

Method and kit for measuring of analytes in bi-component systems and uses thereof

5 The present invention relates to a method and kit of measuring parameters of an analyte using bi-component detection methods and uses thereof. The claimed method comprises measurements of dilutions of the analyte with an extended measurable concentration range of measurements. The measurements are based on the predetermined non-bijective standard curve of the measurements, thereby providing a method to read the non-bijective standard curve of a bi-component detection
10 method for each measurement. Thereby the mathematical relationship to calculate the concentration of the analyte is correctly determined over a wider measurement range. In another aspect, the present invention is related to the application of said non-bijective predetermined standard curve of the measurements method in compartmentalized bi-component detection methods. In again another aspect, the present invention is related to the application of said non-bijective predetermined standard curve of the measurements method using unique molecular
15 identified components in compartmentalized bi-component detection methods. In again another aspect, the present invention is related to the determination of the dissociation constants of analyte-specific binding components of bi-component methods. The claimed method relates to an extended measurable concentration range of measurements, determination of dissociation constants of
20 components and better quantitation of bi-component detection methods, also in a parallel manner.

BACKGROUND OF THE INVENTION

Technical Field

The present invention relates to methods and kits of measuring parameters of an analyte using bi-component detection method and uses thereof. Applications of the methods and kits are also
25 disclosed.

Discussion of Related Art

In particular, the methods and kits are well suited for determining concentration of an analyte using bi-component detection methods and relate to a higher magnitude of measurement range using bi-component detection methods and uses thereof.

30 The saturation effect or "hook effect" is a phenomenon common to detection systems involving saturation of reagent components used to capture specific binding partners or analytes, generally. However, the mathematical background is different in the case of mono-component or bi-component detection methods.

Bi-component detection systems are typically exploited in homogeneous assays where two
35 components are applied to produce detection signals. Many of these assays apply a proximity

concept and the analyte and the components need to be brought in proximity to produce a signal. Proximity assay technologies such as FRET (fluorescence resonance energy transfer), BRET (bioluminescence resonance energy transfer) (Pfleger, Seeber, & Eidne, 2006), cyan fluorescent protein (CFP) – yellow fluorescent protein (YFP) pair, PCA (protein complementation assays) (Morell, Ventura, & Avilés, 2009), Alphascreen (Taouji, Dahan, Bosse, & Chevet, 2009), and DNA labeled proximity methods (PLA – proximity ligation assay, PEA – proximity extension assay) (Söderberg et al., 2006), and a not proximity based assay called emulsion coupling are representative examples of bimolecular (bi-component) detection systems and methods.

In mono-component detection systems, only one assay component, called the tracer, is required to produce a signal. Filtration binding assays involving radioactive or fluorescent tracers are good examples of mono-component detection systems. Standard curves obtained using mono-component detection systems show a typical sigmoid shape ending by a plateau (also commonly called 'hook') obtained at saturating tracer concentrations. These curves are also called saturations curves.

Standard curves produced with bimolecular detection systems are atypical. These curves are non-bijective and characterized by a signal decrease ('hook effect') following a plateau obtained after an initial concentration-dependent signal increase. The range of target molecules concentrations where the signal starts to drop is called the hook point. Below the hook point, the assay components become progressively saturated by the target molecule and a signal increase is measured. At the hook point, both components are saturated with the target molecule and a maximum signal is detected. Above the hook point, an excess of target molecules oversaturates the components, which inhibits their association and causes a progressive signal decrease.

In the prior art it is necessary to determine the hook point of a two-segment non-bijective standard curve before carrying out a measurement of an analyte concentration in order to ensure that all measurements are carried out in the range of a progressively saturated segment of the two-segment non-bijective standard curve. Over-saturation generally considered as a failure of measurement.

A failure of this requirement can result in false measurements since the determined values are not uniquely corresponding to an analyte concentration on the non-bijective standard curve.

In order to avoid such errors, typically the dynamic range of the measurement is limited to the dynamic range of the progressively saturated segment of the non-bijective standard curve.

In the prior art some approaches are known to address the Hook-Effect in particular in sandwich immunoassays.

Wu et al. 2018 discusses the occurrence of the Hook-Effect in a sandwich immunoassay due to an excess of capture and detection antibodies and suggests dilution of the sample to avoid misinterpretation of the results.

JP H06 109740 A relates to a turbidmetry immunoassay (TIA) for a quantitative analysis of target component in a sample using an antibody-antigen reaction. JP H06 109740 A discusses the occurrence of a prozone for an excess of antigen in known methods, which in such cases repeat the test in a sample dilution. As an improvement JP H06 109740 A suggests obtaining two or more calibration curves for samples having different concentrations and deciding which calibration curve is applicable based upon the deviation from a virtual curve. The disclosure of JP H06 109740 A is however conceptionally restricted to preventing the prozone effect to avoid false negative measurements, without allowing for a broader dynamic range of the assay from defined mathematical relationships.

