

When generating a calibration curve for an agglutination assay based on measurements of the turbidity of the reaction mixture, the selection of the wavelength plays, beside the reaction time,

a crucial role for the slope (analytical sensitivity) of the curve and the achievable upper measuring range. For the direct assay format small wavelengths may lead to calibration curves with high slopes and high signals, whereas for the high concentration region the curves may become early a flat leading to comparable signal values for high concentrations and in consequence also to low upper detection limits. On the other hand larger wavelengths may lead to curves with small slopes but to distinguishable signal values for the high concentration region. The selection of the one wavelength and a corresponding reaction time for the signal calculation aimed at the generation of a calibration curve may therefore be a compromise between analytical sensitivity and upper measuring range. Similar situation is encountered in colorimetric assays. The selection of a wavelength near to the absorption maximum of the formed colored product ensures a high signal and high sensitivity, on the other hand the signals for high analyte concentrations may be outside of the specified optical range of the detector.

In the present invention two calibration curves are simultaneously generated for two different wavelengths and reaction times which surprisingly allow the quantification of a specific analyte in a sample with an increased dynamic range. For this purpose the sample is also measured using at least at these two different wavelengths and reaction times. At least one optical signal of the sample or at least one a signal value calculated hereof decides which calibration curve is to be used for its quantitation.

The term “first calibration curve” as used herein is generated from the optical signals of the reaction mixture at a first wavelength and a first reaction time, both optimized for achieving a low detection limit. The first calibration curve recorded at a first wavelength and a first reaction time is optimized for low concentrations of the specific analyte thereby maximizing the lower detection limit.

The term “second calibration curve” as used herein is generated from the optical signals of the reaction mixture at a second wavelength and a second reaction time, both optimized for achieving a high upper detection limit. The second calibration curve recorded at a second wavelength and a second reaction time is optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit. Working with two instead with one calibration curve as it is done in the present invention may also show further benefits such as to alleviate issues related with the number and concentrations of required calibrators as well as the curve-fitting procedure for the calibration curve. The calibration curves of the present invention are predetermined for the specific analyte by using a standard sample.

After preparing the measuring sample by mixing the sample with the analyte specific reaction partner and the analyte, which may be chemically modified, the reaction mixture is allowed to react for a given complete reaction time. The optical signal for the specific analyte of the sample to be determined in the reaction mixture is measured simultaneously at least at the first and second wavelengths over the complete reaction time.

Either the first or the second calibration curve, recorded at the first wavelength and a first reaction time or recorded at the second wavelength and second reaction time respectively, is selected for quantification of the specific analyte, wherein based on at least one of the optical signals of the sample or based on at least one signal value calculated hereof either the first or the second calibration curve is selected for the calculation of the analyte concentration, by the following criteria:

For increasing calibration curves:
If the optical signal of the first and / or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength and a first reaction time;
If at least one optical signal of the first and / or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength and second reaction time.

For decreasing calibration curves:

If the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength and second reaction time;

if the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength and first reaction time. The amount of the specific analyte is quantified by comparison with the selected calibration curve.

The present invention provides a method for determining the amount of a specific analyte by photometric assays, wherein the specific analyte in a sample reacts with an analyte specific reaction partner in a reaction mixture. Depending on the spectrophotometer and the available wavelengths at least two calibration curves for a first and a second wavelengths at a first and

second reaction time are generated, which were predetermined for the specific analyte. Thereby the optical signal for the specific analyte of a sample to be determined is measured simultaneously in the reaction mixture at the first and second wavelengths.

The term "first wavelength" and first reaction time as used herein are optimized for low concentrations of the specific analyte thereby maximizing the lower detection limit. That means the first wavelength in combination with the first reaction time generates e.g. high signals in case of the direct assay format, leading to a calibration curve which has a high analytical sensitivity. Sensitivity, analytical sensitivity, lower detection limit (LDL), limit of blank (LOB), limit of detection (LOD) and limit of quantitation (LOQ) are terms used to describe the smallest concentration of a measurand that can be reliably measured by an analytical method. All of these terms are related but have distinct definitions (siehe Lit. clin biochem rev 2008, 29, 49). For example the term "analytical sensitivity" is defined as the slope of the calibration curve.

The term "lower detection limit" (LDL) as used herein is also called lower measuring range. A typical approach to estimate the LDL consists of measuring replicates, such as n= 21, of a zero calibrator or blank sample, determining the mean value x and standard deviation (SD). The LDL is calculated as x+2SD or x+3SD. This method for the LDL determination is according to the method described by Kaiser (H. Kaiser, Fresenius Zeitschrift für analytische Chemie, 1965, 209, Nr.1, pages 1-18). If the least one optical signal of a sample (out of the optical signals of the sample simultaneously measured at least at the first and second wavelengths over the complete reaction time) or the at least one signal value calculated hereof is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by comparison with the calibration curve of the first wavelength and the first reaction time for assays with increasing calibration curves. In case of competitive agglutination assays or in case of colorimetric assay with falling calibration curves, the calibration curve of the second wavelength and the second reaction time would be used, if the least one optical signal of a sample (out of the optical signals of the sample simultaneously measured at least at the first and second wavelengths over the complete reaction time) or the at least one signal value calculated hereof is lower than a corresponding predetermined threshold value.

The term "second wavelength" and second reaction time as used herein is optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit. That means the second wavelength and the second reaction time generates e.g. distinguishable signals for

different analyte concentrations in the upper measuring range in case of the direct assay format leading to a calibration curve with a high upper measuring range.

The first and second wavelengths are different, ideally by at least 5nm, while the first and second reaction times may be different or identical.

The term "upper detection limit" (UDL) as used herein is also called the upper measuring range. The UDL is the highest amount of the analyte in a sample that can reliably be determined. In the present invention the UDL was determined by evaluating the linearity of the method and then selecting the highest concentration value within the linear range as the UDL. The method is said to be linear when the analyte recovery from a series of sample solutions (measured value) is proportional to the actual concentration of the analyte (true value) in the sample solutions (Arch Pathol Lab Med 2004, 128, pages 44-48). The form of the calibration curve, which can be parabolic or sigmoid-shaped, should not be confused with the linearity of the method which describes the relationship between the measured value and the true value. The calibration curve describes the relationship between signal and concentration.

The term "dynamic range" in the context of the present invention describes the magnitude of the measuring range of an assay and is here defined as ratio of upper detection limit (UDL) to lower detection limit (LDL). If not indicated otherwise we use the term measuring range as concentration values starting at the LDL and ending at the UDL. Principally other sensitivity terms may be used than the LDL, like LOD or LOQ, and also other terms describing the upper measuring range than the UDL may be used to calculate the dynamic range.

Both wavelengths, the first and the second wavelength, are the so called "main wavelengths" according to the state of the art for measuring the analyte.

The term "optical signal" as used herein describes the signal that is obtained by performing a absorbance measurement of the reaction mixture. The optical signal may be an absorbance value in case of turbidimetric and colorimetric assays or a scattered light signal for nephelometric assays.

The term "signal value calculated hereof" in the context of the present invention means a change of the optical signal of the reaction mixture, and may be the difference between two absorbance measurements, an initial and a final measurement, or may be a kinetic parameter expressing the

absorbance change per time. Usually, the initial measurement may be performed before or shortly after the final reagent is added to the reaction mixture.

The term "reaction time" as used herein is the time period between the first (or initial) and second (or final) measurement of the optical signal which is used for the calculation of a signal value hereof. The first (or initial) measurement is performed before or shortly after the final reagent is added to the reaction mixture. In case of kinetic measurements the reaction time may be the time period used for the calculation of the value expressing the absorbance change per time. The "reaction time" may be identical or shorter that the "complete reaction time".

The term "specific analyte" in the context of the present invention means that for each analyte in a sample to be measured, specific calibration curves and specific wavelengths and reaction times may be determined which are optimized for each specific analyte to quantify the concentration and which may differ from analyte to analyte. Common photometers are spectrophotometers or turbidimeters for turbidimetric immunoassays and nephelometers for nephelometric immunoassays. A multiple wavelength spectrophotometer known in the art is used for measuring the optical signal for the specific analyte of a sample to be determined in the reaction mixture according to the present invention. Preferably used for colorimetric assays and turbidimetric and nephelometric immunoassays is a spectrophotometer.

Depending on the architecture of the chosen spectrophotometer and the available wavelengths, which may differ from device to device, a first and a second wavelength is selected out of multiple wavelengths. The photometer as used herein is the Roche´s analyzer cobas c® 311 having the ability to measure 12 wavelengths between 300 nm to 800 ± 2 nm simultaneously. Preferably used are the wavelengths 340, 376, 415, 450, 480, 505, 546, 570, 600, 660, 700, 800 ± 2 nm. The method of the present invention is especially advantageous if used in automated analyzers, such as the cobas c 311, having the capability of measuring multiple wavelengths simultaneously. The measurements are preferably performed at a defined temperature, preferably between 20 and 40 degree Celsius.

As an example, the first and second wavelengths and the first and second reaction times for the generation of the first and second the calibration curves aimed at the quantification of several specific analytes were determined according to the present invention on Roche´s analyzer cobas

c® 311 under standard conditions. The instrument automatically pipettes the sample and the assay reagents into reaction cells (= cuvette). The instrument measures the absorbance simultaneously at 12 different wavelengths: 340, 376, 415, 450, 480, 505, 546, 570, 600, 660, 700, 800 ± 2 nm. For each reaction cell, a water-blank is measured and then absorbance readings are taken 57 times in 10 minutes, thus yielding a total of 57 measure points for the absorbance at each wavelength. The concentration can be calculated by using at least one of these measurement points. The measurements are performed at 37 degree Celsius.

For the analyte CRP (direct assay format) the first wavelength was determined by 505 nm and second wavelength by 600 nm, while the standard wavelength is 570 nm. The first reaction time was determined by 2.0min and second reaction time by 1.2min, while the standard reaction time is 2.0 min. For the first and second calibration curves as well as for the standard calibration curve 800nm was recorded and used for correction purposes by blanking.

For the analyte Ferritin (direct assay format) the first wavelength was determined by 505 nm and the second wavelength by 570 nm, while the standard wavelength is 570 nm. The first reaction time was determined by 4.9min and second reaction time by 5.1min, while the standard reaction time is 5.1 min. For the first and second calibration curves as well as for the standard calibration curve 800nm was recorded and used for correction purposes by blanking.

For the analyte Myoglobin (direct assay format) the first wavelength was determined by 505 nm and the second wavelength by 660 nm, while the standard wavelength is 570 nm. The first reaction time was determined by 6.7min and second reaction time by 2.6min, while the standard reaction time is 4.7 min. For the first and second calibration curves as well as for the standard calibration curve 800nm was recorded and used for correction purposes by blanking.

For the analyte D-Dimer (direct assay format) the first wavelength was determined by 800 nm and the second wavelength by 700 nm, while the standard wavelength is 800 nm. The first reaction time was determined by 4.3min and second reaction time by 1.4min, while the standard reaction time is 4.3 min.

For the analyte Rheuma-factor II (direct assay format) the first wavelength was determined by 546 nm and the second wavelength by 660 nm, while the standard wavelength is 570 nm. The

first reaction time was determined by 8.5min and second reaction time by 1.4min, while the standard reaction time is 2.2 min. For the first and second calibration curves as well as for the standard calibration curve 800nm was recorded and used for correction purposes by blanking.

For the analyte DIG (digoxin) (competitive assay format) the first wavelength was determined by 660 nm and the second wavelength by 505 nm, while the standard wavelength is 660 nm. The first reaction time was determined by 4.7min and second reaction time by 4.7min, while the standard reaction time is 4.7 min. For the second calibration curve 800nm was recorded and used for correction purposes by blanking.

For the analyte Phenobarbital (competitive assay format) the first wavelength was determined by 600 nm and the second wavelength by 505 nm, while the standard wavelength is 600 nm. The first reaction time was determined by 6.5min and second reaction time by 7.9min, while the standard reaction time is 6.5 min. For the first and second calibration curves as well as for the standard calibration curve 800nm was recorded and used for correction purposes by blanking.
In most cases the dynamic range was improved by the use of the approach from the present invention, either by improving the LDL and/or the UDL of the assay. Only in the case of digoxin and D-Dimer the improvement is moderate.

From these assay examples depicted above some trends can be deduced for the direct assay format. The first wavelength, used for the first calibration curve and for the sensitive detection of the analyte, is usually smaller than the second wavelength, used for the second calibration curve and the detection of high analyte concentrations. An exception is shown for the D-Dimer assay. In addition, the first reaction time is larger or identical to the second reaction time in most cases An exception is identified for the Ferritin assay, showing a first reaction time which is smaller than the second reaction time. Similar trends are observed for the assay with the competitive assay format which seem to behave vice versa to the assays with the direct format.

One embodiment of the present invention is that optionally a further wavelength is determined as a blank value for the correction of interferences and compensation of photometric noise, also known as bichromatic measurement (clin. Chem. 1979, 25, 1482 - 1484). For each of the two main wavelengths it is optional if a further wavelength is recorded for correction purposes by

subtraction of the signal at the correction wavelength from the signal at the main wavelength. Preferably, for the analyte CRP this further wavelength for the corrections is recorded at 800 nm.

For the "first wavelength" and first reaction time and for the "second wavelength" and second reaction time calibration curves are constructed with standard samples for a specific analyte which are simultaneously measured. According to the present invention, the first calibration curve recording at the first wavelength (λ1) and first reaction time (t1) is optimized for sensitivity or lower detection limit (LDL). The second calibration curve recording at the second wavelength (λ2) and the second reaction time (t2) is optimized for high upper detection limit (UDL). As an example, for the CRP-assay standard samples are simultaneously measured at all 12 wavelengths available on the cobas c 311, including λ1 (=505 nm) and λ2 (=600nm) and the correction wavelength (800nm), over a time period of 10 min (=complete reaction time). By correcting all the absorbance values at 505nm measured during the complete reaction time (approximately 10 min) via subtraction of the corresponding absorbance values at 800nm, followed by the calculation of the signal values for the first reaction time (t1) hereof by calculating the difference between the absorbance value at the time shortly after the final reagent is added to the reaction mixture and the absorbance value recorded 2.0min after the final reagent was added to the reaction mixture, calibration curve 1 is obtained; and simultaneously by correcting all the absorbance values at 600nm measured during the complete reaction time (approximately 10 min) via subtraction of the corresponding absorbance values at 800nm followed by the calculation of the signal values for the second reaction time (t2) hereof by calculating the difference between the absorbance value at the time shortly after the final reagent is added to the reaction mixture and the absorbance value recorded 1.2min after the final reagent was added to the reaction mixture calibration curve 2 is obtained.

A further embodiment is the method according of the present invention wherein two calibration curves are generated which are recorded at the same wavelengths and at different reaction times.

After constructing calibration curves 1 and 2 for the specific analyte, the concentration of the samples can be determined. Therefore, a sample containing a specific analyte to be determined is added to the test reagents into a cuvette. This mixture is incubated for a certain reaction time, here the complete reaction time, e.g. 10 min.

The term "optical signal" for this analyte, also called absorbance as used herein, is measured simultaneously in the reaction mixture at least at the two predetermined wavelengths, the first

and second wavelengths, in one run over the complete reaction time. Optionally, the optical signal at the correction wavelength may be recorded as well.

The term "threshold value" as used herein, also called "cut-off value", is used for defined absorbance values or a defined amount of the analyte of the present method; absorbance values are preferably used. Ideally the threshold value is taken from the point where the two calibration curves of the present method change from the first calibration curve to the second calibration curve. Only one absorbance value may be defined as threshold, either a value (A[λ1,t1]) for the first wavelength and first reaction time or a value (A[λ2,t2]) for the second wavelength and the second reaction time, or two absorbance values (A[λ1,t1] and A[λ2,t2]) may be considered in parallel. As an example, for the CRP assay A[λ1,t1] and/or A[λ2,t2] may be defined as threshold values which correspond to a concentration of 73 mg/l for CRP. In case of assays with increasing calibration curves as it is the case with CRP, the value A[λ1,t1] is the highest signal value of calibration curve 1, and A[λ2,t2] is the lowest signal value of calibration curve 2. Also the raw absorbance signals corresponding to the absorbance values A[λ1,t1] and/or A[λ2,t2] may be defined as threshold. If at least one optical signal of a sample or at least one signal value calculated hereof exceeds the corresponding predetermined threshold value, the concentration of the analyte is determined by comparison with the second calibration curve of the second wavelength and second reaction time. It is important for an IVD assay that the threshold values are chosen at concentration which does not coincide with the clinical decision value.

For the selection of the point where the two calibration curves of the present method change from the first calibration curve to the second calibration curve there is usually a broad flexibility. Important is that at the selected point both curves fulfill the requirements related with the linearity of the method and with the precision and with the sensitivity. For example calibration curve 2 ideally should have an LDL which at least covers the concentration at the selected point; and calibration curve 1 ideally should show linearity of the method at least up to the said selected point.

The selection of the optimal first wavelength and first reaction time for the first calibration curve, characterized by yielding an optimal LDL, as well as the selection of the optimal second wavelength and second reaction time for the second calibration curve, characterized by yielding an optimal UDL, were performed by a trial-and-error procedure. Although there are some trends for choosing the optimal wavelengths and reaction times, there are also exceptions from the

trends, and as consequence all combinations of wavelengths and reaction times have to be checked for their capacity to yield an optimal LDL or UDL. However, when doing these calculations manually, i.e. without the aid of automated calculation tools or algorithms, it is not possible to make the LDL- and UDL- calculations for all combinations of wavelengths and reaction times with reasonable efforts and in a reasonable time period. For this reason the following procedure was used in the present invention for the development of a method for a certain assay, with other words for the selection of the optimal first wavelength and reaction time as well as for the second wavelength and reaction time as well for the threshold:

1) Measurement of the absorbance values of the following samples simultaneously at the 12 wavelengths available e.g. on the cobas c 311 analyzer over the complete reaction time:
   - at least 6 standards in duplicates for the calibration,
   - a blank sample in 21 replicates for determination of LDL,
   - at least 2 samples in 21 replicates for the determination of the precision (coefficient of variation),
   - dilution series covering concentrations e.g. 2-4 fold higher than the known UDL for the determination of the UDL and the linearity of the method.