EP 0 576 879 A2 relates to methods for determining the concentration of a component in a medical sample and discusses the Hook effect in immunological detection schemes at antigen excess as well a resulting non-monotonic, non-bijective calibration curve, which is referred to as a "Heidelberger-Kurve". EP 0 576 879 A2 discloses that it is known in the prior art to overcome the ambiguity of the non-monotonic calibration curve using two different sample dilutions. As a further improvement EP 0 576 879 A2 suggests the use of a learning algorithm and multivariate statistics to correlate a measured input value with a correct regime of a calibration curve. However, EP 0 576 879 A2 relates to the analysis of signaling in a precipitation assay and thus cannot be straightforward extended to bi-component assays, especially as precipitation is a variable/multi-component reaction with undefined stoichiometry. Moreover, using the learning algorithm the results may vary for different experimental conditions, such that different calibrations curves may, become necessary for each experimental condition.

CN 106 226 516 B relates to detection methods involving hyperbolic calibration curves and also discusses strategies to cope with the Hook effect. CN 106 226 516 B suggests performing separate reaction of standards of different concentration of analytes with equal amounts of reactant in order to assemble a calibration curve at the point of inflection from a pre-calibration and post-calibration curve.

Binder et al 2008 discloses calibration algorithms for a GeneChip microarray that may take into account a Hook-Effect and comprise co-processing the log difference and sum of perfect match and mismatch probe intensities.

Given the teaching of the prior art there is thus room for improvement for providing methods and kits suitable for determining the concentration of an analyte using bi-component detection assays, which may reliable, effectively and with few additional effort broaden the dynamic range of such bi-component detection assays and provide a defined mathematical relationship that eliminates the need for using approximate methods.

In another aspect, the methods and kits are suitable for determining the dissociation constants of analyte binding components using bi-component detection methods and relate to the measurement

of dissociation constants in parallel manner using bi-component detection methods and uses thereof.

In chemistry, biochemistry, medical technologies and pharmacology, a dissociation constant (K_d) is a specific type of equilibrium constant that measures the propensity of a larger complex to dissociate reversibly into smaller components (Friguet, Chaffotte, Djavadi-Ohanian, & Goldberg, 1985). The dissociation constant is the inverse of the association constant. In the special case of bi-component measurement methods, the dissociation constants are the dissociation constants between the two components and the analyte. As the components can be antibodies, the determination of the dissociation constants has a broad interest.

In the prior art some approaches are known for determining dissociation constants.

Rossant ET AL 2015 and Xiong Y. et al. (2017) disclose approaches for determining equilibrium parameters (K_d) of affinity complexes using FRET-based assays, but relate to binary complexes.

Rey et al. 2017 explore the role of cooperativity in the design of potent proteolysis targeting chimeras (PROTACs) for degradation of Bruton's tyrosine kinase (BTK). Rey et al. proposes that maximizing PROTAC activity on BTK is associated with maximizing ternary complex of BTK:PROTAC:CRBN and discloses a TR-FRET analysis of the said ternary complexes with members of a PROTAC library serving as an analyte and BTK and CRBN as ligands, which are each labeled with a FRET (donor/acceptor) fluorescent molecule. Rey et al. further discloses non-bijective curves for ternary complexes formation (and resulting FRET signal) over concentration of an analyte as well as numerical fitting for deriving dissociation constants (K_D values) for the binding of PROTAC with BTK or CRBN.

EP 2 837 937 A1 discloses a mathematical model for the binding equilibria in ternary affinity complexes. EP 2 837 937 A1 discusses features of ternary complex formation, including the "bell-shaped" binding curve or "Hook effect". Equations for the relationships between equilibrium dissociations constants and concentrations of analyte, binding components and/or ternary complex concentrations are also disclosed.

While Rey et al. 2017 and EP 2 837 937 A1 address an estimation of equilibrium parameters in ternary complex formation and mention the occurrence of a Hook effect, they do not provide a solution to properly take into account these effects.

EP 1 460 414 A1 relates to homogenous time resolved FRET assay for the identification of potential lead antibodies in drug discovery processes. EP 1 460 414 A1 discloses examples for using HFRET assay to analyze protein-protein interactions. As a disadvantage of direct binding assays EP 1 460 414 A1 discloses that screening of undiluted samples may increase the number of false negatives as a result of the "Hook" effect. Screening at multiple sample dilutions is proposed to limit the effect, but may affect time and costs.

Zorba et al. discloses principles of the amplified luminescent proximity homogenous assay (AlphaLISA) which was modified from a binding competition assay to detect the interactions between the transcription-activation domain p53 and its ligands. Zorba et al. discusses the occurrence of the Hook effect at exceeding concentrations and proposes suitable titration for avoidance.

Douglass et al. 2013 discuss different assays for monitoring protein-protein interaction and identifying small molecules as inhibitors including AlphaScreen or AlphaLISA. Douglass et al. also discusses the hook effect and suggest titrations to choose a protein concentration avoiding the hook point for the assay.

Neither EP 1 460 414 A1, Zorba nor Douglass et al. 2013 address however the estimation of dissociation constants in these assays.

In light of the teachings of the prior art it would be desirable to provide improved methods and kits suitable for measuring dissociation constants using bi-component detection methods, especially in in parallel manner or high throughput format.

Furthermore, methods for determining the dissociation constants of bi-component detection methods in the prior art are elaborate and expensive, however especially in parallel manner they are seriously limited. Most frequently used methods like displacement ELISA, fluorescence polarization method, and surface plasma resonance need the parallelization of standalone measurements. A need for a simplified and reaction-level parallel yet robust method therefore exists.