2) Selection of the first reaction time t1 and first wavelength λ1 for the first calibration curve is performed by analyzing the 12 kinetic curves, recorded at the 12 different main wavelengths over the complete reaction time, of the blank sample (21 replicates) and a low concentrated sample. Optionally correction wavelengths may be additionally considered thus increasing the number of kinetic curves to be analyzed for the selection of the best wavelengths and reaction times. The low concentrated sample has a concentration in the range of 1-5-fold of the known LOQ value and may be one of the standard samples or one of the precision samples measured under point 1). The kinetic curves describe the absorbance values in the y axis and the time (in case of the cobas c 311 the time is depicted as measure point) in the x axis. Only absorbance values within the specified optical range of the detector (0.0000-3.0000 absorbance) are considered. In the first step some optimal reaction times are determined for each wavelength from the corresponding kinetic curve by choosing an initial time point (or measure point), before or shortly after the final reagent is added, and by choosing a final time point (or measure point), fulfilling the following guidelines:
the signal variation of the blank sample (21 replicates) is minimal and the signal of the blank

sample is low at the initial time point

the absorbance reading for the low concentrated sample is low at the initial time point and high at the final time point, i.e. the difference between the initial absorbance reading and final absorbance reading is maximal.

The term "reaction time" as used herein is the difference from the initial time point from the final time point. After having selected some optimal reaction times for each wavelength, the LDL for all 12 wavelengths at these reaction times are calculated. The wavelength and reaction time yielding the best LDL are selected as the first wavelength and first reaction time for the generation of the first calibration curve.

3) Selection of the second reaction time t2 and second wavelength λ2 for the second calibration curve is performed by analyzing the 12 kinetic curves, recorded at the 12 different main wavelengths over the complete reaction time, of the dilution series covering concentrations e.g. 2-4 fold higher than the known UDL. Optionally correction wavelengths may be additionally considered thus increasing the number of kinetic curves to be analyzed for the selection of the best wavelengths and reaction times. Only absorbance values within the specified optical range of the detector (0.0000-3.0000) are considered. In the first step some optimal reaction times are determined for each wavelength from the corresponding kinetic curve by choosing an initial time point (or measure point), before or shortly after the final reagent is added, and by choosing a final time point (or measure point), fulfilling the following criteria:

- the initial time point is chosen at a point before or shortly after the final reagent is added
- the final time point is chosen in such way that the highest concentrated samples of the dilution series show distinguishable and increasing signals with increasing concentrations, i.e. the absolute value of the difference between the initial absorbance reading and final absorbance reading for a certain concentrated sample is higher than the corresponding absolute value of the difference value for the next lower concentrated sample.

After having selected some optimal reaction times (difference from the initial time point from the final time point) for each wavelength, the UDL for all 12 wavelengths at these reaction times are calculated. The wavelength and reaction time yielding the best UDL are

selected as the second wavelength and second reaction time for the generation of the second calibration curve.

4) Threshold setting: the threshold values are set at a concentration where the calibration curves change from the first to the second calibration curve, i.e. for lower concentrations the first calibration curve is used for the analyte quantification and for higher concentrations the second calibration curve is used for the analyte quantification. For the selection of the point or the concentration where the calibration curves change, there usually exist a broad flexibility.

At this point it is important that the following criteria are fulfilled:

- that both calibration curves show linearity of the method, the first calibration curve in the range from the LDL to the concentration at the threshold and the second calibration curve in the range from the concentration at the threshold to the UDL,
- that the second calibration curve shows a LDL which at least covers the concentration at the threshold,
- that the concentration at the threshold is not identical with the clinical decision value of the IVD test.

An embodiment is therefore a computer-implemented method of determining the optimal wavelength, reaction time and threshold according to the present invention, comprising the steps of:

a) receiving a dataset representing the absorbance values of the samples simultaneously available on an analyzer, wherein said dataset including a plurality of data points:

   - standards in duplicates for the calibration,
   - a blank sample for determination of LDL,
   - at least 2 samples in replicates for the determination of the precision,

b) dilution series covering concentrations determining of the first reaction time and first wavelength for the first calibration curve by analyzing the kinetic curves, recorded at different main wavelengths over the complete reaction time, of the blank sample and a low concentrated sample.

c) determining of the second reaction time and second wavelength for the second calibration curve by analyzing the kinetic curves, recorded at different main wavelengths over the complete reaction time,

d) setting the threshold values at a concentration where the calibration curves change from the first to the second calibration curve, by using for lower concentrations the first calibration curve for the analyte quantification and by using for higher concentrations the second calibration curve for the analyte quantification.

A further embodiment is a computer readable medium including code controlling a processor to determining the optimal wavelength, reaction time and threshold according to the present invention, the code including instructions to:

a) receiving a dataset representing the absorbance values of the samples simultaneously available on an analyzer, wherein said dataset including a plurality of data points:

- standards in duplicates for the calibration,
- a blank sample for determination of LDL,
- at least 2 samples in replicates for the determination of the precision,

b) dilution series covering concentrations determining of the first reaction time and first wavelength for the first calibration curve by analyzing the kinetic curves, recorded at different main wavelengths over the complete reaction time, of the blank sample and a low concentrated sample.

c) determining of the second reaction time and second wavelength for the second calibration curve by analyzing the kinetic curves, recorded at different main wavelengths over the complete reaction time,

d) setting the threshold values at a concentration where the calibration curves change from the first to the second calibration curve, by using for lower concentrations the first calibration curve for the analyte quantification and by using for higher concentrations the second calibration curve for the analyte quantification.

In an exemplary embodiment of the present invention, the method may be implemented by using conventional personal computer systems including, but not limited to, an input device to input a data set, such as a keyboard, mouse, and the like; a display device to represent a specific point of

interest in a region of a curve, such as a monitor; a processing device necessary to carry out each step in the method, such as a CPU; a network interface such as a modem, a data storage device to store the data set, a computer code running on the processor and the like. Furthermore, the method may also be implemented in an analyzer e.g. cobas c 311 analyzer.

In the case that these criteria are not fulfilled for the threshold at any concentration, either the conditions for the first calibration curve (first wavelength and first reaction time) and/or the conditions for the second calibration curve (second wavelength and second reaction time) have to be changed so that the criteria are fulfilled. Such changes may compromise the achieved improvement factor for the dynamic range. In the procedure described above for the selection of the optimal wavelengths and reaction times for calibration curves 1 and 2 only the two-point-end method was applied for calculating the signals. A two-point-end method is an end-point-assay which performs a sample blank. Here two absorbance readings at two different measure points are considered: the first reading is usually taken before or shortly after the final reagent is added; the second reading is taken at any time point after the final reagent was added. Principally, for the selection of the optimal first wavelength and first reaction time as well as for the second wavelength and second reaction time a kinetic signal calculation method be additionally considered and which may yield for certain assays better results compared to the two-point-end method.

By selecting either the first or the second calibration curve for the quantification of the specific analyte, which depends of the optical signal of the sample to be determined, an extended dynamic range or measuring range is achieved.

The term “extended dynamic range” as used herein means that samples with a very low concentration of the specific analyte and/or samples with a very high concentration of the specific analyte can be determined, which is not the case standard methods using only one main wavelength and one calibration curve. According to the present invention, the improvement of the lower detection limit and/or of the upper detection limit results in an improved dynamic range.

According to the present invention “a high concentration of a specific analyte” as used herein is not causing a High-dose “Hook-effect”. The hook effect is common phenomenon in everyday work of a clinical laboratory. Primarily, the hook effect depends on analyte concentration, it is an excessive amount of analyte which overwhelms the binding capacity of the capture antibody.

This results in an inappropriately low signal that causes erroneous low or normal result ("hooked" result) for a sample with an excessively elevated analyte concentration. It implies the presence of huge excess of analyte which saturates all binding sites on antibody. A hook-effect is existent, if in diluted samples higher values are measured than in nondiluted sample. Some automated analysers have a system for recognizing excess of an analyte while the sample is being simultaneously diluting. According to the present invention an excessive amount of a specific analyte causing a High-dose "Hook-effect" cannot be prevented by comparison with the second calibration curve.

One embodiment of the present invention is the provision of cases for correcting calibration curves. The analyte CRP is measured at 505nm for the first wavelength for the sensitive detection and at 600 nm for the second wavelength for the upper detection limit. The third wavelength is recorded at 800 nm for correcting interferences and compensations of most of the photometric noise in the first and second calibration curve.

The term "complete reaction time" as used herein is the time period of measuring a specific analyte at a plurality of wavelengths. For the selection of the best two wavelengths aimed at the generation of the two calibration curves standards were simultaneously measured at the 12 different wavelengths available on the cobas c® instrument. Only absorbance values lying within the optical range of the detector (0.0000 – 3.0000 absorbance) were taken into consideration.

The typical complete reaction time of the present immunoassay time varies between 1 and 20 minutes. Preferably, the complete reaction time of a multiple wavelength spectrophotometer photometer preferably is around 10 minutes. It is an embodiment of the present invention that the optical signals of the specific analyte are measured during the complete reaction time. Most preferably, the optical signal of the specific analyte is measured simultaneously at least at the first and at the second main wavelengths. The term "time delay" as used herein is the time period between the first and at the second main wavelength for the detection of the specific analyte. The term "simultaneously" as used in the present invention, may imply a time delay smaller 60x seconds, e.g. a time delay smaller 10x seconds, preferably smaller 1x second, most preferably smaller 1 ms, or even smaller 0.1x ms. Most preferably, the term "simultaneously" means no time delay.

A further aspect of the present invention is a method for increasing the sensitivity and/or dynamic range of photometric assays, wherein the specific analyte in a sample reacts with an

analyte specific reaction partner in a reaction mixture, comprising the steps of generating at least two calibration curves, wherein the first calibration curve recorded at a first wavelength is optimized for low concentrations of the specific analyte thereby maximizing the lower detection limit and, the second calibration curve recorded at a second wavelength is optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit the optical signal is measured simultaneously for the specific analyte of a sample to be determined in the reaction mixture at least at the first and second wavelengths. Either the first or the second calibration curve is selected for quantification of the specific analyte, by the following criteria:

For increasing calibration curves:

If the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength. If the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength, and finally quantifying the amount of the specific analyte.

For decreasing calibration curves:

If the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength. If the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength and finally quantifying the amount of the specific analyte. Preferably, two calibration curves for a first and a second wavelength and reaction times are generated, which were predetermined for a specific analyte. According to the present invention, the lower detection limit and the upper detection limit both results in an increase of the sensitivity and/or dynamic range. The benefit is a reduced number of re-runs leading to a higher throughput of samples and a reduced turn-around-times. Furthermore, reduced test costs and improved reliability.

The present invention leads to an improvement shown for the lower detection limit (LDL) and/or upper detection limit (UDL). Preferably, a 1 to 2 times improvement is shown for the lower

detection limit (LDL) and/or upper detection limit (UDL). More preferably, a 3 to 4 times improvement is shown for the lower detection limit (LDL) and/or upper detection limit (UDL). Most preferably, the present invention leads to a 5 times improvement shown for the lower detection limit (LDL) and/or upper detection limit (UDL). Preferably, the improvement factor is higher than factor 1, more preferably the factor is higher than factor 3, most preferably the improvement factor is higher than factor 5 as shown for the RF II direct assay.

A further aspect of the present invention is a method for determining the amount of CRP by turbidimetric and nephelometric immunoassays, wherein CRP in a sample is quantified from the change in the turbidity of the reaction mixture based on the aggregation of CRP and a CRP binding partner. Two calibration curves are generated for a first and a second wavelength and corresponding first and second reaction time, which were predetermined for CRP. The optical signal for CRP of a sample to be determined is measured simultaneously in the reaction mixture at least at the first and second wavelengths during the complete reaction time. The first wavelength is optimized for low concentrations of CRP thereby maximizing the lower detection limit, the second wavelength is used which has is optimized for high concentrations of CRP thereby maximizing the upper detection limit. Either the first or the second wavelengths and the first or second reaction time are selected for quantification of CRP. If at least one of the optical signal of a sample measured during the complete reaction time or at least one signal value calculated hereof is lower than a corresponding predetermined threshold value, the concentration of CRP is determined by comparison with the calibration curve of the first wavelength and first reaction time. On the other hand, if at least one of the optical signal of a sample or at least one signal value calculated hereof exceeds the corresponding predetermined threshold value, the concentration of CRP is determined by comparison with the calibration curve of the second wavelength and second reaction time.

In the case of the CRP test the calibration curve recorded at 505 nm with a reaction time of 2.0min and corrected at 800nm yielded the best LDL (0,036 mg/L). The comparison between the standard performance and the approach of the invention results in an increase of the sensitivity (LDL) by a factor of 1.8 and an extension of the dynamic range by a factor of approximately 3.1. The calibration curve 2 recorded at 600 nm with 1.2min reaction time and corrected at 800nm resulted the best UDL (660 mg/L). The UDL shows an increase of a factor of 1.8 compared to standard conditions.

Furthermore, for particle-enhanced turbidimetric assays the combination of the approach of the present invention with adapted particle sizes and antibody loading degrees was assessed using the CRP L3 assay as lead parameter. The CRP L3 assay includes two different types of particles as described in EP 0898169, large latex particles (MP2) coated with highly affine antibodies and small latex particles (MP1) coated with less affine antibodies (clone 21F12, concentration for latex coating: 45mg/g latex).

An additional improvement of the CRP-assay sensitivity was yielded by using large particles between 230- 400 nm, more preferably 250-350 nm, most preferably 280-320 nm diameter for increased light scattering efficiency and using a high loading degrees of the particles with antibody, obtained by using a concentration of the antibody between 55-70 mg IgG/gram particles, preferably 60-70 mg IgG/gram particles for their conjugation to the particles.

The combination of the method of the present invention with large particle sizes between 230-330 nm and an antibody concentration between 55-70 mg IgG/gram particles for conjugation reaction leads to a further increase of the sensitivity (LDL) in comparison to the standard assay (size of large particles: 220nm, antibody concentration of 50 mg IgG/gram particles for the conjugation of the large particles; size of small particles: 110-140nm,) by a factor of approximately eight and therefore to an extension of the dynamic range by a factor of approximately eight.

A further embodiment of the present invention is therefore the method according to present invention, wherein for the specific analyte CRP a particle size for large particles between 230-400 nm and for small particles around 110-140 nm and an antibody concentration of 55-70 mg IgG/gram particles for conjugation reaction for the conjugation of the large particles is used. The first calibration curve was recorded at a first wavelength of 505nm with 800nm as correction wavelength and a first reaction time of 2.8min; the second calibration curve was measured at 570nm as main wavelength and 800nm as correction wavelength and a reaction time of 2.0min. The standard method has only one calibration curve, measured at 570nm as main wavelength and 800nm as correction wavelength and a reaction time of 2.0min.

A further aspect of the present invention is the use of at least two wavelengths and at least two reaction times predetermined for a specific analyte for the generation of at least two calibration curves for quantifying the amount of a specific analyte of photometric assays, wherein a first calibration curve recorded at a first wavelength is optimized for low concentrations of the

specific analyte thereby maximizing the lower detection limit and second calibration curve recorded at a second wavelength is optimized for high concentrations of the specific analyte thereby maximizing the upper detection limit and the optical signal of the specific analyte at the first and second wavelengths is measured simultaneously during the complete reaction time.

Another aspect of the present invention is a system analyzer comprising a sample container and an assay reagent container, a reaction container for receiving and mixing the sample and the assay reagents, a photometer for measuring the reaction mixture of step b and means for outputting the analysis result for determining the amount of a specific analyte according to the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the Calibration curves at different wavelengths of the CRP-assay. Six standards were simultaneously measured at the 12 different wavelengths available on the cobas c® instrument. Only absorbance values lying within the optical range of the detector (0.0000 – 3.0000 absorbance) were taken into consideration. Here, five calibration curves are shown as examples which were recorded at 5 different main wavelengths out of the 12 possible wavelengths, for the main wavelengths 480-, 505nm, 546nm, 570-nm and 600- nm. All curves were corrected at the wavelength 800nm and taken at a reaction time of 2.0 min.

Figure 2 shows the improvement obtained with the CRP assay by application of the method of the present invention in combination with different particle sizes and different antibody loadings of the particles.

In the first example the new approach was combined with particle adaptation aimed at the increase of the sensitivity (LDL). The combination of the new approach with larger particle sizes (290nm instead of 220nm diameter of MP2 particles) for increased light scattering efficiency and higher antibody loading degrees (antibody concentration of 60 mg IgG/g particles for conjugation reaction of the large particles) for increased immune reactivity leads to an increase of the sensitivity (LDL) by a factor of approximately eight and an extension of the dynamic range by a factor of eight.

In the second example the sizes and antibody loading of both particles, the large particles MP2 as well as the small particles MP1, were modified:

- larger MP2 particles (290nm instead of 220nm), conjugated with an antibody concentration of 70 mg IgG/g particles, and
- smaller MP1 particles (90nm instead of 110-140nm) conjugated with an antibody concentration of 50 mg IgG/g particles.

These new particles yielded an improved LDL by a factor of 3 and an improved UDL of a factor of approximately 2 compared to the standard assay (MP1: 110-140nm with 45mg IgG/g and MP2: 220nm with 50mgIgG/g).

Figure 3 shows schematically how the threshold values may be placed for the selection of one of the two calibration curves of the present invention.

Figure 4 shows calibration curves for the CRP-assay. The standard calibration curve is obtained by measuring at 570- 800 nm. Calibration curve 1 (Cal 1) recorded at 505-800 nm and a reaction time of 2.0min yielded the best LDL (0,036 mg/L). The calibration curve 2 (Cal 2) recorded at 600-800 nm and 1.2min reaction time resulted in the best UDL (660 mg/L). The threshold value for CRP may be defined as at least one of the two absorbance values at 73 mg/L.

Figure 5 shows the benefit of the new approach by using commercial tests out of Roche's product portfolio. The analytes which were determined are Ferritin, Myoglobin, D-Dimer, Rheuma factor II (RF II), CRP, Digoxin (DIG), Phenobarbital (PHENO). In the second column the conditions for standard calibration curves are shown. Columns three and four show the new conditions for calibration curves 1 and 2. For each specific analyte the best wavelengths for LDL and UDL and the improvement factors for LDL, UDL were determined. In most cases the new approach improves the dynamic range without any modification of reagents or formulations.