DISCLOSURE OF THE INVENTION

In light of the prior art the technical problem underlying the present invention is to provide alternative and/or improved means for the determining the concentration of an analyte using a bi-component detection as well as determining the dissociation constants of analyte-specific binding components in a bi-component detection method.

In particular, an object of the present invention is to provide for a method or kit of measuring an extended range of concentration of an analyte using bi-component detection methods.

An object of the present invention is also to provide for a method and kit of measuring concentration of an analyte and dissociation constants of analyte-bi-component complexes using compartmentalized bi-component detection methods of measurement and uses thereof.

In particular, a purpose of the invention is to provide for a method of measuring concentration of an analyte with absolute quantified, extended dynamic range and parallel detection in compartmentalized bi-component detection methods and uses thereof.

This object is solved by the features of the independent claims. Preferred embodiments of the present invention are provided by the dependent claims.

The invention therefore relates to a method for determining the concentration of an analyte, preferably in a sample with an unknown concentration of analyte, using a bi-component detection method comprising:

- 5 a. providing two analyte-specific binding components at known concentrations for conducting a bi-component detection method in a solution comprising bringing said two analyte-specific binding components in contact with said analyte to produce a signal that depends on the concentration of bi-component/analyte complexes formed in said solution
- 10 b. providing an analyte concentration reference curve for said bi-component detection method which is a mathematical function reflecting the dependence of said signal on the concentration of analyte, wherein the analyte concentration reference curve is not a bijective function and preferably exhibits an increasing and a decreasing monotonic segment
- c. preparing one or more dilutions of said sample using defined dilutions factors,
- d. applying the bi-component detection method to the sample and the one or more dilutions
- 15 e. determining the concentration of the analyte in the sample using said signal detected in the sample and in the one or more dilutions as a constraining input for a mathematical fit for said non-bijective analyte concentration reference curve at different analyte concentrations.

In some embodiments the method encompasses after applying the bicomponent detection method a step of comparing the signals detected in the sample and in the one or more dilutions with the analyte concentration reference curve in order to determine which point of the analyte concentration reference curve is applicable for the concentration of the analyte in the sample, wherein the defined dilutions factors are taken into account. In some embodiments determining the concentration of the analyte in the sample comprises comparing the signal reflecting the formation of bi-component/analyte complexes in the sample with the applicable point of the analyte concentration reference curve.

25 Preferably the two analyte-specific binding components as well as the analyte are non-immobilized and in solution, such that the of bi-component/analyte complexes likewise form in solution. In some embodiments the method comprises a step of providing a sample with an unknown concentration of analyte. In some embodiments the method is an *in-vitro* method, wherein preferably a step of providing a sample does not comprise a surgical or invasive treatment of a living human or animal body.

The method of the invention allows for determining an unknown concentration of a known analyte using uncompartimentalized or compartimentalized bi-component detection methods with extended dynamic range.

35 As discussed above, reference curves for saturable bi-component detection methods are non-bijective functions with two-segments separated by the so-called hook point. Bijective function

means that a one-to-one correspondence is possible. In mathematical terms a bijective function, is a function between the elements of two sets, where each element of one set is paired with exactly one element of the other set, and vice versa. For the reference curves or standard curves of saturable bi-component detection methods, this is not the case. The same signal reflecting the formation of the concentration of bi-component/analyte complexes may result in at least two different analyte concentrations. The same detectable signal produced by the formation of bi-component/analyte complexes may therefore stem from two different actual analyte concentrations.

To avoid ambiguities there are approaches in the prior art therefore to estimate the hook point of the two-segmented non-bijective reference curve before carrying out a measurement of an analyte concentration. Subsequently, the measurements are typically carried out only in the range of one of the segments thus considerably limiting the dynamic range.

Using the present method, such limitations are eliminated. Instead, the method prepares one or more dilutions of the sample with known dilution factors, and applies the bi-component detection method to the sample as well as the one or more dilutions. Applying the bi-component detection method preferably means that the two analyte-specific binding components at known concentrations are brought into contact with the sample and one or more dilutions in order to produce a signal that depends on the concentration of bi-component/analyte complexes formed in said solution.

As detailed herein, the signal can take a variety of forms and depends on the bi-component detection system and method used. For instance, in case of proximity assays such as a FRET assay, the signal may refer to a fluorescence signal from a FRET-donor-acceptor pair indicating the formation bi-component/analyte complexes. In case of a compartmentalized bi-component method such as emulsion coupling, the signal may also refer to signals resulting from a ddPCR using fluorescently tagged PCR products or NGS-sequencing that indicate the presence of a bi-component/analyte complex based upon a deviation from the expected Poisson distribution in the compartmentalized droplets.

Since, the signal reflecting the formation of bi-component/analyte complexes is acquired for the sample as well as one or more dilutions the signals and dilution factors can be used to determine, which point of the analyte concentration reference curve is applicable for the concentration of the analyte in the sample. To this end a comparison of the signals stemming from the sample as well as one or more dilutions with the reference curve is performed.

As shown in Fig. 1 the reference curve for a bi-component detection method will typically present a bell shape curve with an increasing monotonic segment for lower analyte concentrations and a decreasing monotonic segment for higher analyte concentration (saturation regime). Increasing and decreasing thus relates to a signal alternation in dependence of analyte concentration.