Figure 6 shows exemplary kinetic curves for the ferritin assay showing the signal magnitude (absorbance) of a low concentrated sample (10µg/ml ferritin; 21 replicates) and a blank sample (0µg/ml ferritin; 21 replicates) at 2 different main wavelengths, 505nm (figure 6 a) and 570nm (figure 6 b). The kinetic curves describe the absorbance values in the y axis and the time (in case of the cobas c 311 the time is depicted as measure point) in the x axis. Optimal reaction times for the first calibration curve are determined by choosing an initial time point (or measure point), before or shortly after the final reagent is added, and by choosing a final time point (or measure point), fulfilling the following guidelines:

- the signal variation of the blank sample (21 replicates) is minimal and the signal of the blank sample is low at the initial time point

- the absorbance reading for the low concentrated sample is low at the initial time point and high at the final time point, i.e. the difference between the initial absorbance reading and final absorbance reading is maximal.

The reaction time is the difference from the initial time point from the final time point. In most cases, the higher the signal magnitude of the low concentrated sample the better LDL values are obtained provided the standard deviation of the blank sample is comparable.

Figure 7 shows exemplary kinetic curves for the ferritin assay showing the signal magnitude (absorbance) of different highly concentrated samples (981-4950 µg/L ferritin) recorded at the main wavelength 570nm, corrected by the absorbance at the wavelength 800nm. The kinetic curves describe the absorbance values in the y axis and the time (in case of the cobas c 311 the time is depicted as measure point) in the x axis. Optimal reaction times for the second calibration curve are determined by choosing an initial time point (or measure point), before or shortly after the final reagent is added, and by choosing a final time point (or measure point), fulfilling the following criteria:

- the initial time point is chosen at a point before or shortly after the final reagent is added
- the final time point is chosen in such way that the highest concentrated samples of the dilution series show distinguishable and increasing signals with increasing concentrations, i.e. the difference between the initial absorbance reading and final absorbance reading for a certain concentrated sample is higher than the corresponding difference value for the next lower concentrated sample.

The reaction time is the difference from the initial time point from the final time point. Usually, good UDL values are obtained when the signals of the highly concentrated samples are distinguishable and when the signals increase with the sample concentration.

Figure 8: as shown in table 4 and the examples 6,7,8,9 the application of the invention leads to an extension of the measuring range of commercially available assays.

One benefit of this measuring range extension is that for assays running on automated lab analyzers the re-run rate, which is caused by samples with analyte concentrations exceeding the measuring range, can be reduced. In order to make a rough estimation of the scale of the achievable rerun rate reduction the results obtained for at least 1000 clinical samples per assay on a cobas c501 analyzer were analyzed with the standard method an the method of the present invention. Samples with analyte concentrations falling out of the measuring range cause an

automatic rerun on the analyzer. The achievable measuring range with the standard method is depicted in package insert document of commercial test.

ALTL example: by using the method of the present invention the measuring range of the assay was extended by a factor of 12.1 compared to the standard method. This dynamic range improvement leads to a reduction of the reruns in comparison to the standard method: 87% of all reruns caused by samples with analyte concentrations below the standard measuring range can be avoided by applying the method of the present invention; and 99% of all reruns caused by samples exceeding the standard measuring range can be avoided. The samples used for this study are samples from donors with a health profile comparable with that of patients that occur in standard central labs.

EXAMPLES

Example 1: Determination of the dynamic range of the CRP L3 test

1.1 Instrument:

Roche´s (Roche Diagnostics GmbH) cobas c311 analyzer, which has a multiple wavelength spectrophotometer as detection unit, was used for the experiments. The instrument automatically pipettes the sample and the assay reagents into reaction cells. Up to 3 different reagents, R1, R2 and R3, may be added to the sample. The instrument uses a tungsten halogen lamp as irradiation source (12 V / 50 W) and measures the absorbance simultaneously at 12 different wavelengths (at 340, 376, 415, 450, 480, 505, 546, 570, 600, 660, 700 and 800 ± 2 nm) with a photodiode array consisting of 12 photodiodes. The optical path length is 5.6 mm and the optical range of the detector is 0.0000 – 3.0000 absorbance. For each reaction cell, a water-blank is measured and then absorbance readings are taken 57 times in 10 minutes, here also called the complete reaction time, thus yielding a total of 57 measure points for the absorbance at each wavelength, also called photometric points or assay points. The concentration can be calculated by using at least one of these measurement points. There are two fundamental types of photometric assays on this instrument: endpoint assays and rate assays. The measurements are performed at 37 degree Celsius.

1.2 Procedure for the CRP-assay using the standard approach:

Roche´s CRP L3 test (CRPL3, Cat.No. 04956842), a particle-enhanced immunoturbidimetric assay, was selected for this study. Human CRP agglutinates with latex particles coated with monoclonal anti-CRP antibodies; the aggregates are determined turbidimetrically. Reagents for all Roche tests are provided in cobas c packs. These cassettes contain from one to three specially designed reagent bottles and have barcode labels with detailed reagent and test-related. For CRP L3 tests two reagents are used in the cassette: R1 (TRIS buffer with bovine serum albumin and preservatives) and R2 (Latex particles coated with anti-CRP (mouse) in glycine buffer, immunoglobulins (mouse) and preservative). The procedure described in the package insert from the CRP L3 test was used as standard method.

1.3 Pipetting scheme:

2µL sample and 150µL assay buffer (R1) were added subsequently to the reaction cell, followed by the addition of the latex reagent (R2), diluted with 24µl diluent (water), and mixing of the reaction mixture.

1.4 Conditions for the generation of the calibration curve:

For the measurements 570nm was used as main wavelength and 800nm as correction wavelength. The assay type was a two-point-end assay. A two-point-end assay is an end-point-assay which performs a sample blank. Here two absorbance readings at two different measure points are considered: the first reading is usually taken before or shortly after the final reagent is added; the second reading is taken at any time point after the final reagent was added. The absorbance value for the calibration curve and therefore for the concentration calculation is obtained by subtraction of the first reading from the second reading. For CRP L3 the first reading is at measure point 8 and means shortly after the final reagent addition, and the second reading at measure point 18, which corresponds to a reaction time of 2.0 minutes. For the generation of the calibration curve 6 standards from Roche (Cat. No. 11355279) are measured as duplicates with spline as calibration type, which fits the ranges between the data points of the measured calibrators approximated by third degree polynomials so that a smooth calibration curve is obtained.

1.5 Procedure for the CRP-assay according to the invention:

The reagents used for these experiments were identical to those used for the standard method: see example 1.2. The procedure described in the package insert from the CRP L3 test was used with exception of the wavelengths and reaction times.

1.6 Pipetting scheme: identical to pipetting scheme 1.3

1.7 Conditions for the generation of the calibration curve:

The best conditions for both calibration curves were determined as described earlier and the following result was obtained: 505nm was used as first wavelength, 800nm as correction wavelength and 2.0 minutes as first reaction time; 600nm was used as second wavelength, 800nm as correction wavelength and 1.2 minutes as second reaction time. The assay type was a two-point-end assay as described under example 1.4.

For the first calibration curve the first reading is at measure point 8 and means shortly after the final reagent addition, and the second reading at measure point 18, which corresponds to a reaction time of 2.0 minutes; for the second calibration curve the first reading is at measure point 8 and means shortly after the final reagent addition, and the second reading at measure point 14, which corresponds to a reaction time of 1.2 minutes. For the generation of both calibration curves each 6 standards covering the corresponding concentrations ranges are measured as duplicates with spline as calibration type, which fits the ranges between the data points of the measured calibrators approximated by third degree polynomials so that a smooth calibration curve is obtained. Similar results were obtained when for both calibration curves the 6 standards from the standard method (Cat. No. 11355279) were used.

The first calibration curve is used which is optimized for a low concentration of the specific analyte thereby maximizing the lower detection limit, the second calibration curve is used which is optimized for a high concentration of the specific analyte thereby maximizing the upper detection limit. Either the first calibration curve or the second calibration curve is to be selected for quantification of CRP, depending on the analyte content or corresponding absorbance of the sample to be quantified. For this purpose the signal of the sample is compared with a predetermined threshold value. The threshold value for CRP may be defined as one of the two or both absorbance values in the calibration curves for CRP which correspond to a concentration of 73 mg/L (see figure 3 or figure new), with other words the absorbance at 505nm-800nm for the reaction time of 2.0 minutes (A[λ1,t1] = A[505nm-800nm,2.0 minutes]) and/or the absorbance at 600nm-800nm for the reaction time of 1.2 minutes (A[λ2,t2] = A[600nm-800nm,1.2 minutes]).

If the optical signal of the sample calculated for 505nm-800nm and 2.0 minutes reaction time is below the predetermined threshold value A[505nm-800nm,2.0 minutes] and/or if the optical

signal of the sample calculated for 600nm-800nm and 1.2 minutes reaction time is below the predetermined threshold value A[600nm-800nm,1.2 minutes], the first calibration curve is used for its quantitation.

If the optical signal of the sample calculated for 505nm-800nm and 2.0 minutes reaction time exceeds the predetermined threshold value A[505nm-800nm,2.0 minutes] and/or if the optical signal of the sample calculated for 600nm-800nm and 1.2 minutes reaction time exceeds the predetermined threshold value A[600nm-800nm,1.2 minutes], the second calibration curve is used for its quantitation. As result two calibration curves are obtained for covering the measuring range for CRP determination, the first covering analyte concentrations from 0 to 73mg/L and the second covering the range from 73 mg/L to the UDL.

Results for the CRP-assay using the standard and the approach according to the invention:

1.8 Determination of LDL:

21 replicates of a blank sample were generated and the corresponding signal values x1, x2, …x21 calculated, by correcting the absorbance values recorded at the main wavelength of 570nm by subtraction of the absorbance at 800nm followed by the subtraction of the value taken at measure point 8 from the value taken at measure point 18. Finally, the mean value x of the 21 signal values x1, x2, …x21 and the corresponding standard deviation is calculated. The LDL is determined as the concentration corresponding to the signal value (x + 3 SD). For the new approach only the data generated under the first wavelength and first reaction time as well as the corresponding first calibration curve are used.

1.9 Determination of the UDL:

Sample dilution series, covering concentrations approx. 2 to 4 fold higher than the UDL known from the Roche assay, were measured followed by the calculation of the analyte recovery by forming the ratio of measured value to theoretical value. For values between 90% and 110% the method was considered to be linear. The UDL was selected as the highest concentration value within the linear range. For the new approach only the data generated under the second wavelength and second reaction time as well as the corresponding second calibration curve are used.

1.10 Determination of the coefficient of variation (CV):

21 replicates of serum samples of certain concentration were measured and the CV of the found

concentration values calculated by the ratio of the standard deviation to the mean. For the new approach, depending on the analyte concentration to be assessed, either

- the data generated under the first wavelength and first reaction time as well as the corresponding first calibration curve or
- the data generated under the second wavelength and second reaction time as well as the corresponding second calibration curve are used. Two low concentrated samples (1 and 5 mg/L CRP) were assessed for the assay.

1.11 Result overview and conclusions:

The result overview is depicted in Table 1. The application of the new approach of CRP test shows an increase of the sensitivity (LDL, lower detection limit) by a factor of 1.8 and an extension of the upper detection limit UDL by a factor of 1.8. The improvement of the corresponding dynamic range is by a factor of 3.1. The precision (CV) obtained with the new method is comparable with the standard method. The new approach affords minimal implementation; the application of the approach does not need any changes of reagents and their formulations. Only the number and concentration of standards for the calibration may be adapted.

Table 1: Result overview for CRP L3

	Standard method (570 nm wavelength, 800nm correction wavelength, 2.0 min. reaction time)	**New method (calibration curve 1: 505 nm wavelength, 800nm correction wavelength, 2.0 min. reaction time; calibration curve 2: 600nm wavelength, 800nm correction wavelength, 1.2 min reaction time)**
LDL (mg/L)	0,063	0,036
UDL (mg/L)	370	660
Dynamic range	5870	18330
Measuring range (mg/L)	0,063 - 370	0,036 - 660
CV at 1 mg/L (%)	5,3	5,0
CV at 5 mg/L (%)	1,8	1,7

Example 2: Determination of the dynamic range of further assays

2.1 Instrument: See example 1.

2.2 Procedure for the ferritin assay using the standard approach:

Roche´s ferritin test (FERR4, Cat.No. 04885317), a particle-enhanced immunoturbidimetric assay, was selected for this study. Human ferritin agglutinates with latex particles coated with anti-ferritin antibodies; the aggregates are determined turbidimetrically. Reagents for all Roche tests are provided in cobas c packs. These cassettes contain from one to three specially designed reagent bottles and have barcode labels with detailed reagent and test-related. For Ferritin tests two reagents are used in the cassette: R1 (TRIS buffer pH 7.5, immunoglobulins (rabbit) and preservative) and R3 (Latex particles coated with anti-human ferritin antibodies (rabbit), preservative). The procedure described in the package insert from the Ferritin test was used as standard method.

2.3 Pipetting scheme:

10µL sample and 80µL assay buffer (R1) were added subsequently to the reaction cell, followed by the addition of the latex reagent (R3) and mixing of the reaction mixture.

2.4 Conditions for the generation of the calibration curve:

For the measurements 570nm was used as main wavelength and 800nm as correction wavelength. The assay type was a two-point-end assay as described under example 1.4. For ferritin the first reading is at measure point 24 and means shortly after the final reagent addition, and the second reading at measure point 57, which corresponds to a reaction time of 5.1 minutes. For the generation of the calibration curve 6 standards from Roche (Cat. No. 11355279) are measured as duplicates with spline as calibration type, which fits the ranges between the data points of the measured calibrators approximated by third degree polynomials so that a smooth calibration curve is obtained.

2.5 Procedure for the ferritin assay according to the invention:

The reagents used for these experiments were identical to those used for the standard method as described in example 2.2. The procedure described in the package insert from the ferritin test was used with exception of the wavelengths and reaction times.

2.6 Pipetting scheme: identical to pipetting scheme as described in 2.3.

2.7 Conditions for the generation of the calibration curve:

The best conditions for both calibration curves were determined as described earlier and the following result was obtained: 505nm was used as first wavelength, 800nm as correction wavelength and 4.9 minutes as first reaction time; 570nm was used as second wavelength, 800nm as correction wavelength and 5.1 minutes as second reaction time. The assay type was a two-point-end assay as described under example 1.4. For the first calibration curve the first reading is at measure point 25 and means shortly after the final reagent addition, and the second reading at measure point 57, which corresponds to a reaction time of 4.9 minutes; for the second calibration curve the first reading is at measure point 24 and means shortly after the final reagent addition, and the second reading at measure point 57, which corresponds to a reaction time of 5.1 minutes. For the generation of both calibration curves each 6 standards covering the corresponding concentrations ranges are measured as duplicates with spline as calibration type, which fits the ranges between the data points of the measured calibrators approximated by third degree polynomials so that a smooth calibration curve is obtained. Similar results were obtained when for both calibration curves the 6 standards from the standard method (Cat. No. 11355279) were used. The threshold may be defined as one of the two or both absorbance values in the calibration curves for ferritin at a concentration of 100 µg/L.

Results for the ferritin assay using the standard and the approach according to the invention:

2.8 Determination of LDL / UDL / CV: See example 1.

2.9 Result overview and conclusions:

The result overview is depicted in Table 2. The application of the new approach to Roche´s ferritin test shows an increase of the sensitivity (LDL, lower detection limit) by a factor of 1.7 and a retained upper detection limit UDL. The improvement of the corresponding dynamic range is by a factor of 1.7. The precision (CV) obtained with the new method is comparable with the standard method. The new approach affords minimal implementation; the application of the approach does not need any changes of reagents and their formulations. Only the number and concentration of standards for the calibration may be adapted.

Table 2: Result overview for FERR4

	Standard method (570 nm wavelength, 800nm correction wavelength, 5.1 min. reaction time)	**New method (calibration curve 1: 505 nm wavelength, 800nm correction wavelength, 4.9 min. reaction time; calibration curve 2: 570nm wavelength, 800nm correction wavelength, 5.1 min reaction time)**
LDL (µg/L)	2.0	1.2
UDL (µg/L)	1940	1940
Dynamic range	970	1617
Measuring range (µg/L)	2 - 1940	1.2 - 1940
CV at 10 µg/L (%)	8.9	6.7
CV at 30 µg/L (%)	2.9	1,7
CV at 145 µg/L	0.7	0.7

Example 3: Assessment of the dynamic range extension potential of further tests from Roche

3.1 Instrument: See example 1.

3.2 Procedure for the assays using the standard approach:

The following particle-enhanced immunoturbidimetric assays from Roche were assessed: three assays with the direct test format, myoglobin (MYO2, Cat. No. 04580010), D dimer (D-DI2, No. 04912551) and rheumatoid factors (RF-II, Cat. No. 20764574), as well as two assays with a competitive test format (KIMS: kinetic interaction of microparticles in solution), digoxin (DIG, Cat. No. 20737836) and phenobarbital (PHNO2, Cat. No. 04490924). In the DIG assay the particles are coated with digoxin and rapidly aggregate in the presence of antibody solution depending on the digoxin concentration of the sample. In the PHNO2 assay the phenobarbital antibody is covalently coupled to microparticles and the phenobarbital derivative is linked to a macromolecule; the binding of the phenobarbital derivative to the antibody on the microparticles

is inhibited by the presence of phenobarbital in the sample. The procedure described in the corresponding package inserts from the above mentioned tests was used as standard method.

3.3 Overview on reagents, pipetting and calibration procedure:

The procedure was as described in the package insert document from the tests and summarized in the table below (table 3).

	Reagents	Pipetting scheme	Wavelength, reaction time**	Calibration
Myoglobin	R1: Glycine buffer, preservatives. R2: Latex particles coated with anti-myoglobin antibodies, buffer with preservatives.	3 µL sample, 90 µL R1, 30 µL R2	570nm – 800nm, 4.7 min (7-29)	Standards: 6 point calibration, Cat. No. 04580044. Calibration type: RCM*. Assay type: Two-point-end.
D Dimer	R1: Tris buffer, preservatives. R3: Latex particles coated with anti D Dimer antibodies, buffer with preservatives.	5 µL sample, 90 µL R1, 90 µL R3	800nm, 4.3 min (27-57)	Standards: 6 point calibration, Cat. No. 05050901. Calibration type: Spline. Assay type: Two-point-end.
RF II	R1: Glycine buffer, preservatives. R2: Latex particles coated with human IgG, buffer with preservatives.	3 µL sample, 90 µL R1, 30 µL R2	570nm – 800nm, 2.2 min (7-18)	Standards: 6 point calibration, Cat. No. 12172828. Calibration type: RCM*. Assay type: Two-point-end.
DIG	R1: Anti-digoxin antibody in buffer with preservatives. R2: Conjugated digoxin derivative microparticles, buffer with preservatives.	5.5 µL sample, 84 µL R1, 22 µl R2 (diluted with 20 µl water)	660nm, 4.7 min (9-31)	Standards: 6 point calibration, Cat. No. 03375790. Calibration type: RCM*. Assay type: Two-point-end.
PHENO	R1: Phenobarbital conjugate in buffer with preservatives. R2: Latex particles coated with anti-phenobarbital antibody, buffer with preservatives.	2 µL sample, 93 µL R1, 93 µL R2	600nm – 800nm, 6.5 min (10-49)	Standards: 6 point calibration, Cat. No. 03375790. Calibration type: RCM*. Assay type: Two-point-end.

Table 3: Test overview

* RCM: Rodbard fitting; ** main wavelength – correction wavelength; reaction time in minutes; in brackets measure point for first and second reading

3.4 Procedure for the assays according to the present invention:

The reagents used for these experiments were identical to those used for the standard method as described in example 3.2. The procedure described in the package insert from the ferritin test was used with exception of the wavelengths and reaction times.

3.5 Overview on reagents, pipetting and calibration procedure:

The procedure was as described in the package insert document from the tests and summarized in the table 3, if not mentioned otherwise.

Results for the assays myoglobin, D dimer, RF II, DIG and PHENO using the standard and new approach of the invention:

3.6 Determination of LDL / UDL / CV: See example 1.

3.7 Result overview and conclusions:

The result overview is depicted in Table 4. Optimal wavelengths and reaction times for the first and second calibration curves were determined as described earlier. The application of the new approach to the commercial Roche´s tests leads in most cases an improvement of the sensitivity (LDL, lower detection limit) and/or to an improvement of the upper measuring range. The improvement of the dynamic range is by a factor of 1.5 to 5.8. In two cases, D dimer and Dig, the improvement is moderate. In cases where the improvement was moderate the improvement factor was set into brackets. The precision (CV) obtained with the new method is comparable with the standard method. The new approach affords minimal implementation; the application of the approach does not need any changes of reagents and their formulations. Only the number and concentration of standards for the calibration may be adapted.

Table 4: Result overview

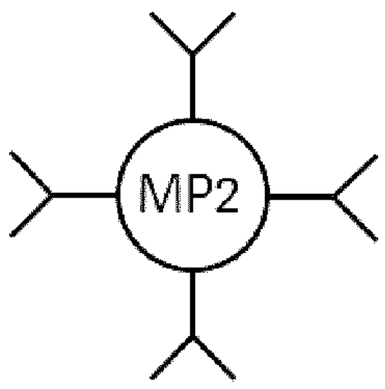
Roche test	Standard conditions [d]	New conditions [d] Cal 1	Cal 2	Improvement factor [c] LDL	UDL	dyn. range
Myoglobin Gen. 2	570 nm – 800 nm 4.7min (7 – 29)	505 nm – 800 nm 6.7min (9 – 50)	660 nm – 800 nm 2.6min (7 – 20)	2 [a]	2	4
D-Dimer Gen. 2	800 nm 4.3min (27 – 57)	800 nm 4.3min (27 – 57)	700 nm 1.4min (26 – 33)	-	(1.14)	(1.14)
RF II	570 nm – 800 nm 2.2min (7 – 18)	546 nm – 800 nm 8.5min (7 – 57)	660 nm – 800 nm 1.4min (7 – 14)	5 [b]	(1.15)	5.8
DIG	660 nm 4.7min (9 – 31)	660 nm 4.7min (9 – 31)	505 nm – 800 nm 4.7min (9 – 31)	-	(1.15)	(1.15)
PHENO	600 nm – 800 nm 6.5min (10 – 49)	600 nm – 800 nm 6.5min (10 – 49)	505 nm – 800 nm 7.9min (10 – 57)	-	1.5	1.5

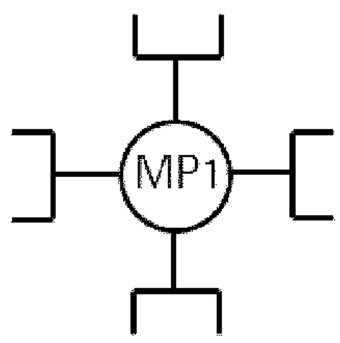
[a] Without instrument factor, [b] Change calibration to spline, [c] Good precision from standard conditions is retained in new conditions, [d] First row:main wavelength – correction wavelength; second row: reaction time in minutes, in brackets: measure points

<u>Example 4: Additional improvement of the dynamic range for the combination assay of invention with modified particles MP2</u>

4.1 In addition, the combination of the assay of the present invention with adapted particle sizes and antibody loading degrees was assessed using the CRP L3 assay as lead parameter. The CRP L3 assay includes two different types of particles as described in EP 0898169, large latex particles (MP2, approx.. 220nm diameter) coated with highly affine antibodies (clone 36F12; concentration for latex coating: 50mg/g latex) and small latex particles (MP1, approx.. 110-140 nm diameter) coated with less affine antibodies (clone 21F12, concentration for latex coating: 45mg/g latex). Here, the MP2 particles were modified by using larger sizes and antibody loading

degrees in order to increase the analytical sensitivity, and finally the achievable LDL and dynamic range determined in the assay as described





• ~ 220 nm ∅ latex	• 110-140 nm ∅ latex
• Anti-CRP 36F12 (high affinity)	• Anti-CRP 21F12 (lower affinity)
• 50 mg 36F12/g latex	• 45 mg 21F12/g latex

below.

4.2 Synthesis of the modified MP2 particles:

To latex particles (carboxylate-modified microparticles, 290nm sized) with a concentration of 2% solids in 750µl MES buffer (20mM, pH 6.1) 30µl of a sulfo-NHS (sulfo- N-hydroxysulfosuccinimide, 100mM in MES buffer) solution and 30µl of an EDC (1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide, 100mM in MES buffer) solution were successively added. The reaction mixture was shortly sonicated and allowed to react for 1 hour at room temperature on a mixing wheel. Afterwards 60mg antibody 36F12 per gram latex in 750µl MES buffer (20mM, pH 6.1) containing 37µl of Synperonic P85 solution (1% in MES) were mixed with the sulfo-NHS/EDC-activated latex particles. The reaction mixture was shortly sonicated and allowed to react for 2 hours at room temperature on a mixing wheel. To the reaction mixture 45µl glycine solution (2M, pH 11) were added, sonicated and mixed for 15 minutes on a mixing wheel. Finally the particles were washed 2 times by centrifugation and resuspension in glycine (50mM, pH 8) containing 0.03% Synperonic P85. After a final centrifugation and resuspension step the latex particles (1% solids) were stored in glycine (50mM, pH 8) containing 0.03% Synperonic P85, 0.1% BSA and 0.05% sodium azide.

4.3 Synthesis of the standard MP1 particles:

To latex particles (carboxylate-modified microparticles, size 110-140nm) with a concentration of 2% solids in 750µl MES buffer (20mM, pH 6.1) 30µl of a sulfo-NHS (sulfo- N-hydroxysulfosuccinimide, 100mM in MES buffer) solution and 30µl of an EDC (1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide, 100mM in MES buffer) solution were successively added.

The reaction mixture was shortly sonicated and allowed to react for 1 hour at room temperature on a mixing wheel. Afterwards 45mg antibody 21F12 per gram latex in 750µl MES buffer (20mM, pH 6.1) were mixed with the sulfo-NHS/EDC-activated latex particles. After 20 minutes 37µl of Synperonic P85 solution (1% in MES) was added to the mixture. The reaction mixture was shortly sonicated and allowed to react for 100 minutes at room temperature on a mixing wheel. To the reaction mixture 45µl glycine solution (2M, pH 11) were added, sonicated and mixed for 15 minutes on a mixing wheel. Finally the particles were washed 2 times by centrifugation and resuspension in glycine (50mM, pH 8) containing 0.03% Synperonic P85. After a final centrifugation and resuspension step the latex particles (1% solids) were stored in glycine (50mM, pH 8) containing 0.03% Synperonic P85, 0.1% BSA and 0.05% sodium azide.

4.4 Preparation of the reagents R1 and R2 for the analyser assay:

The reagents R1 and R2 are as described in the package insert document from the tests. The R2 reagent is prepared by mixing the MP1 and MP2 particles synthesized in steps a) and b). The Reagent is filled in a cobas c pack.

4.5 Instrument: See example 1.

4.6 Procedure for the CRP-assay using the standard approach is identical to the procedure of example 1.2.

4.7 Pipetting scheme: identical to pipetting scheme of example 1.3.

4.8 Conditions for the generation of the calibration curve are identical to example 1.4.

4.9 Procedure for the CRP-assay using the new approach of the invention:

The reagents used for these experiments were prepared as described above in example 1.2. The procedure described in the package insert from the CRP L3 test was used with exception of the wavelengths and reaction times.

4.10 Pipetting scheme: identical to the pipetting scheme of example 1.3.

4.11 Conditions for the generation of the calibration curve:

The best conditions for both calibration curves were determined as described earlier and the following result was obtained: 505nm was used as first wavelength, 800nm as correction

wavelength and 2.8 minutes as first reaction time; 570nm was used as second wavelength, 800nm as correction wavelength and 2.0 minutes as second reaction time. The assay type was a two-point-end assay as described under example 1.4. For the first calibration curve the first reading is at measure point 8 and means shortly after the final reagent addition, and the second reading at measure point 22, which corresponds to a reaction time of 2.8 minutes; for the second calibration curve the first reading is at measure point 8 and means shortly after the final reagent addition, and the second reading at measure point 18, which corresponds to a reaction time of 2.0 minutes. For the generation of both calibration curves each 6 standards covering the corresponding concentrations ranges are measured as duplicates with spline as calibration type, which fits the ranges between the data points of the measured calibrators approximated by third degree polynomials so that a smooth calibration curve is obtained. Similar results were obtained when for both calibration curves the 6 standards from the standard method (Cat. No. 11355279) were used. The first calibration curve is used which is optimized for a low concentration of the specific analyte thereby maximizing the lower detection limit, the second calibration curve is used which is optimized for a high concentration of the specific analyte thereby maximizing the upper detection limit. Either the first calibration curve or the second calibration curve is to be selected for quantification of CRP, depending on the analyte content or corresponding absorbance of the sample to be quantified. For this purpose the signal of the sample is compared with a predetermined threshold value. The threshold value for CRP may be defined as one of the two or both absorbance values in the calibration curves for CRP which correspond to a concentration of 10 mg/L, with other words the absorbance at 505nm-800nm for the reaction time of 2.8 minutes (A[λ1,t1] = A[505nm-800nm,2.8 minutes]) and/or the absorbance at 570nm-800nm for the reaction time of 2.0 minutes (A[λ2,t2] = A[570nm-800nm,2.0 minutes]).

If the optical signal of the sample calculated for 505nm-800nm and 2.8 minutes reaction time is below the predetermined threshold value A[505nm-800nm,2.8 minutes] and/or if the optical signal of the sample calculated for 570nm-800nm and 2.0 minutes reaction time is below the predetermined threshold value A[570nm-800nm,2.0 minutes],the first calibration curve is used for its quantitation.

If the optical signal of the sample calculated for 505nm-800nm and 2.8 minutes reaction time exceeds the predetermined threshold value A[505nm-800nm,2.8 minutes] and/or if the optical signal of the sample calculated for 570nm-800nm and 2.0 minutes reaction time exceeds the predetermined threshold value A[570nm-800nm,2.0 minutes], the second calibration curve is used for its quantitation. As result two calibration curves are obtained for covering the measuring

range for CRP determination, the first covering analyte concentrations from 0 to 10mg/L and the second covering the range from 10 mg/L to the UDL.

Results for the CRP-assay using the standard and the approach according to the invention:

4.12 Determination of LDL, UDL and CV: See example 1.

4.13 Result overview and conclusions:

As depicted in figure 2 and table 5, the use of larger MP2 particles (290nm instead of 220nm) with higher antibody loading degrees (60mg 36F12/g latex instead of 50 mg/g) in combination with the multi-application approach leads to an improvement of the LDL and dynamic range by a factor of 8 compared with the commercial standard CRP L3 assay using the standard conditions (latex reagent with unmodified MP1 and MP2 particles; measurement at one main wavelength at 570nm, corrected by 800nm, 2.0 min reaction time). The precision (CV) obtained with the new method is comparable with the standard method. The conditions for the new approach are a first calibration curve, recorded at 505nm with 800nm as correction wavelength and 2.8 min reaction time, and a second calibration curve, recorded at 570nm with 800nm as correction wavelength and 2.0 min reaction time.

Table 5: Result overview for CRP with adapted particles MP2

	Standard reagents (MP1: 110-140nm, 45mg 21F12/g latex; MP2: 220nm, 50mg 36F12/g latex), Standard method (570 nm - 800nm, 2.0 min)	**Adapted reagents (MP1: 110-140nm, 45mg 21F12/g latex; MP2: 290nm, 60mg 36F12/g latex), New method (Cal 1: 505 nm - 800nm, 2.8 min; Cal 2: 570nm – 800nm, 2.0 min)**
LDL (mg/L)	0.091	0.011
UDL (mg/L)	350	350
Dynamic range	3846	31818
Measuring range (mg/L)	0.091- 350	0.011- 350
CV at 1 mg/L (%)	4,6	3,4
CV at 5 mg/L (%)	1,5	0,9

Example 5: Additional improvement of the dynamic range for the combination of the multi-application approach with modified particles MP1 and MP2

5.1 In addition, the combination of the multi-application approach with adapted particle sizes and antibody loading degrees was assessed using the CRP L3 assay as lead parameter. The CRP L3 assay includes two different types of particles as described in EP 0898169, large latex particles (MP2, approx.. 220nm diameter) coated with highly affine antibodies (clone 36F12; concentration for latex coating: 50mg/g latex) and small latex particles (MP1, approx.. 110-140 nm diameter) coated with less affine antibodies (clone 21F12, concentration for latex coating: 45mg/g latex). Here, the MP2 particles were modified by using larger sizes and antibody loading degrees and the small MP1 particles were modified by using smaller particles, and finally the achievable LDL, UDL and dynamic range determined in the assay as described below.

5.2 Synthesis of the modified MP2 particles:

To latex particles (carboxylate-modified microparticles, 290nm sized) with a concentration of 2% solids in 750µl MES buffer (20mM, pH 6.1) 30µl of a sulfo-NHS (sulfo- N-hydroxysulfosuccinimide, 100mM in MES buffer) solution and 30µl of an EDC (1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide, 100mM in MES buffer) solution were successively added. The reaction mixture was shortly sonicated and allowed to react for 1 hour at room temperature on a mixing wheel. Afterwards 70mg antibody 36F12 per gram latex in 750µl MES buffer (20mM, pH 6.1) containing 37µl of Synperonic P85 solution (1% in MES) were mixed with the sulfo-NHS/EDC-activated latex particles. The reaction mixture was shortly sonicated and allowed to react for 2 hours at room temperature on a mixing wheel. To the reaction mixture 45µl glycine solution (2M, pH 11) were added, sonicated and mixed for 15 minutes on a mixing wheel. Finally the particles were washed 2 times by centrifugation and resuspension in glycine (50mM, pH 8) containing 0.03% Synperonic P85. After a final centrifugation and resuspension step the latex particles (1% solids) were stored in glycine (50mM, pH 8) containing 0.03% Synperonic P85, 0.1% BSA and 0.05% sodium azide.

5.3 Synthesis of the modified MP1 particles:

To latex particles (carboxylate-modified microparticles, size 90nm) with a concentration of 2% solids in 750µl MES buffer (20mM, pH 6.1) 30µl of a sulfo-NHS (sulfo- N-hydroxysulfosuccinimide, 100mM in MES buffer) solution and 30µl of an EDC (1-Ethyl-3-(3-

dimethyllaminopropyl)carbodiimide, 100mM in MES buffer) solution were successively added. The reaction mixture was shortly sonicated and allowed to react for 1 hour at room temperature on a mixing wheel. Afterwards 50mg antibody 21F12 per gram latex in 750µl MES buffer (20mM, pH 6.1) were mixed with the sulfo-NHS/EDC-activated latex particles. After 20 minutes 37µl of Synperonic P85 solution (1% in MES) was added to the mixture. The reaction mixture was shortly sonicated and allowed to react for 100 minutes at room temperature on a mixing wheel. To the reaction mixture 45µl glycine solution (2M, pH 11) were added, sonicated and mixed for 15 minutes on a mixing wheel. Finally the particles were washed 2 times by centrifugation and resuspension in glycine (50mM, pH 8) containing 0.03% Synperonic P85. After a final centrifugation and resuspension step the latex particles (1% solids) were stored in glycine (50mM, pH 8) containing 0.03% Synperonic P85, 0.1% BSA and 0.05% sodium azide.

5.4 Preparation of the reagents R1 and R2 for the analyser assay:

The reagents R1 and R2 are as described in the package insert document from the tests. The R2 reagent is prepared by mixing the MP1 and MP2 particles synthesized in steps a) and b). The Reagents is filled in a cobas c pack.

5.5 Instrument: See example 1.

5.6 Procedure for the CRP-assay using the standard approach is identical to the procedure of example 1.2.

5.7 Pipetting scheme: identical to pipetting scheme of example 1.3.

5.8 Conditions for the generation of the calibration curve: is identical to example 1.4.

5.9 Procedure for the CRP-assay using the new approach of the invention:

The reagents used for these experiments were prepared as described above and afterwards filled in cobas c packs: R1 (TRIS buffer) and R2 (Latex particles coated with anti-CRP (mouse) in glycine buffer). The procedure described in the package insert from the CRP L3 test was used with exception of the wavelengths and reaction times.