Different scenarios for signals detected in the sample and dilutions can be envisioned. For instance, the signal stemming from the sample may be higher than a signal stemming from a dilution (having

a lower analyte concentration). From this information, it can be deduced either that both signals stem from the monotonic increasing segment or that the signal from the sample stems from the monotonic decreasing segment and the signal from the dilution stems from the monotonic increasing segment. The difference between the two scenarios can be determined by fitting the dilution factor between the sample and the dilution (see Example 3).

It is noted that based upon the dilution factors, the expected concentration ratio between the analyte concentrations of the sample and one or more dilutions are known. In the depicted curve of Fig. 1 this corresponds to a distance of the horizontal axis.

Advantageously, a mathematical fit for two signals of a known difference/ratio of analyte concentration will faithfully result in an optimal positioning of the signals on to the non-bijective reference curve, such that the applicable concentration can be determined. In other words, by diluting the sample in order to detect multiple signals with known dilution factors provides additional constraints for a mathematical fit of the acquired data, which robustly allows to deduce the applicable and corresponding concentration value of a signal stemming from the sample.

It is noted, that further signals stemming from one or more dilution can be determined and the information can be used to enhance the accuracy of the analyte concentration measurement.

Determining the non-bijective mathematical signal-concentration relationship of a bi-component method, i.e. the reference or standard curve, and using defined dilutions of the analyte it is thus possible to constrain the measurement to avoid failures of a hook point determination of the two-segment non-bijective standard curve. In a typical case, the dynamic range of the signal of bi-component methods can be thus used on both segments of the non-bijective reference curve. Since for methods of the prior art, it is important to leave a considerable distance from the hook point, this effectively leads to well more than a doubling of the dynamic range.

While the prior art to some extent suggests a dilution concept to avoid an ambiguity associated with the Hook effect, the method described herein preferably allows to determine the concentration considering the calculation requirement of the non-bijective curve, for which preferably two independent inputs for a mathematical fit are necessary. In other words beyond a general broad-sense dilution concept, the invention preferably provides a mathematical understanding, which leads to significant additional advantages. In particular, preferably the entire non-bijective analyte concentration reference curve may be used as a calibration curve for determining the concentration of an analyte. While a generally dilution concept is restrained to empirically finding dilutions, which enable a measurement avoiding an ambiguity, they do not consider the whole non-bijective curve as a calibration curve. Instead, the methods described herein may preferably provide essential parameters to calculate a non-bijective curve and considering the whole non-bijective curve as calibration curve, for determining an analyte concentration using bi-component detection reactions.

In one embodiment the reference curve is obtained experimentally by providing a reference sample of known analyte concentration, generating a series of known dilutions of said reference sample

and conducting for the reference sample and each known dilution thereof said bi-component detection method.

Advantageously, the reference curve for a given bi-component detection method has to be determined only once experimentally. To this end, a bi-component detection system with components with a defined affinity for the analyte at a given concentration is provided as well as a reference sample of known analyte concentration. For the reference sample a series of dilutions is generated and on each of the dilutions the bi-component detection method is applied generating thus a series of signals reflecting the formation of bi-component/analyte complex for a given analyte concentration. Said experimentally obtained dependence of the signal reflecting the formation of bi-component/analyte complex on the analyte concentration can be interpolated in order to form a reference or standard curve for use as described herein.

However, it is also possible to provide the reference curve by analytically methods or numerical simulations.

In one embodiment, the analyte concentration reference curve is calculated analytically by solving chemical balance and mass conservation equations or is provided by numerical solutions based upon the provision of dissociation constants for each of the two analyte-specific binding components with the analyte.

A demonstration of the provision of a reference curve based upon analytical methods or numerical simulations is provided in Example 1.

Generally, analytical and numerical methods for providing the reference curve will necessitate some additional parameters defining the bi-component-detection method in relation to the analyte. In particular, in order to numerically provide a reference curve, the dissociation constants for each of the two analyte-specific binding components with the analyte have to be estimated. As detailed in the examples below, essentially with this information a reference curve for the signal reflecting the formation of bi-component/analyte complexes can be provided in an efficient and robust manner.

In one embodiment, the method may be used for determining the concentration of an analyte using compartmentalized bi-component detection methods and relates to a higher magnitude of measurement range using compartmentalized bi-component detection methods and uses thereof. Compartmentalized bi-component detection methods are highly preferred as they allow to determine the absolute concentration of the analyte.

A typical compartmentalized method is the digital PCR (ddPCR) (Quan, Sauzade, & Brouzes, 2018) method utilizing a water-oil emulsion droplet system. Droplets are formed in a water-oil emulsion to form partitions (compartments) that separate the template DNA molecules. The droplets serve essentially the same function as individual test tubes or wells in a plate in which the PCR reaction takes place. The massive sample partitioning is a key aspect of the ddPCR technique.

The ddPCR technology is digital, in the regard that, the droplets support PCR amplification of the template molecules they contain and generate signals based on individual DNA template molecules. Following PCR, each droplet is analyzed or read to determine the fraction of PCR-positive droplets in the original sample. These data are then analyzed using Poisson statistics to determine the absolute DNA template concentration in the original sample.

In another embodiment the method may be used to apply the absolute quantitation principle (Quan et al., 2018) to bi-component measurement methods. The signal of the analyte is based on detection of the individual molecules of the compartmentalized analyte-bi-component complexes and Poisson statistics, wherein the analyte-bi-component complexes are determined by the chemical balances based on the concentration of the molecules and their analytical properties, namely dissociation constants.