5.10 Pipetting scheme: identical to pipetting scheme of example 1.3.

5.11 Conditions for the generation of the calibration curve:

The best conditions for both calibration curves were determined as described earlier and the following result was obtained: 546nm was used as first wavelength, 800nm as correction wavelength and 7.1 minutes as first reaction time; 660nm was used as second wavelength, 800nm as correction wavelength and 0.8 minutes as second reaction time. The assay type was a two-point-end assay as described under example 1.4. For the first calibration curve the first reading is at measure point 7 and means shortly after the final reagent addition, and the second reading at measure point 50, which corresponds to a reaction time of 7.1 minutes; for the second calibration curve the first reading is at measure point 7 and means shortly after the final reagent addition, and the second reading at measure point 11, which corresponds to a reaction time of 0.8 minutes. For the generation of both calibration curves each 6 standards covering the corresponding concentrations ranges are measured as duplicates with spline as calibration type, which fits the ranges between the data points of the measured calibrators approximated by third degree polynomials so that a smooth calibration curve is obtained. Similar results were obtained when for both calibration curves the 6 standards from the standard method (Cat. No. 11355279) were used.

The first calibration curve is used which is optimized for a low concentration of the specific analyte thereby maximizing the lower detection limit, the second calibration curve is used which is optimized for a high concentration of the specific analyte thereby maximizing the upper detection limit. Either the first calibration curve or the second calibration curve is to be selected for quantification of CRP, depending on the analyte content or corresponding absorbance of the sample to be quantified. For this purpose the signal of the sample is compared with a predetermined threshold value. The threshold value for CRP may be defined as one of the two or both absorbance values in the calibration curves for CRP which correspond to a concentration of 50 mg/L, with other words the absorbance at 546nm-800nm for the reaction time of 7.1 minutes ($A[\lambda 1,t1]$ = A[546nm-800nm,7.1 minutes]) and/or the absorbance at 660nm-800nm for the reaction time of 0.8 minutes ($A[\lambda 2,t2]$ = A[660nm-800nm,0.8 minutes]).

If the optical signal of the sample calculated for 546nm-800nm and 7.1 minutes reaction time is below the predetermined threshold value A[546nm-800nm,7.1 minutes] and/or if the optical signal of the sample calculated for 660nm-800nm and 0.8 minutes reaction time is below the predetermined threshold value A[660nm-800nm,0.8 minutes], the first calibration curve is used for its quantitation.

If the optical signal of the sample calculated for 546nm-800nm and 7.1 minutes reaction time exceeds the predetermined threshold value A[546nm-800nm,7.1 minutes] and/or if the optical signal of the sample calculated for 660nm-800nm and 0.8 minutes reaction time exceeds the predetermined threshold value A[660nm-800nm,0.8 minutes],the second calibration curve is used for its quantitation. As result two calibration curves are obtained for covering the measuring range for CRP determination, the first covering analyte concentrations from 0 to 50mg/L and the second covering the range from 50 mg/L to the UDL.

Results for the CRP-assay using the standard and new approach of the invention:

5.12 Determination of LDL/ UDL / CV: See example 1.

5.13 Result overview and conclusions:

As depicted in figure 2 and table 6, the use of larger MP2 particles (290nm instead of 220nm) with higher antibody loading degrees (70mg 36F12/g latex instead of 50 mg/g) and of smaller MP1 particles (90nm instead of 110-140nm; 50mg 21F12/g latex the for latex conjugation reaction) in combination with the multi-application approach leads to an improvement of the LDL, UDL and dynamic range by a factor of 3.0, 2.3 and 6.9 respectively compared with the commercial standard CRP L3 assay using the standard conditions (latex reagent with unmodified MP1 and MP2 particles; measurement at one main wavelength at 570nm, corrected by 800nm, 2.0 min reaction time). The precision (CV) obtained with the new method is comparable with the standard method. The conditions for the new approach are a first calibration curve, recorded at 546nm with 800nm as correction wavelength and 7.1 min reaction time, and a second calibration curve, recorded at 660nm with 800nm as correction wavelength and 0.8 min reaction time.

Table 6: Result overview for CRP with adapted particles MP1 and MP2

	Standard reagents (MP1: 110-140nm, 45mg 21F12/g latex; MP2: 220nm, 50mg 36F12/g latex), Standard method (570 nm - 800nm, 2.0 min)	**Adapted reagents (MP1: 90nm, 50mg 21F12/g latex; MP2: 290nm, 70mg 36F12/g latex), New method (Cal 1: 546 nm - 800nm, 7.1 min; Cal 2: 660nm – 800nm, 0.8 min)**
LDL (mg/L)	0,060	0,020
UDL (mg/L)	367	844

Dynamic range	6117	42200
Measuring range (mg/L)	0,060 - 367	0,020 - 844
CV at 1 mg/L (%)	8,3	2,7
CV at 5 mg/L (%)	1,9	0,9
CV at 10 mg/L (%)	1,3	1,3
CV at 100 mg/L (%)	1,3	1,3

Example 6: Determination of the dynamic range of colorimetric assays: Alanine Aminotransferase (ALTL)

6.1 Instrument: See example 1.

6.2 Procedure for the ALTL (Alanine Aminotransferase) assay using the standard approach:

Roche´s Alanine Aminotransferase test (ALTL, Cat.No. 20764957), a colorimetric assay, was selected for this study. The enzyme alanine aminotransferase (ALT) catalyzes the reaction between L-alanine and 2-oxoglutarate. The pyruvate formed is reduced by NADH in a reaction catalyzed by lactate dehydrogenase (LDH) to form L-lactate and NAD^+. The rate of NADH oxidation is directly proportional to the catalytic ALT activity which is measured photometrically. It is determined by measuring the decrease in absorbance.

$$\text{L-Alanine} + \text{2-oxoglutarate} \xrightarrow{\text{ALT}} \text{pyruvate} + \text{L-glutamate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$

Reagents for all Roche tests are provided in cobas c packs. These cassettes contain one to three specially designed reagent bottles and have barcode labels with detailed reagent and test-related information. For ALTL tests the cassette is composed of two reagents: R1 (TRIS buffer, pH 7.3, L-alanine, albumin (bovine), LDH (microorganisms), stabilizers and preservative) and R2 (2-

Oxoglutarate, NADH, additives and preservative). The procedure described in the package insert of the ALTL test was used as standard method.

6.3 Pipetting scheme:

9µL sample and 59µL assay reagent R1 diluted with 32µL diluent (water) were subsequently added to the reaction cell, followed by the addition of 17µl 2-Oxoglutarate and NADH reagent (R2) diluted with 20µL diluent (water). Afterwards all reaction components were mixed.

6.4 Conditions for the generation of the calibration curve:

Absorbance readings were carried out at 340nm as main wavelength and at 700nm as correction wavelength. The assay type was a kinetic A assay, also called rate A assay.

For rate assays, the time course of the reaction is followed by measuring the absorbance as a function of time. That is, measurements are taken as the reaction proceeds. Rate assays use these measurements because their concentration calculations are based on the determination of the rate of change in absorbance. A rate A assay is programmed for multiple measure points. This means, there is a measuring window and every photometric measurement within this window is taken into account for the rate calculation - beginning with the reading at the first programmed measure point through the reading at the second programmed measure point. The absorbance values are converted into the rate of change in absorbance by least squares analysis. For ALTL the first programmed measure point (initial measure point) was measure point 12 which means shortly after the sample and the final reagent were pipetted into the reaction cell (66 seconds after the final reagent addition). The second programmed measure point (final measure point) was taken at measure point 31 which corresponds to a reaction time of 4.1 minutes. The signal was calculated from every reading between the initial and the final measure points. The rate of change in absorbance (slope) between these two measure points was calculated by least squares method. For the generation of the calibration curve two standards from Roche (standard 1: water for the blank measurement; standard 2, Cat. No. 10759350) were measured as duplicates with linear calibration type, which fits the data points of the measured calibrators by a linear mode so that a linear calibration curve is obtained.

6.5 Procedure for the ALTL assay according to the invention:

The reagents used for these experiments were identical to those used for the standard method as described in example 6.2. The procedure described in the package insert from the ALTL test was used with exception of the wavelengths and reaction times.

6.6 Pipetting scheme: identical to the pipetting scheme 6.3.

6.7 Conditions for the generation of the calibration curve:

For the generation of the two calibration curves 2 standards were measured as duplicates with linear as calibration type, which fits the data points of the measured calibrators by a linear mode so that a linear calibration curve was obtained (standard 1: water for the blank measurement; standard 2: from Roche Cat.No.10759350). The best conditions for both calibration curves were determined as described earlier and the following result was obtained: 340nm was used as first wavelength, 800nm as correction wavelength and 8.5 minutes as first reaction time; 376nm was used as second wavelength, 700nm as correction wavelength and 1.4 minutes as second reaction time. The assay type was a two-point-end assay for both, the first and second calibration curves as described under example 1.4. For the first calibration curve the first reading was at measure point 7 and means shortly after the final reagent addition, and the second reading at measure point 57, which corresponds to a reaction time of 8.5 minutes; for the second calibration curve the first reading was at measure point 7 and means shortly after the final reagent addition, and the second reading at measure point 14, which corresponds to a reaction time of 1.4 minutes. Similar results were obtained when using other assay types, such as kinetic A, for the calculation of the first and/or second calibration curves. Furthermore, similar results were achieved when using other standard concentrations than those used here (commercially available calibrators from Roche, Cat. No. 10759350), as well as when using more than 2 standards, e.g. up to six standards for the generation of each calibration curve, and different calibration types for each of the two calibration curves (e.g. spline).

The first calibration curve is used which is optimized for a low concentration of the specific analyte thereby maximizing the lower detection limit, the second calibration curve is used which is optimized for a high concentration of the specific analyte thereby maximizing the upper detection limit. Either the first calibration curve or the second calibration curve is to be selected for quantification of ALTL, depending on the analyte content or corresponding absorbance of the sample to be quantified. For this purpose the signal of the sample is compared with a predetermined threshold value. The threshold value for ALTL may be defined as one of the two

or both absorbance values in the calibration curves for ALTL which correspond to a concentration of 200 U/L, with other words the absorbance at 340nm-800nm for the reaction time of 8.5 minutes (A[λ1,t1] = A[340nm-800nm,8.5 minutes]) and/or the absorbance at 376nm-700nm for the reaction time of 1.4 minutes (A[λ2,t2] = A[376nm-700nm,1.4 minutes]).

If the optical signal of the sample calculated for 340nm-800nm and 8.5 minutes reaction time is below the predetermined threshold value A[340nm-800nm,8.5 minutes] and/or if the optical signal of the sample calculated for 376nm-700nm and 1.4 minutes reaction time is below the predetermined threshold value A[376nm-700nm,1.4 minutes],the second calibration curve is used for its quantitation.

If the optical signal of the sample calculated for 340nm-800nm and 8.5 minutes reaction time exceeds the predetermined threshold value A[340nm-800nm,8.5 minutes] and/or if the optical signal of the sample calculated for 376nm-700nm and 1.4 minutes reaction time exceeds the predetermined threshold value A[376nm-700nm,1.4 minutes], the first calibration curve is used for its quantitation. As result two calibration curves are obtained for covering the measuring range for ALTL determination, the first covering analyte concentrations from 0 to 200 U/L and the second covering the range from 200 U/L to the UDL.

Results for the ALTL assay using the standard and the approach according to the invention:

6.8 Determination of LDL / UDL / CV: See example 1.

6.9 Result overview and conclusions:

The result overview is depicted in Table 7. The application of the new approach to Roche´s ALTL test shows an increase of the sensitivity (LDL, lower detection limit) by a factor of 2.9 and an extension of the upper detection limit UDL by a factor of 4.2. Finally the dynamic range was extended by a factor of 12.2. The precision (CV) obtained with the new method is comparable with the standard method. The new approach affords minimal implementation; the application of the approach does not need any changes of reagents and their formulations. Only the number and/or concentration of standards for the calibration may be adapted if needed.

Table 7: Result overview for ALTL

	Standard method	**New method**

	(340 nm wavelength, 700nm correction wavelength, 4.1 min. reaction time)	(calibration curve 1: 340 nm wavelength, 800nm correction wavelength, 8.5 min. reaction time; calibration curve 2: 376nm wavelength, 700nm correction wavelength, 1.4 min reaction time)
LDL (U/L)	1.6	0.55
UDL (U/L)	811	3383
Dynamic range	507	6151
Measuring range (U/L)	1.6 - 811	0.55 - 3383
CV at 10 U/L (%)	8.37	5.00
CV at 150 U/L (%)	0.53	0.30
CV at 2000 U/L (%)	0.47	0.44

Example 7: Determination of the dynamic range of colorimetric assays: Aspartate Aminotransferase (ASTL)

7.1 Instrument: See example 1.

7.2 Procedure for the ASTL (Aspartate Aminotransferase) assay using the standard approach:

Roche´s Aspartate Aminotransferase test (ASTL, Cat.No. 20764949), a colorimetric assay, was selected for this study. The enzyme aspartate aminotransferase (AST) catalyzes the reaction between L-aspartate and 2-oxoglutarate. The oxalacetate formed is reduced by NADH in a reaction catalyzed by malate dehydrogenase (MDH) to form L-malate and NAD^+. The rate of NADH oxidation is directly proportional to the catalytic AST activity which is measured photometrically. It is determined by measuring the decrease in absorbance.

$$\text{L-Aspartate} + \text{2-oxoglutarate} \xrightarrow{\text{AST}} \text{oxalacetate} + \text{L-glutamate}$$

$$\text{Oxalacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-malate} + NAD^+$$

Reagents for all Roche tests are provided in cobas c packs. These cassettes contain one to three specially designed reagent bottles and have barcode labels with detailed reagent and test-related information. For ASTL tests the cassette is composed of two reagents: R1 (TRIS buffer, pH 7.8, L-aspartate, albumin (bovine), MDH (microorganisms), LDH (microorganisms), stabilizers and preservative) and R2 (2-Oxoglutarate, NADH and preservative). The procedure described in the package insert of the ASTL test was used as standard method.

7.3 Pipetting scheme:

9µL sample and 40µL assay reagent R1 diluted with 51µL diluent (water) were subsequently added to the reaction cell, followed by the addition of 17µl reagent R2 diluted with 20µL diluent (water). Afterwards the reaction mixture was mixed.

7.4 Conditions for the generation of the calibration curve:

Absorbance readings were carried out at 340nm as main wavelength and at 700nm as correction wavelength. The assay type was a kinetic A assay, also called rate A assay.

For rate assays, the time course of the reaction is followed by measuring the absorbance as a function of time. That is, measurements are taken as the reaction proceeds. Rate assays use these measurements because their concentration calculations are based on the determination of the rate of change in absorbance. A rate A assay is programmed for multiple measure points. This means, there is a measuring window and every photometric measurement within this window is taken into account for the rate calculation - beginning with the reading at the first programmed measure point through the reading at the second programmed measure point. The absorbance values are converted into the rate of change in absorbance by least squares analysis. For ALTL the first programmed measure point (initial measure point) was measure point 12 which means shortly after the sample and the final reagent were pipetted into the reaction cell (66 seconds after the final reagent addition). The second programmed measure point (final measure point) was taken at measure point 31 which corresponds to a reaction time of 4.1 minutes. The signal was calculated from every reading between the initial and the final measure points. The rate of change in absorbance (slope) between these two measure points was calculated by least squares method. For the generation of the calibration curve two standards from Roche (standard 1: water for the blank measurement; standard 2, Cat. No. 10759350) were measured as duplicates with linear calibration type, which fits the data points of the measured calibrators by a linear mode so that a linear calibration curve is obtained.

7.5 Procedure for the ASTL assay according to the present invention:

The reagents used for these experiments were identical to those used for the standard method as described in example 7.2. The procedure described in the package insert from the ASTL test was used with exception of the wavelengths and reaction times.

7.6 Pipetting scheme: identical to the pipetting scheme 7.3.

7.7 Conditions for the generation of the calibration curve:

For the generation of the two calibration curves 2 standards were measured as duplicates with linear as calibration type, which fits the data points of the measured calibrators by a linear mode so that a linear calibration curve was obtained (standard 1: water for the blank measurement; standard 2: from Roche Cat.No.10759350). The best conditions for both calibration curves were determined as described earlier and the following result was obtained: 340nm was used as first wavelength, 700nm as correction wavelength and 8.3 minutes as first reaction time; 376nm was used as second wavelength, 800nm as correction wavelength and 1.2 minutes as second reaction time. The assay type was a two-point-end assay for both, the first and second calibration curves as described under example 1.4. For the first calibration curve the first reading was at measure point 8 and means shortly after the final reagent addition, and the second reading at measure point 57, which corresponds to a reaction time of 8.3 minutes; for the second calibration curve the first reading was at measure point 8 and means shortly after the final reagent addition, and the second reading at measure point 14, which corresponds to a reaction time of 1.2 minutes. Similar results were obtained when using other assay types, such as kinetic A, for the calculation of the first and/or second calibration curves. Furthermore, similar results were achieved when using other standard concentrations than those used here (commercially available calibrators from Roche, Cat. No. 10759350), as well as when using more than 2 standards, e.g. up to six standards for the generation of each calibration curve, and different calibration types for each of the two calibration curves (e.g. spline).

The first calibration curve is used which is optimized for a low concentration of the specific analyte thereby maximizing the lower detection limit, the second calibration curve is used which is optimized for a high concentration of the specific analyte thereby maximizing the upper detection limit. Either the first calibration curve or the second calibration curve is to be selected for quantification of ASTL, depending on the analyte content or corresponding absorbance of the sample to be quantified. For this purpose the signal of the sample is compared with a

predetermined threshold value. The threshold value for ASTL may be defined as one of the two or both absorbance values in the calibration curves for ASTL which correspond to a concentration of 200 U/L, with other words the absorbance at 340nm-700nm for the reaction time of 8.3 minutes (A[λ1,t1] = A[340nm-700nm,8.3 minutes]) and/or the absorbance at 376nm-800nm for the reaction time of 1.2 minutes (A[λ2,t2] = A[376nm-800nm,1.2 minutes]).

If the optical signal of the sample calculated for 340nm-700nm and 8.3 minutes reaction time is below the predetermined threshold value A[340nm-700nm,8.3 minutes] and/or if the optical signal of the sample calculated for 376nm-800nm and 1.2 minutes reaction time is below the predetermined threshold value A[376nm-800nm,1.2 minutes],the second calibration curve is used for its quantitation.

If the optical signal of the sample calculated for 340nm-700nm and 8.3 minutes reaction time exceeds the predetermined threshold value A[340nm-700nm,8.3 minutes] and/or if the optical signal of the sample calculated for 376nm-800nm and 1.2 minutes reaction time exceeds the predetermined threshold value A[376nm-800nm,1.2 minutes], the first calibration curve is used for its quantitation. As result two calibration curves are obtained for covering the measuring range for ASTL determination, the first covering analyte concentrations from 0 to 200 U/L and the second covering the range from 200 U/L to the UDL.