In this embodiment, the creation of tens, thousands or millions (or even higher number) of droplets and their application for bi-competent detection method means that a single sample measurement having the number of droplets as the primary dynamic range of the bi-component method, in an absolute quantitative, highly linear manner, rather than using a single, inherently non-linear signal as in the case of non-compartmentalized measurements obtained. Thereby bringing the absolute quantitativity and statistical features inherent to compartmentalized methods into the detection of concentration of analytes using bi-competent methods. These compartmentalization approaches can be combined with the methods described herein in order to provide an extended range of a non-bijective two-segment reference curve of bi-component measurement methods and effectively double the available dynamic range of the measurements.

In some embodiments, these methods are preferred for multi-analyte measurements as they usually have vast concentration differences. In such embodiments one can measure DNA copy number (low-abundance) and RNA/protein copy number (high abundance) in the same sample using suitable compartmentalized bi-component method such as emulsion coupling (see examples below and EP 3224360).

The number of compartments has no upper limits, regarding the signal generation principle, if one applies a digital, linear signal generation method. Due to the extended range of measurement by using the method described herein, a doubled signal based dynamic range is available for the measurement, while maintaining sensitivity and precision that are the hallmarks of compartmentalized bi-components methods.

Therefore the compartmentalized bi-components methods in combination with the method described herein provides unparalleled precision as the massive sample partitioning enables the reliable measurement of small fold differences in target analytes. This leads to increased signal-to-noise ratio, as it the dominating template in a sample do not hamper the detection of rare targets. Compartmentalized bi-components methods provide further low signal drop-out error rates as the

inherent high dilution of the methods is removing substances that may interfere with effective signal generation.

According to the invention, the above-mentioned methods are also suitable for determining the dissociation constants of the bi-component/analyte complexes.

5 In another aspect the invention relates to a method for determining the dissociation constants of analyte-specific binding components in a bi-component detection method comprising:

- 10 a. providing two analyte-specific binding components at known concentrations for conducting a bi-component detection method comprising bringing said two analyte-specific binding components in contact with a solution containing said analyte to produce a signal that depends on the concentration of bi-component/analyte complexes formed in said solution
- 15 b. providing a dissociation constant relationship for said bi-component detection method which is a mathematical functions for the relationship between the dissociation constants of said analyte-specific binding components in dependence of said signal reflecting the concentration of bi-component/analyte complexes and the concentration of analytes and/or analyte-specific binding components
- c. preparing one or more dilutions of said sample or said analyte-specific binding components,
- d. applying the bi-component detection method to the sample and the one or more dilutions
- 20 e. determining the dissociations constants of said two analyte-specific binding component using said signal detected of the sample and the one or more dilutions, as constraining inputs for a mathematical fit for said dissociation constant relationship at different analyte concentrations and/or analyte-specific binding components concentrations.

The method of the invention allows the determination of dissociation constants for bi-component detection systems by preparing one or more dilutions of the sample with known dilution factors and
 25 applies the bi-component detection method to the sample as well as the one or more dilutions. Applying the bi-component detection method for determining the dissociation constants of analyte-specific binding components preferably means that two analyte-specific binding components at known concentrations are brought into contact with the sample at known concentration in order to produce a signal that depends on the concentration of bi-component/analyte complexes formed in
 30 said solution. Either the concentration of the analyte-specific binding components or the sample is varied by dilution providing dilutions with known dilution factors. The signal detected of in sample and in the one or more dilutions as well as the concentration of analytes and/or analyte-specific binding components can be used as a constraining input for the mathematical fit.

35 Preferably the two analyte-specific binding components as well as the analyte are non-immobilized and in solution, such that the bi-component/analyte complexes likewise form in solution. In some

embodiments the method comprises a step of providing a sample with a known concentration of analyte. In some embodiments the method is an *in-vitro* method, wherein preferably a step of providing a sample does not comprise a surgical or invasive treatment of a living human or animal body. As detailed above the signal can take a variety of forms and depends on the bi-component detection system used. For instance, in case of proximity assays such as a FRET assay, the signal may refer to a fluorescence signal from a FRET-donor-acceptor pair indicating the formation bi-component/analyte complexes. In case of a compartmentalized bi-component method such as emulsion coupling, the signal may also refer to signals resulting from a ddPCR and/or NGS-sequencing that indicate the presence of a bi-component/analyte complex based upon a deviation from the expected Poisson distribution in the compartmentalized droplets.

As shown in the examples, (see e.g. Example 2, 5 and Fig. 2) using the signal detected in the sample and in one or more dilutions as a constraining input for a mathematical fit allows for a robust determination of dissociations constants of two analyte-specific binding components with the analyte.

The dissociation constant relationship may be provided by analytically solving chemical balance, and mass conservation equations (see Example 2; Eq. 3). In generally terms, it can be described as $f(K_{d1}, c_{12}, a_0) = K_{d2}$, wherein “ K_{d1} ” and “ K_{d2} ” are dissociation constants, “ c_{12} ” is the measured concentration of ternary complex, as determined by the signals reflecting the formation of bi-component/analyte complexes and “ a_0 ” is the known concentration of the analyte. As a further input $f(K_{d1}, c_{12}, a_0) = K_{d2}$ may further comprises the concentration of analyte, end both bi-components, which are also known. These terms are valid in the case of known and not varied concentration of analyte-specific binding components.