Results for the ASTL assay using the standard and the approach according to the invention:

7.8 Determination of LDL / UDL / CV: See example 1.

7.9 Result overview and conclusions:

The result overview is depicted in Table 8. The application of the new approach to Roche´s ASTL test shows an increase of the sensitivity (LDL, lower detection limit) by a factor of 2.2 and an extension of the upper detection limit UDL by a factor of 3.4. Finally the dynamic range was extended by a factor of 7.5. The precision (CV) obtained with the new method is comparable with the standard method. The new approach affords minimal implementation; the application of the approach does not need any changes of reagents and their formulations. Only the number and/or concentration of standards for the calibration may be adapted if needed.

Table 8: Result overview for ASTL

	Standard method (340 nm wavelength, 700nm correction wavelength, 4.1 min. reaction time)	**New method (calibration curve 1: 340 nm wavelength, 700nm correction wavelength, 8.3 min. reaction time; calibration curve 2: 376nm wavelength, 800nm correction wavelength, 1.2 min reaction time)**
LDL (U/L)	1.71	0.78
UDL (U/L)	854	2868
Dynamic range	502	3677
Measuring range (U/L)	1.71-854	0.78-2868
CV at 20 U/L (%)	4.46	1.67
CV at 150 U/L (%)	0.58	0.27
CV at 2000 U/L (%)	1.12	0.31

Example 8: Determination of the dynamic range of colorimetric assays: Blood-Urea-Nitrogen (U-BUN)

8.1 Instrument: See example 1.

8.2 Procedure for the U-BUN (Blood-Urea-Nitrogen) assay using the standard approach:

Roche´s Blood-Urea-Nitrogen test (UREAL, Cat.No. 04460715), a colorimetric assay, was selected for this study. The enzyme urease hydrolyzes urea in the presence of urease to form ammonium and carbonate. In the following reaction glutamate dehydrogenase (GLDH) catalyzes the reaction between 2-oxoglutarate, ammonium and NADH resulting in L-glutamate and NAD^+ formation. The rate of decrease in the NADH concentration is directly proportional to the urea concentration in the specimen and is measured photometrically. It is determined by measuring the decrease in absorbance.

$$\text{Urea} + 2\,H_2O \xrightarrow{\text{UREASE}} 2\,NH_4^+ + CO_3^{2-}$$

$$NH_4^+ + \text{2-oxoglutarate} + \text{NADH} \xrightarrow{\text{GLDH}} \text{L-glutamate} + NAD^+ + H_2O$$

Reagents for all Roche tests are provided in cobas c packs. These cassettes contain one to three specially designed reagent bottles and have barcode labels with detailed reagent and test-related information. For U-BUN tests the cassette is composed of two reagents: R1 (NaCl 9%) and R2 (TRIS buffer, pH 8.6, 2-oxoglutarate, NADH, ADP, urease (jack bean), GLDH (bovine liver), nonreactive stabilizers and preservatives). The procedure described in the package insert of the U-BUN (UREAL) test was used as standard method.

8.3 Pipetting scheme:

2µL sample and 10µL assay reagent R1 diluted with 90µL diluent (water) were subsequently added to the reaction cell, followed by the addition of 38µl of reagent R2 diluted with 110µL diluent (water). Afterwards the reaction components were mixed.

8.4 Conditions for the generation of the calibration curve:

Absorbance readings were carried out at 340nm as main wavelength and at 700nm as correction wavelength. The assay type was a kinetic A assay, also called rate A assay.

For rate assays, the time course of the reaction is followed by measuring the absorbance as a function of time. That is, measurements are taken as the reaction proceeds. Rate assays use these measurements because their concentration calculations are based on the determination of the rate of change in absorbance. A rate A assay is programmed for multiple measure points. This means, there is a measuring window and every photometric measurement within this window is taken into account for the rate calculation - beginning with the reading at the first programmed measure point through the reading at the second programmed measure point. The absorbance values are converted into the rate of change in absorbance by least squares analysis. For U-BUN the first programmed measure point (initial measure point) was measure point 10 which means shortly after the sample and the final reagent were pipetted into the reaction cell (42 seconds after the final reagent addition). The second programmed measure point (final measure point) was taken at measure point 19 which corresponds to a reaction time of 1.8 minutes. The signal was calculated from every reading between the initial and the final measure points. The rate of change in absorbance (slope) between these two measure points was calculated by least squares method. For the generation of the calibration curve two standards from Roche (standard 1: water for the blank measurement; standard 2, Cat. No. 10759350) were measured as duplicates with linear calibration type, which fits the data points of the measured calibrators by a linear mode so that a linear calibration curve is obtained.

8.5 Procedure for the U-BUN assay according to the present invention:

The reagents used for these experiments were identical to those used for the standard method as described in example 8.2. The procedure described in the package insert of the U-BUN (UREAL) test was used with exception of the wavelengths and reaction times.

8.6 Pipetting scheme: identical to the pipetting scheme 8.3.

8.7 Conditions for the generation of the calibration curve:

For the generation of the two calibration curves 2 standards were measured as duplicates with linear as calibration type, which fits the data points of the measured calibrators by a linear mode so that a linear calibration curve was obtained (standard 1: water for the blank measurement; standard 2: from Roche Cat.No.10759350). The best conditions for both calibration curves were determined as described earlier and the following result was obtained: for the first calibration curve 340nm was used as first wavelength, 800nm as correction wavelength and 8.3 minutes as first reaction time; for the second calibration curve 376nm was used as second wavelength, 700nm as correction wavelength and 1.4 minutes as second reaction time. The assay type was a two-point-end assay for both, the first and second calibration curves as described under example 1.4. For the first calibration curve the first reading was at measure point 8 and means shortly after the final reagent addition, and the second reading at measure point 57, which corresponds to a reaction time of 8.3 minutes; for the second calibration curve the first reading was at measure point 8 and means shortly after the final reagent addition, and the second reading at measure point 15, which corresponds to a reaction time of 1.4 minutes. Similar results were obtained when using other assay types, such as kinetic A, for the calculation of the first and/or second calibration curves. Furthermore, similar results were achieved when using other standard concentrations than those used here (commercially available calibrators from Roche, Cat. No. 10759350), as well as when using more than 2 standards, e.g. up to six standards for the generation of each calibration curve, and different calibration types for each of the two calibration curves (e.g. spline).

The first calibration curve is used which is optimized for a low concentration of the specific analyte thereby maximizing the lower detection limit, the second calibration curve is used which is optimized for a high concentration of the specific analyte thereby maximizing the upper detection limit. Either the first calibration curve or the second calibration curve is to be selected for quantification of U-BUN, depending on the analyte content or corresponding absorbance of

the sample to be quantified. For this purpose the signal of the sample is compared with a predetermined threshold value. The threshold value for U-BUN may be defined as one of the two or both absorbance values in the calibration curves for U-BUN which correspond to a concentration of 10 mmol/L, with other words the absorbance at 340nm-800nm for the reaction time of 8.3 minutes (A[λ1,t1] = A[340nm-800nm,8.3 minutes]) and/or the absorbance at 376nm-700nm for the reaction time of 1.4 minutes (A[λ2,t2] = A[376nm-700nm,1.4 minutes]).

If the optical signal of the sample calculated for 340nm-800nm and 8.3 minutes reaction time is below the predetermined threshold value A[340nm-800nm,8.3 minutes] and/or if the optical signal of the sample calculated for 376nm-700nm and 1.4 minutes reaction time is below the predetermined threshold value A[376nm-700nm,1.4 minutes],the second calibration curve is used for its quantitation.

If the optical signal of the sample calculated for 340nm-800nm and 8.3 minutes reaction time exceeds the predetermined threshold value A[340nm-800nm,8.3 minutes] and/or if the optical signal of the sample calculated for 376nm-700nm and 1.4 minutes reaction time exceeds the predetermined threshold value A[376nm-700nm,1.4 minutes], the first calibration curve is used for its quantitation. As result two calibration curves are obtained for covering the measuring range for U-BUN determination, the first covering analyte concentrations from 0 to 10 mmol/L and the second covering the range from 10 mmol/L to the UDL.

Results for the U-BUN (UREAL) assay using the standard and the approach according to the invention:

8.8 Determination of LDL / UDL / CV: See example 1.

8.9 Result overview and conclusions:

The result overview is depicted in Table 9. The application of the new approach to Roche´s U-BUN test shows an increase of the sensitivity (LDL, lower detection limit) by a factor of 2.2 and an extension of the upper detection limit UDL by a factor of 1.4. Finally the dynamic range was extended by a factor of 3. The precision (CV) obtained with the new method is comparable with the standard method. The new approach affords minimal implementation; the application of the approach does not need any changes of reagents and their formulations. Only the number and/or concentration of standards for the calibration may be adapted if needed.

Table 9: Result overview for U-BUN

	Standard method (340 nm wavelength, 700nm correction wavelength, 1.8 min. reaction time)	**New method (calibration curve 1: 340 nm wavelength, 800nm correction wavelength, 8.3 min. reaction time; calibration curve 2: 376nm wavelength, 700nm correction wavelength, 1.4 min reaction time)**
LDL (mmol/L)	0.071	0.032
UDL (mmol/L)	57	78
Dynamic range	803	2438
Measuring range (mmol/L)	0.071-57	0.032-78
CV at 2 mmol/L (%)	1.74	1.35
CV at 10 mmol/L (%)	0.99	0.91
CV at 60 mmol/L (%)	0.72	0.92

Example 9: Determination of the dynamic range of colorimetric assays: Glucose (Gluc3)

9.1 Instrument: See example 1.

9.2 Procedure for the Glucose (Gluc3) assay using the standard approach:

Roche´s Glucose test (Gluc3, Cat.No. 04404483), a colorimetric assay, was selected for this study. The enzyme hexokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate (G-6-P) by ATP. In the following reaction glucose-6-phosphate dehydrogenase (G-6-PDH) oxidizes glucose-6-phosphate in presence of $NADP^+$ resulting in gluconate-6-phosphate and NADPH formation. The rate of NADPH formation is directly proportional to the glucose concentration in the specimen and is measured photometrically. It is determined by measuring the increase in absorbance.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G-6-PDH}} \text{gluconate-6-P} + \text{NADPH} + \text{H}^+$$

Reagents for all Roche tests are provided in cobas c packs. These cassettes contain one to three specially designed reagent bottles and have barcode labels with detailed reagent and test-related information. For Gluc3 tests the cassette is composed of two reagents: R1 (MES buffer, pH 6.0, Mg^{2+}, ATP, NADP and preservative) and R2 (HEPES buffer, pH 8.0, Mg^{2+}, hexokinase (yeast), glucose-6-phosphate dehydrogenase (E.coli) and preservatives). The procedure described in the package insert of the Gluc3 test was used as standard method.

9.3 Pipetting scheme:

2µL sample and 28µL assay reagent R1 diluted with 141µL diluent (water) were subsequently added to the reaction cell, followed by the addition of 10µl of reagent R2 diluted with 20µL diluent (water). Afterwards the reaction components were mixed.

9.4 Conditions for the generation of the calibration curve:

Absorbance readings were carried out at 340nm as main wavelength and at 700nm as correction wavelength. The absorbance readings were performed at two different measure points. The assay type was a two-point-end assay. A two-point-end assay is an end-point-assay which performs a sample blank. Here two absorbance readings at two different measure points are considered: the first reading is usually taken before or shortly after the final reagent is added; the second reading is taken at any time point after the final reagent was added. The absorbance value for the calibration curve and therefore for the concentration calculation is obtained by subtraction of the first reading from the second reading.

For Gluc3 the first reading is at measure point 6 and means shortly before the final reagent addition, and the second reading at measure point 32, which corresponds to a reaction time of 5.5 minutes. For the generation of the calibration curve two standards were measured as duplicates with linear as calibration type, which fits the data points of the measured calibrators by a linear mode so that a linear calibration curve is obtained (standard 1: water for the blank measurement; standard 2: from Roche Cat.No.10759350).

9.5 Procedure for the Gluc3 assay according to the invention:

The reagents used for these experiments were identical to those used for the standard method as described in example 9.2. The procedure described in the package insert of the Gluc3 test was used with exception of the wavelengths and reaction times.

9.6 Pipetting scheme: identical to the pipetting scheme 9.3.

9.7 Conditions for the generation of the calibration curve:

For the generation of the two calibration curves two standards were measured as duplicates with linear as calibration type, which fits the data points of the measured calibrators by a linear mode so that a linear calibration curve was obtained (standard 1: water for the blank measurement; standard 2: from Roche Cat.No.10759350). The best conditions for both calibration curves were determined as described earlier and the following result was obtained: for the first calibration curve 340nm was used as first wavelength, 700nm as correction wavelength and 8.7 minutes as first reaction time; for the second calibration curve 376nm was used as second wavelength, 700nm as correction wavelength and 5.5 minutes as second reaction time. The assay type was a two-point-end assay which was already described in the standard method for Gluc3 test. For the first calibration curve the first reading was performed at measure point 6 (6 seconds before the final reagent addition) and the second reading at measure point 57, which corresponds to a reaction time of 8.7 minutes; for the second calibration curve the first reading was also at measure point 6 and the second reading at measure point 32, which corresponds to a reaction time of 5.5 minutes. Similar results were obtained when using other assay types, such as kinetic A, for the calculation of the first and/or second calibration curves. Furthermore, similar results were achieved when using other standard concentrations than those used here (commercially available calibrators from Roche, Cat. No. 10759350), as well as when using more than 2 standards, e.g. up to six standards for the generation of each calibration curve, and different calibration types for each of the two calibration curves (e.g. spline).

The first calibration curve is used which is optimized for a low concentration of the specific analyte thereby maximizing the lower detection limit, the second calibration curve is used which is optimized for a high concentration of the specific analyte thereby maximizing the upper detection limit. Either the first calibration curve or the second calibration curve is to be selected for quantification of Gluc3, depending on the analyte content or corresponding absorbance of the sample to be quantified. For this purpose the signal of the sample is compared with a predetermined threshold value. The threshold value for Gluc3 may be defined as one of the two or both absorbance values in the calibration curves for Gluc3 which correspond to a concentration of 10 mmol/L, with other words the absorbance at 340nm-700nm for the reaction time of 8.7 minutes (A[λ1,t1] = A[340nm-700nm,8.7 minutes]) and/or the absorbance at 376nm-700nm for the reaction time of 5.5 minutes (A[λ2,t2] = A[376nm-700nm,5.5 minutes]).

If the optical signal of the sample calculated for 340nm-700nm and 8.7 minutes reaction time is below the predetermined threshold value A[340nm-700nm,8.7 minutes] and/or if the optical signal of the sample calculated for 376nm-700nm and 5.5 minutes reaction time is below the predetermined threshold value A[376nm-700nm,5.5 minutes], the first calibration curve is used for its quantitation.

If the optical signal of the sample calculated for 340nm-700nm and 8.7 minutes reaction time exceeds the predetermined threshold value A[340nm-700nm,8.7 minutes] and/or if the optical signal of the sample calculated for 376nm-700nm and 5.5 minutes reaction time exceeds the predetermined threshold value A[376nm-700nm,5.5 minutes], the second calibration curve is used for its quantitation. As result two calibration curves are obtained for covering the measuring range for Gluc3 determination, the first covering analyte concentrations from 0 to 10 mmol/L and the second covering the range from 10 mmol/L to the UDL.

Results for the Gluc3 assay using the standard and the approach according to the invention:

9.8 Determination of LDL / UDL / CV: See example 1.

9.9 Result overview and conclusions:

The result overview is depicted in Table 10. The application of the new approach to Roche´s Gluc3 test shows an increase of the sensitivity (LDL, lower detection limit) by a factor of 1.5 and an extension of the upper detection limit UDL by a factor of 1.6. Finally the dynamic range was extended by a factor of 2.4. The precision (CV) obtained with the new method is comparable with the standard method. The new approach affords minimal implementation; the application of the approach does not need any changes of reagents and their formulations. Only the number and/or concentration of standards for the calibration may be adapted if needed.

Table 10: Result overview for Gluc3

	Standard method (340 nm wavelength, 700nm correction wavelength, 5.5 min. reaction time)	**New method (calibration curve 1: 340 nm wavelength, 700nm correction wavelength, 8.7 min. reaction time; calibration curve 2: 376nm wavelength, 700nm correction wavelength, 5.5 min reaction time)**
LDL (mmol/L)	0.017	0.011
UDL (mmol/L)	46	73
Dynamic range	2706	6636
Measuring range (mmol/L)	0.017-46	0.011-73
CV at 0.3 mmol/L (%)	6.51	5.94
CV at 10 mmol/L (%)	0.75	0.71
CV at 100 mmol/L (%)	0.55	0.83

CLAIMS:

1. A method for determining an amount of a specific analyte in a sample by photometric assays, wherein the specific analyte in the sample reacts with an analyte specific reaction partner in a reaction mixture, the method comprising:

generating at least two calibration curves, wherein

a first calibration curve is recorded at a first wavelength and is optimized for low concentrations of the specific analyte thereby maximizing a lower detection limit and, and

a second calibration curve is recorded at a second wavelength and is optimized for high concentrations of the specific analyte thereby maximizing an upper detection limit,

measuring simultaneously an optical signal for the specific analyte of the sample in the reaction mixture at least at the first and second wavelengths,

selecting either the first or the second calibration curve for quantification of the specific analyte, by the following criteria:

for increasing calibration curves:

if the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by use of the first calibration curve recorded at the first wavelength;

if the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by use of the second calibration curve recorded at the second wavelength,

for decreasing calibration curves:

if the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength;

if the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength; and

quantifying the amount of the specific analyte by comparison with the selected calibration curve.

2. The method according to claim 1, wherein in turbidimetric and nephelometric immunoassays the specific analyte is quantified from a change in the turbidity of the reaction mixture based on an agglutination of the specific analyte and the analyte specific binding partner.

3. The method according to claim 1, wherein in colorimetric assays the specific analyte is quantified with the aid of a color reagent.

4. The method according to any one of claims 1 to 3, wherein the first calibration curve recorded at the first wavelength is recorded at a first reaction time.