Another advantageous aspect of determination of dissociation constant relationship may be provided if the concentration of analyte-specific binding components are varied, it can be described as $f(K_{d1}, c_{12}, b_{10}, b_{20}) = K_{d2}$, wherein “ K_{d1} ” and “ K_{d2} ” are dissociation constants, “ c_{12} ” is the measured concentration of ternary complex, as determined by the signals reflecting the formation of bi-component/analyte complexes and “ b_{10} ” and “ b_{20} ” are the known concentration of the analyte-specific binding components. As a further input $f(K_{d1}, c_{12}, b_{10}, b_{20}) = K_{d2}$ may further comprises the concentration of analyte, which is also known. These terms are valid in the case of known and not varied concentration of analyte.

In order to determine the dissociation constants (“ K_{d1} ” and “ K_{d2} ”) of the binding components Eq 3. may be applied. Having more than one solution containing a known amount of analyte, bi-components and a determined amount of ternary antibody/bi-component complex, the two K_d can be calculated according to the Example 2.

As different dilutions of the sample containing the analyte corresponds to different concentration of “ c_{12} ”, the “ f ” function has different forms (see Fig. 2). However since the K_d s are material constants, all forms of the “ f ” function must be satisfied by a certain pair of values of K_d s.

In practice, graphing the dissociation constant relationships according to K_{d1} and K_{d2} , the curves of the different known concentrations intersect close to one point which are the applicable pair of K_{ds} to be determined (see Example 2). In other words, the mathematical solutions are preferably to find an intersection between of curves, which reflect the dissociation constants (K_{d1} - K_{d2}) relationships varied by the different input concentrations as provided by applying the bi-component detection method to the sample as well as one or more dilutions.

In one embodiment determining more than one relationship of the dissociation constants using varied experimental conditions, e.g. by providing different dilutions of the analyte, can help to determine the value of the dissociation constants, which satisfy these relationships.

10 In a preferred embodiment, one can determine the value of the dissociation constants using statistical models and/or other mathematical tools, since measurement errors are confounded in different measurements.

In another aspect, the methods are suitable for determining the dissociation constants of analyte binding components using a bi-component detection method in parallel manner using bi-component detection methods and uses thereof.

15 In one embodiment, the methods for determining the dissociation constants of analyte binding components is based on absolute quantifiable compartmentalized bi-component detection methods.

20 In another embodiment of the methods described herein, it is preferred to use parallel reading technologies. Next-generation DNA sequencing is preferred in some embodiments as a bi-component readout technology. Particularly those bi-component methods are suitable for DNA sequencing based readout, which generate unique DNA signals as a part of the detection principles. These methods include, but are not limited to proximity ligation, extension assay and emulsion coupling.

25 In another embodiment DNA sequencing readout based bi-component measurement methods are preferred, if the bi-component methods is a compartmentalized bi-component method. Under compartmentalized conditions, the generation of unique DNA signals is unbiased, compared to the un compartmentalized measurements.

30 In another aspect of compartmentalized generation of DNA signals means unbiased DNA signals that enabled by the usage of unique molecular identifiers (UMI) (Parekh, Ziegenhain, Vieth, Enard, & Hellmann, 2017) signal readout.

Various methods have been developed to improve accuracy of quantification of different polynucleotides in a sample using next-generation sequencing technologies, including such methods as competitive polymerase chain reaction (PCR), described in U.S. Patent Number 35 5,213,961 and deep barcode sequencing using unique molecular identifiers (UMI), as described by (Smith et al., 2009).

Unique molecular identifiers, or molecular barcodes, provide an advantage in quantifying unique DNA signals of the bi-component measurement methods in a sample. However, if UMIs are involved in more than the first round of detection, the same UMI may be introduced into different targets, resulting in counting errors. Also, the original UMI method is based on an ideal, but unrealistic, situation— that is, where both PCR and sequencing technologies are both perfect and no errors are introduced. The UMI strategy operates on the assumption that both PCR and sequencing steps report the underlying targets and UMI fragments free-of-error. These conditions are difficult to provide under uncompartimentalized conditions, but seamlessly provided under compartmentalized conditions. UMI labeled components are single or few molecules per compartment in a compartmentalized bi-component measurement method, which means effective incorporation of the UMI barcodes into the unique DNA signals.

In another aspect, unique molecular identifiers (UMIs) representing unique DNA signals can be incorporated into unique DNA signals and further amplified in a compartmentalized bi-component method. However, without the usage of the unique molecular identifiers, the quantitation of the unique DNA signal is biased. The amplification can be specific to certain unique DNA signals; to balance their ratio against other not further amplified unique DNA signals.

In another aspect, unique molecular identifiers incorporated unique DNA signals can be used to detect analytes with two or more identical binding sites or analytes with more than two non-identical binding sites. These arrangements are particularly preferred to detect interacting analytes, like proteins or other molecules.

In another aspect, the methods and kits of bi-component measurement methods using parallel reading technologies, can measure both the concentration of analyte and dissociation constants of the components in a parallel manner. Today next-generation sequencing technologies can provide billions of independent reads, which are directly proportional with the achievable parallelism of the readout.