5. The method according to any one of claims 1 to 4, wherein the second calibration curve recorded at the second wavelength is recorded at a second reaction time.

6. The method according to any one of claims 1 to 5, wherein the simultaneous measurement of the optical signal for the specific analyte of a sample in the reaction mixture is performed at least at the first and second wavelengths over the complete reaction time at which the first calibration curve is recorded at the first wavelength and the second calibration curve is recorded at the second wavelength.

7. The method according to any one of claims 1 to 6, wherein the simultaneous measurements are stored in a data management system of an instrument platform.

8. The method according to any one of claims 1 to 7, wherein further wavelengths are determined as a blank value for a correction of interferences and compensation of photometric noise.

9. The method according to any one of claims 1 to 8, wherein the at least two calibration curves are recorded at the same wavelengths and at different reaction times.

10. The method according to any one of claims 1 to 9, wherein the analyte specific reaction partner comprises binding proteins, antigens, antigen fragments, antibodies, antibody fragments, nucleic acids, receptors and particle enhanced binding partners, enzymes, substrates or specific chemical reagents leading to a color change or to a turbidity change in the presence of the specific analyte.

11. A method for increasing the sensitivity and/or dynamic range of photometric assays, wherein a specific analyte in a sample reacts with an analyte specific reaction partner in a reaction mixture, the method comprising:

generating at least two calibration curves, wherein

a first calibration curve is recorded at a first wavelength and is optimized for low concentrations of the specific analyte thereby maximizing a lower detection limit and,

a second calibration curve is recorded at a second wavelength and is optimized for high concentrations of the specific analyte thereby maximizing an upper detection limit,

measuring simultaneously an optical signal for the specific analyte of the sample in the reaction mixture at least at the first and second wavelengths,

selecting either the first or the second calibration curve for quantification of the specific analyte, by the following criteria:

for increasing calibration curves:

if the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by use of the first calibration curve recorded at the first wavelength;

if the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by use of the second calibration curve recorded at the second wavelength,

for decreasing calibration curves:

if the optical signal of the first and/or the second wavelength is lower than a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the second calibration curve recorded at the second wavelength;

if the optical signal of the first and/or the second wavelength exceeds a corresponding predetermined threshold value, the concentration of the analyte is determined by the use of the first calibration curve recorded at the first wavelength, and

quantifying an amount of the specific analyte by comparing the optical signal with the selected calibration curve.

12. A computer-implemented method of determining an optimal wavelength, reaction time and threshold of the first calibration curve and the second calibration curve as defined in any one of claims 1 to 11, the method comprising the steps of:

receiving a dataset representing absorbance values of samples recorded at multiple wavelengths over the complete reaction time at which the first calibration curve is recorded at the first wavelength and the second calibration curve is recorded at the second wavelength simultaneously available on an analyzer, wherein said dataset comprising a plurality of data points for:

standards for the calibration,

a blank sample for determination of LDL,

at least two samples for the determination of precision, and

dilution series

setting the threshold values at a concentration where the calibration curves change from the first to the second calibration curve, by using for lower concentrations the first calibration curve for the analyte quantification and by using for higher concentrations the second calibration curve for the analyte quantification,

determining the first and second wavelength as well as the first and the second reaction time; analyzing kinetic curves from the absorbance values recorded over the reaction time and selecting the optimal set up for the first calibration curve and the second calibration curve.

13. A computer readable medium including code controlling a processor for determining an optimal wavelength, reaction time and threshold of the first calibration curve and the second calibration curve as defined in any one of claims 1 to 11, the code comprising instructions for:

receiving a dataset representing absorbance values of samples recorded at multiple wavelengths over the complete reaction time at which the first calibration curve is recorded at the first wavelength and the second calibration curve is recorded at the second wavelength simultaneously available on an analyzer, wherein said dataset comprises a plurality of data points for:

standards for the calibration;

a blank sample for determination of LDL;

at least two samples for the determination of precision; and

at least one dilution series;

setting the threshold values at a concentration where the calibration curves change from the first to the second calibration curve, by using for lower concentrations the first calibration curve for the analyte quantification and by using for higher concentrations the second calibration curve for the analyte quantification; and

determining the first and second wavelength as well as the first and the second reaction time; analyzing kinetic curves from the absorbance values recorded over the reaction time and selecting the optimal set up for the first calibration curve and the second calibration curve according to any one of claims 1 to 10.

14. A system analyzer comprising:

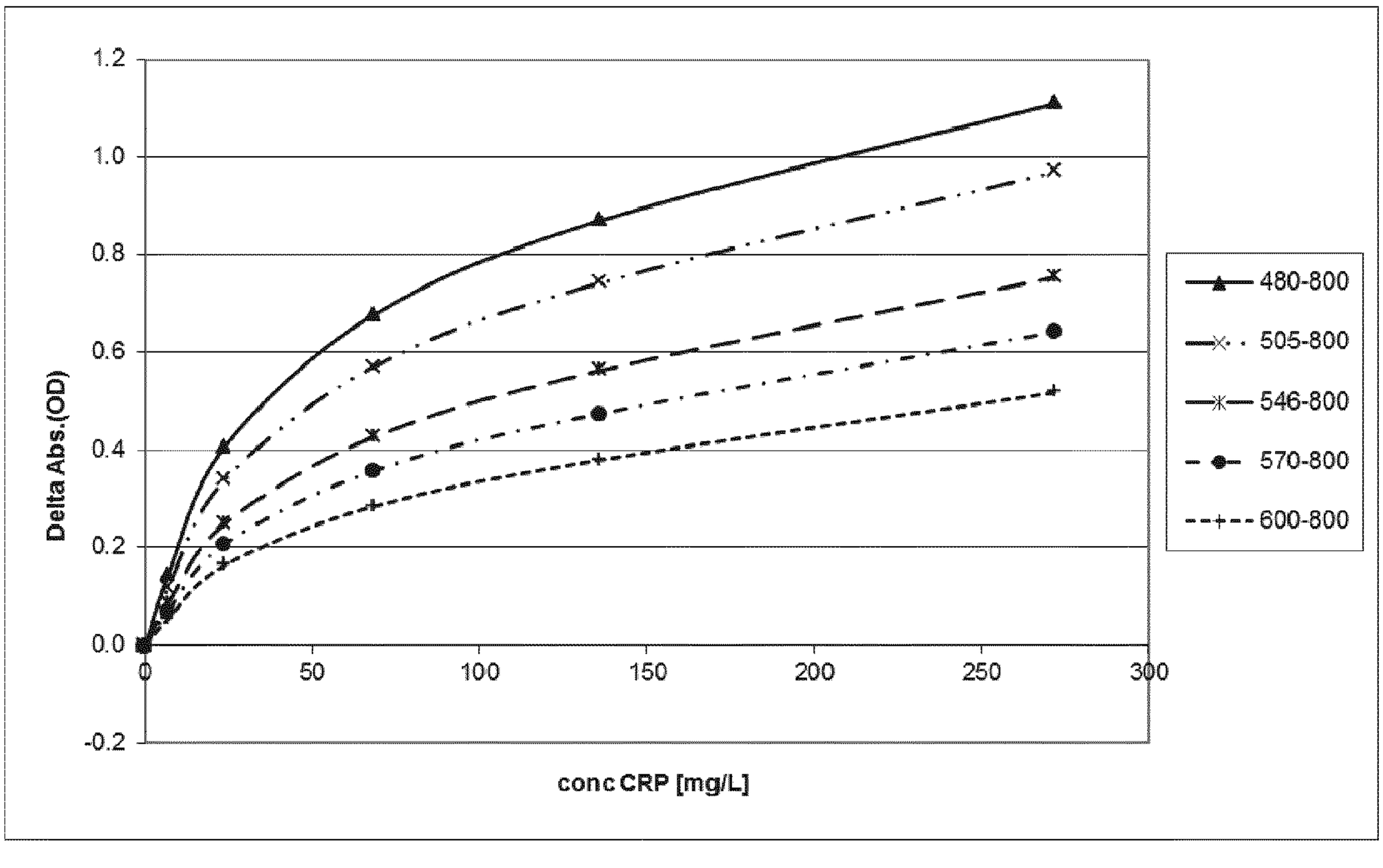
a sample container and an assay reagent container;

a reaction container for receiving and mixing a sample and assay reagents;

a photometer for measuring the reaction mixture of the sample and assay reagents; and

means for outputting an analysis result for determining the amount of a specific analyte according to the method of claim 1.

Figure 1:


1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Delta Abs.(OD)
0
50
100
150
200
250
300
conc CRP [mg/L]
480-800
505-800
546-800
570-800
600-800

CA 02871536 2014-10-24

Test	Standard conditions [a,c]	New conditions [a,c] Cal 1	New conditions [a,c] Cal 2	Improvement factor: LDL	LDL	dyn. range
CRP: direct assay format with selected MP2	570 – 800 nm / 2.0 min MP1: 110-140 nm, 45mg IgG/g MP2: 220 nm, 50mg IgG/g	505 – 800 / 2.8 min MP1: 110-140 nm, 45mg IgG/g MP2: 290 nm, 60mg IgG/g	570 – 800 nm / 2.0 min MP1: 110-140 nm, 45mg IgG/g MP2: 290 nm, 60mg IgG/g	8	-	8
CRP: direct assay format with selected MP1 and MP2	570 – 800 nm / 2.0 min MP1: 110-140 nm, 45mg IgG/g MP2: 220 nm, 50mg IgG/g	545 – 800 / 7.1 min MP1: 90 nm, 50mg IgG/g MP2: 290 nm, 70mg IgG/g	660 – 800 nm / 0.8 min MP1: 90 nm, 50mg IgG/g MP2: 290 nm, 70mg IgG/g	3.0	2.3	6.9

a The CRP L3 assay from Roche includes two different types of particles, large particles MP2 (approx. 220nm) with highly affine anti-CRP antibodies and small particles MP1 (approx. 110-140nm) with less affine anti-CRP antibodies,

b Good precision from standard conditions is retained in new conditions,

c Main wavelength – correction wavelength / reaction time in minutes

Figure 2:

Figure 3:

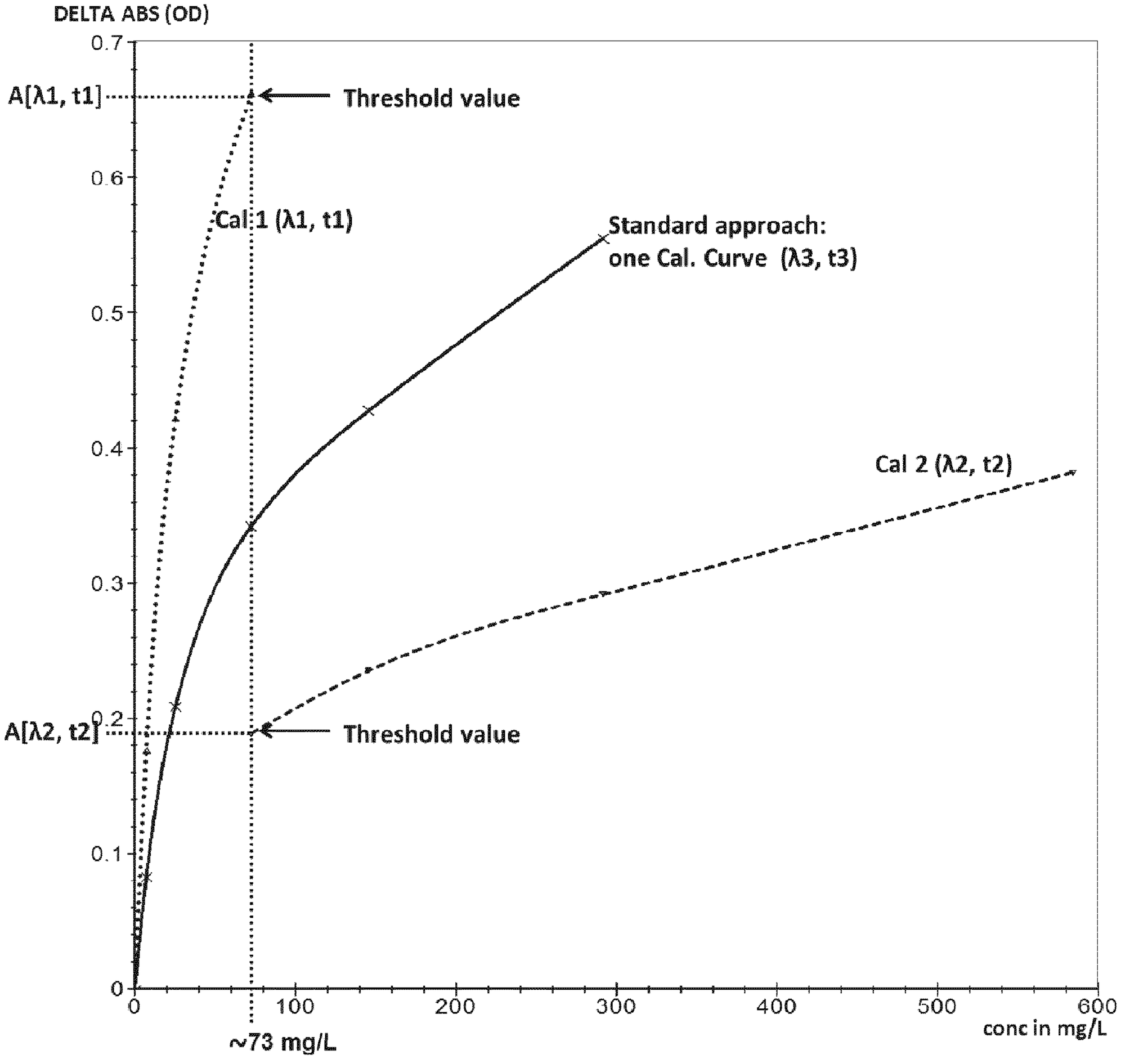

DELTA ABS (OD)
0.7
A[λ1, t1]
Threshold value
0.6
Cal 1 (λ1, t1)
Standard approach:
one Cal. Curve (λ3, t3)
0.5
0.4
Cal 2 (λ2, t2)
0.3
0.2
A[λ2, t2]
Threshold value
0.1
0
0
100
200
300
400
500
600
conc in mg/L
~73 mg/L

Figure 4:

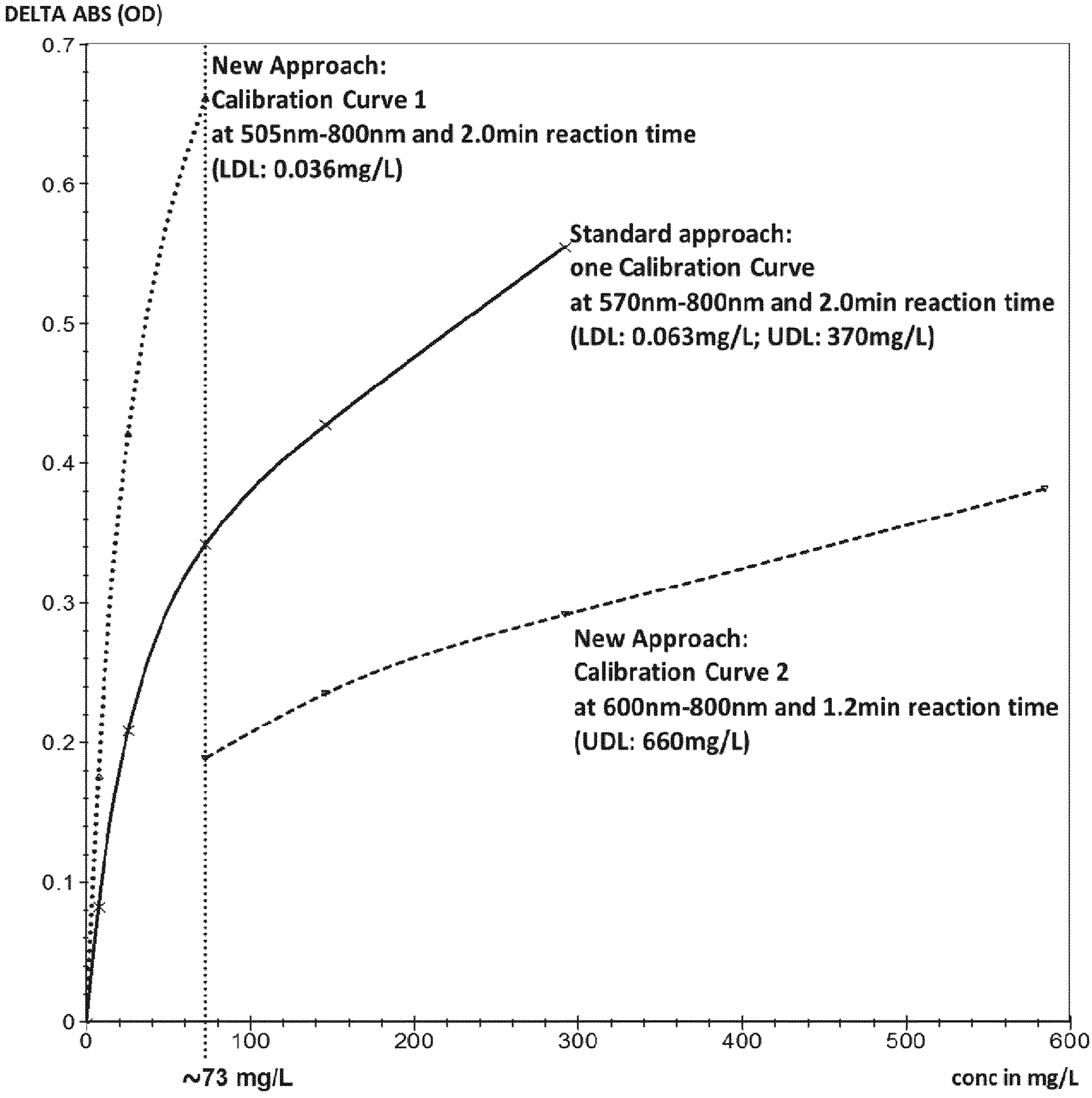

DELTA ABS (OD)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
New Approach:
Calibration Curve 1
at 505nm-800nm and 2.0min reaction time
(LDL: 0.036mg/L)
Standard approach:
one Calibration Curve
at 570nm-800nm and 2.0min reaction time
(LDL: 0.063mg/L; UDL: 370mg/L)
New Approach:
Calibration Curve 2
at 600nm-800nm and 1.2min reaction time
(UDL: 660mg/L)
0
100
200
300
400
500
600
~73 mg/L
conc in mg/L