In one further embodiment, the analyte is selected from a group consisting of proteins, peptides, nucleic acid segments, carbohydrates, lipids, antibodies (monoclonal or polyclonal), antigens, oligonucleotides, specific receptor proteins, ligands, molecules, cells, microorganisms as well as fragments products or combinations of thereof.

In one embodiment the two analyte-specific binding components are selected from a group consisting of nucleic acids, preferably RNA and/or DNA oligonucleotides, antibodies, peptides, proteins, aptamers, molecularly-imprinted polymers, cells or combinations of thereof.

In one embodiment, the sample comprise two or more different types of analytes.

In one embodiment, two or more pairs of different kind of analyte-specific binding components are used.

A particular advantage of the methods described herein is that it is even possible to have more than one kind of analytes (e.g. DNA; RNA; protein, interacting proteins and their chemical modifications) and similarly more than one kind of binding component in the same assay.

5 Using parallel approaches as described herein, the analyte concentration for different kind of analytes can be determined in parallel as well as dissociation constants for different kind of analyte-specific binding components.

10 In one embodiment the bi-component method comprises employing a proximity-based assay to produce the bi-component/analyte complexes concentration depended signal, wherein the proximity-based assay uses two analyte-specific binding components that produce a detectable signal in dependence of their proximity.

15 In one embodiment the bi-component method comprises employing a resonance energy transfer assay, preferably a Förster resonance energy transfer (FRET) assay or a bioluminescence resonance energy transfer (BRET) assay, a protein complementation assay (PCA), Alphascreen or a DNA labeled proximity assay, preferably a proximity ligation assay (PLA) or a proximity extension assay (PEA).

In one embodiment, the bi-component detection method comprises employing a compartmentalized assay to produce a bi-component/analyte complexes concentration depended signal, wherein the signal reflects the presence of said two analyte-specific binding components in a single compartment.

20 In one embodiment, the compartmentalized assay employs an emulsion droplet method, wherein each droplet in the emulsion represents a separate compartment.

25 In one embodiment, the compartmentalized assay is emulsion coupling. As described in detail below emulsion coupling refers to is a digital assay concept based on the detection of double-labelled (bi-component), individual ternary molecular complexes in emulsion, which may be identified, for example, by digital droplet PCR (ddPCR) using fluorescently tagged PCR products or next generation sequencing (NGS).

Advantageously, such an approach allows for an absolute quantification of analyte concentration as well as a parallel determination of the concentration of multiple analytes in a robust and efficient manner.

30 In one embodiment, the bi-component detection method comprises employing an absolute molecular count based analytical method. Such an absolute molecular count based analytical method preferably refers to applying digital detection methods, such as a digital PCR or next-generation sequencing.

35 In one embodiment, the bi-component detection method comprises employing a droplet digital PCR assay.

In one embodiment, the bi-component detection method comprises using analyte-specific binding components associated with unique amplifiable nucleic acid labels and employs a compartmentalized assay, wherein a nucleic acid amplification is performed for each compartment using fluorescently tagged amplification products.

- 5 In preferred embodiments, the nucleic acid amplification is a PCR and the fluorescently tagged amplification products are fluorescently tagged PCR products.

The analyte-specific binding components (such as an antibody pair) may preferably be labelled by unique PCR amplifiable DNA labels, that is the two analyte-specific binding components, e.g. two antibodies, may preferably be labeled with a single stranded DNA that uniquely identifies the binding
10 component (e.g. antibody). The labelled binding components (e.g. antibodies) are added to the sample or the one or more dilutions in order to allow for bi-component/antibody complex formation.

Subsequently the reaction is preferably highly diluted, e.g. by a dilution factor of more than 20 000, preferably more than 50 000, 100 000 to achieve single-complex separation upon a compartmentalization, e.g. by emulsification into droplets.

- 15 For the nucleic acid amplification, fluorescently tagged amplification products are used that recognize the binding component (e.g. antibody) specific unique amplifiable nucleic acid labels. For instance, fluorescently tagged PCR products, e.g. using FAM- or VIC-labelled real-time PCR probes may be used that are complementary to the single stranded DNA that uniquely identify the binding component.

- 20 The nucleic acid amplification is performed in each of the compartments, e.g. emulsion droplets and a signal readout may be performed by detecting the fluorescence single of the compartments, e.g. the 'color' of the droplets.

Preferably, ddPCR is employed and according to a standard evaluation of ddPCR, the clusters of droplets may determine according to the fluorescent signals of the droplets. Herein, the number of
25 labelled binding components (e.g. antibodies) is determined in each reaction (counting all label-positive droplets for a given label, and using the same definition of cluster of droplets for all reactions). Additionally the number of the double-colored (having two different binding component labels) is also determined.

- Without the formation of ternary bi-component/analyte complexes, the partitioning of the labelled
30 antibodies follows Poisson distribution, and results in a calculable number of double-colored droplets (having two binding components in one compartment based upon pure chance). In the case of ternary complexes present in the reaction, the number of the detected double-colored droplets (having additional ternary complexes) is larger than would be expected by Poisson distribution alone. Based upon such an analysis the number of ternary complexes can be thus

calculated. Advantageously, the embodiment allows for an absolute quantitation of the ternary analyte complexes formed.