Figure 5:

Result overview – in most cases the new approach improves/extends the dynamic range ***w/o reagent modification***

Roche test	Standard conditions [d]	New conditions [d]		Improvement factor [c]		
		Cal 1	Cal 2	LDL	UDL	dyn range
Ferritin Gen. 4 direct assay	570 nm – 800 nm / 5.1min	505 nm – 800 nm / 4.9min	570 – 800 nm / 5.1min	1.7	-	1.7
Myoglobin Gen. 2 direct assay	570 nm – 800 nm / 4.7min	505 nm – 800 nm / 6.7min	660 – 800 nm / 2.6min	2 [a]	2	4
D-Dimer Gen. 2 direct assay	800 nm / 4.3min	800 nm / 4.3min	700 nm / 1.4min	-	(1.14)	(1.14)
RF II direct assay	570 nm – 800 nm / 2.2min	546 nm – 800 nm / 8.5min	660 – 800 nm / 1.4min	5 [b]	(1.15)	5.8
CRP L3 (Gen. 3) direct assay	570 nm – 800 nm / 2.0min	505 nm – 800 nm / 2.0min	600 – 800 nm / 1.2min	1.8	1.8	3.1
DIG competitive assay	660 nm / 4.7min	660 nm / 4.7min	505 – 800 nm / 4.7min	-	(1.15)	(1.15)
PHENO competitive assay	600 nm – 800 nm / 6.5min	600 nm – 800 nm / 6.5min	505 – 800 nm / 7.9min	-	1.5	1.5

[a] Without instrument factor, [b] Change calibration to spline, [c] Good precision from standard conditions is retained in new conditions.
[d] Main wavelength – correction wavelength / reaction time in minutes

Figure 6 a:

Ferritin, 0 und 10µ/L (N= 21) bei

A) 505nm and

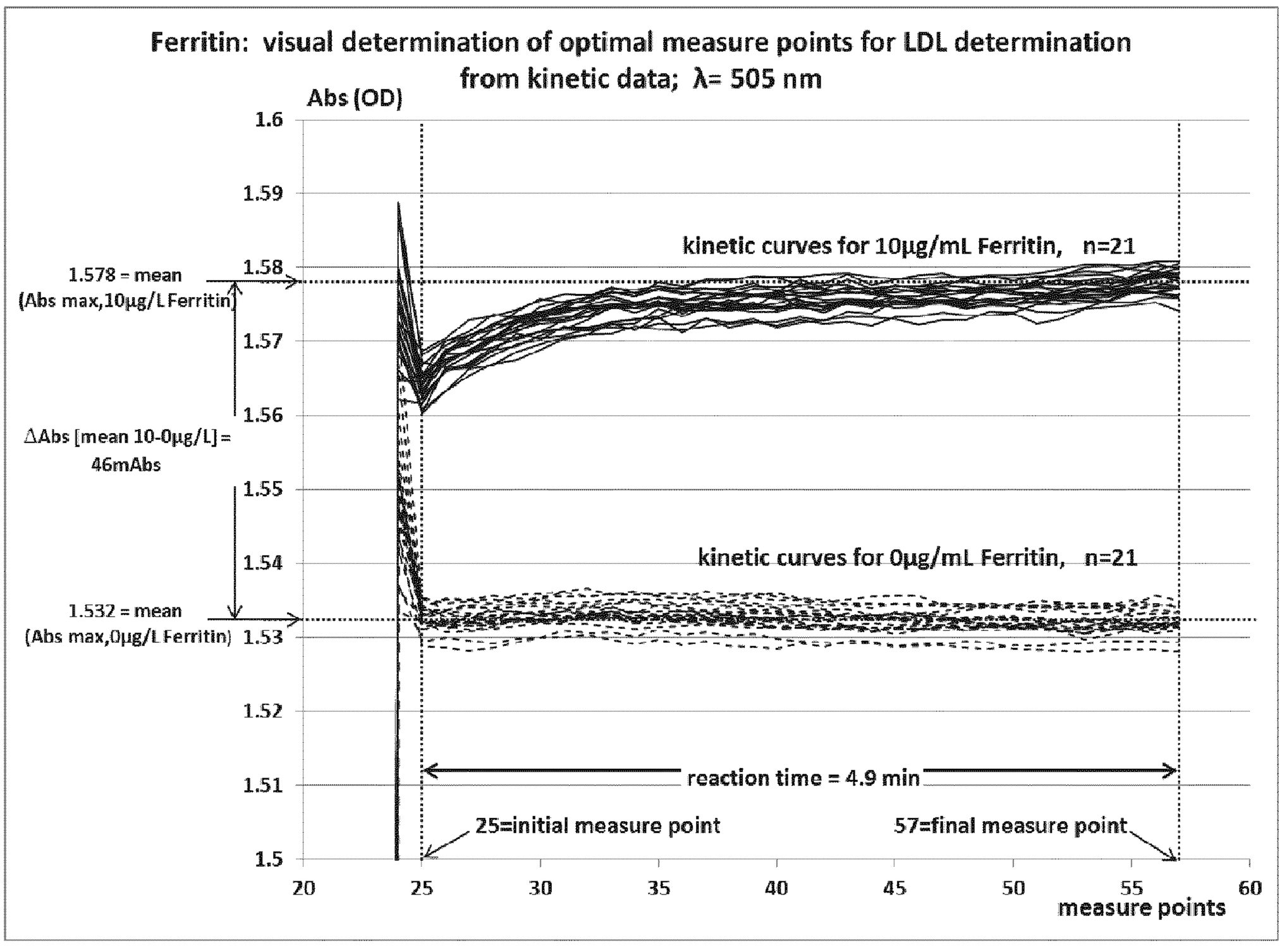


Figure 6 b:

Ferritin, 0 und 10µ/L (N= 21) bei

B) 570nm

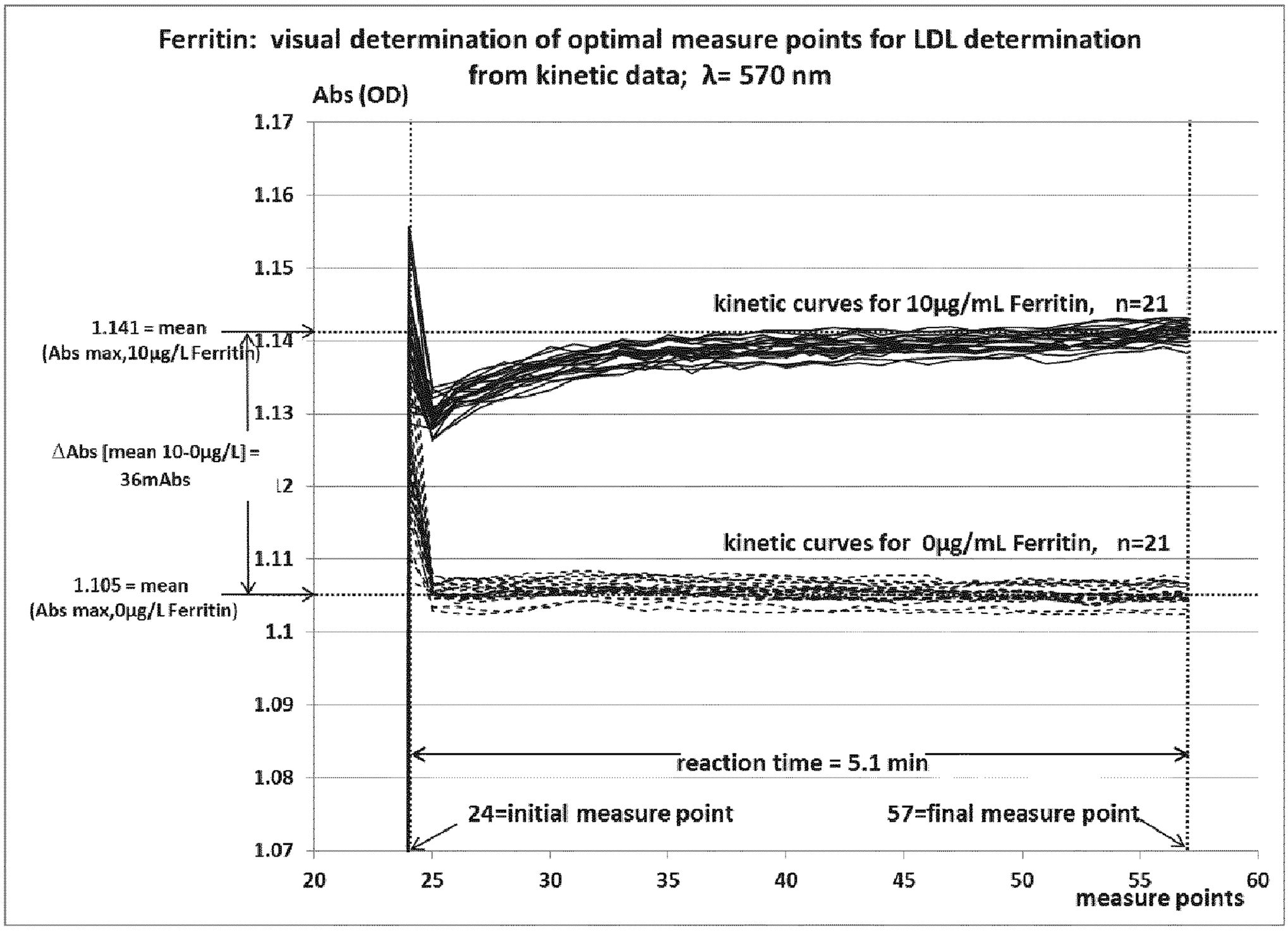


Figure 7:

Ferritin, 981-4950µg/L (N= 1) bei 570-800nm

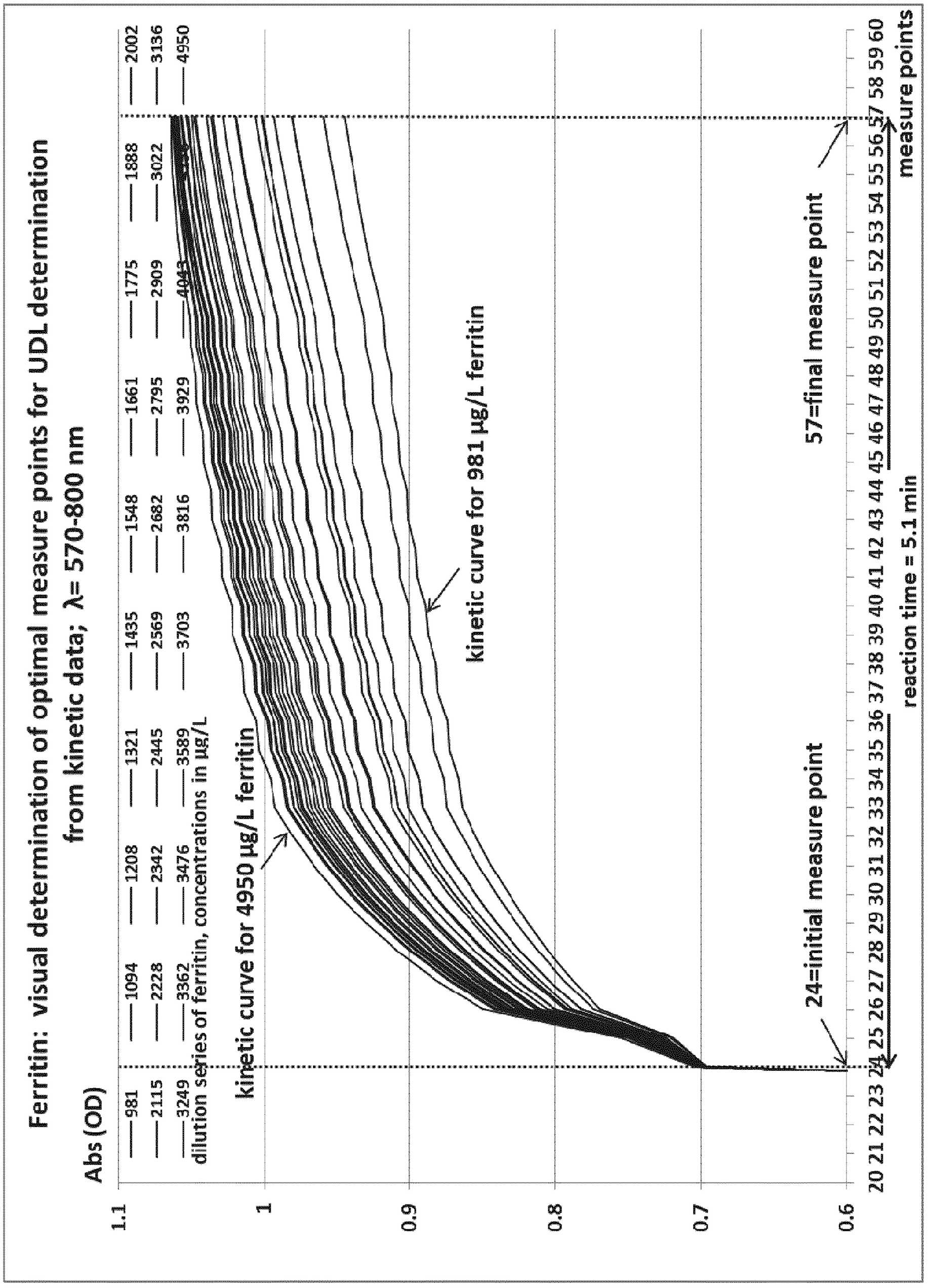
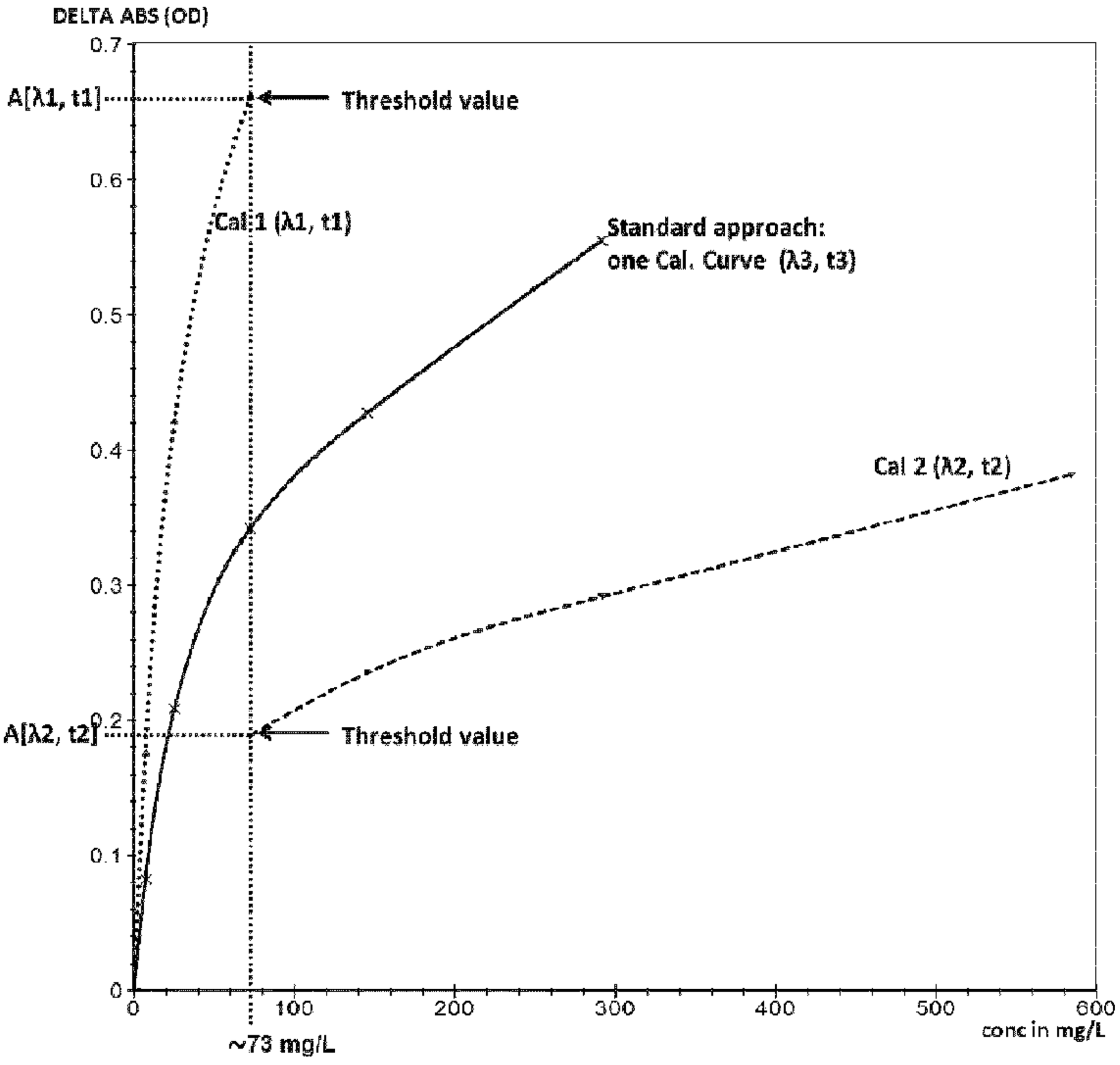


Figure 8:

Assay	Improvement factor for dynamic range based on the invention	Reduction of automatic reruns by the invention	
		Reruns caused by samples below standard measuring range	Reruns caused by samples above standard measuring range
ALTL	12.2	87 %	99 %
ASTL	7.5	87 %	99 %
CRPL3	3.1	57 %	99 %
D-Dimer Gen.2	1.14	no calculation *	0 %
DIG	1.15	0 %	27 %
Ferritin Gen.4	1.7	71 %	0 %
GLUC3	2.4	81 %	96 %
MYO2	4	no calculation *	51 %
PHENO	1.5	3 %	77 %
RF II	5.8	97 %	9 %
U-BUN	3	no calculation *	87 %

* not enough data available for calculation


DELTA ABS (OD)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A[λ1, t1]
Threshold value
Cal 1 (λ1, t1)
Standard approach:
one Cal. Curve (λ3, t3)
Cal 2 (λ2, t2)
A[λ2, t2]
Threshold value
0
100
200
300
400
500
600
conc in mg/L
~73 mg/L