In one embodiment of the method described herein, multiple analytes are determined in parallel.

In one embodiment the bi-component detection method comprises using multiple analyte-specific binding components comprising nucleic acid barcodes and employs a compartmentalized assay, wherein a nucleic acid amplification is performed for each compartment producing linked nucleic acid barcodes, the compartments are reunited in a common pool and a parallel nucleic acid sequencing technique is used to produce the bi-component/analyte complexes concentration depended signal.

A preferred parallel nucleic acid sequencing technique used herein is a next generation sequencing technique.

In preferred embodiments, the binding components comprising nucleic acid barcodes refer to antibodies labelled with unique PCR amplifiable DNA labels, comprising a unique label for the type of antibody termed and also a label for the individual molecule (unique molecular identifier - UMI) (see also Parekh et al., 2017). The thus labelled antibodies are added to the sample in order to allow for bi-component/antibody complex formation.

Performing the nucleic acid amplification for each compartment to produce linked nucleic acid barcodes may be achieved by highly diluting the sample before the nucleic acid amplification, e.g. with a dilution factor of more than 20 000, preferably more than 50 000, more preferably more than 100 000. In preferred embodiments, the nucleic acid amplification is PCR. PCR reagents may be added to achieve single-complex separation and nucleic acid amplification per water-in-oil emulsion droplet. To this end droplet digital PCR standard protocols may be particularly suited.

Afterwards the compartments, e.g. emulsion droplets, may be recombined in a common pool and a parallel nucleic acid sequencing technique can be used to assess antibody specific dimerized UMI labels. Herein, the number of labelled antibodies can be determined in each reaction by counting all unique UMI labels for a given antibody (counting restricted to a given antibody – e.g. in a given label context). A possible multiple labeling of the same antibody can be eliminated using their preferentially dimerized sequences, since multiple labels per antibody exhibit a double UMI label dimers with a given antibody specific label context, as they are co-localizing in the same droplet.

The ternary bi-component/antibody complexes can be counted on the basis of their dimerized double UMI labeled PCR products two different antibody specific labels, called heterodimers) with a correction according to the antibody multiple labels.

Without the formation of ternary complexes, the partitioning of the labelled antibodies follows Poisson distribution, and results in a calculable number of ternary complexes (based on the detection of heterodimers) in droplets. In the case of ternary complexes present in the reaction, the

number of the detected heterodimers is larger than would be expected by pure Poisson distribution. Based on this measurement the number of complexes can be calculated.

In a compartmentalized bi-component method, it is advantageous to incorporate UMIs into different targets as an encoding step for co-localization of unique DNA signals.

5 In a preferred embodiment, the invention relates to a kit, preferably for carrying out the method for determining the concentration of an analyte using a bi-component detection method as described herein, comprising:

- 10 a. two analyte-specific binding components at known concentrations for conducting a bi-component detection method comprising bringing said two analyte-specific binding components in contact said analyte to produce a signal that depends on the concentration of bi-component/analyte complexes formed in said solution
- 15 b. an analyte concentration reference curve for said bi-component detection method, which is a mathematical function reflecting the dependence of said signal on the concentration of analyte, wherein the analyte concentration reference curve is non-bijective function and exhibits an increasing and a decreasing monotonic segment
- 20 c. optionally instructions for preparing one or more dilutions of said sample and applying the bi-component detection method to the sample and the one or more dilutions
- d. a computer program when executed on a computer configured for performing the computational steps of comparing signals detected in the sample and in one or more dilutions with the analyte concentration non-bijective reference curve in order to determine the concentration of the analyte in the sample

25 In a further preferred embodiment, the invention relates to a kit, preferably for carrying out the method for determining the dissociation constants in a bi-component detection method as described herein, comprising:

- a. two analyte-specific binding components at known concentrations for conducting a bi-component detection method comprising bringing said two analyte-specific binding components in contact with an analyte to produce a signal that depends on the concentration of bi-component/analyte complexes formed in said solution
- 30 b. a dissociation constant relationship for said bi-component detection method which is a mathematical functions for the relationship between the dissociation constants of analyte-specific binding components (k_{d1} and K_{d2} , respectively) with the analyte in dependence of said signal reflecting the concentration of bi-component/analyte complexes and the concentration of analytes and/or analyte specific binding components
- 35

c. optionally instructions for preparing one or more dilutions of said sample and applying the bi-component detection method to the sample and the one or more dilutions using defined dilutions factors

5 d. a computer program when executed on a computer configured for performing the computational steps of determining the dissociations constants k_{d1} and k_{d2} of said two analyte-specific binding component using said signal detected in the sample and in the one or more dilutions as a constraining input for a mathematical fit for said dissociation constant relationship at given analyte and/ or analyte specific binding components concentrations.

10 Technical features that have been disclosed for the methods described herein also apply for the kits for use in such methods. A person skilled in the art will, therefore, recognize that preferred features of the methods described herein can be advantageously used as well in the context of the kits.

The method of the present invention, in some embodiments, may also relate to a computer programme product, such as a software product.

15 The software may be configured for execution on common computing devices and is configured for carrying out one or more of steps of the method described herein.

In one embodiment, the computer program may be configured for carrying out step e) of claim 1 that is comparing signals detected in the sample and in one or more dilutions with the analyte concentration reference curve in order to determine the concentration of the analyte in the sample
20 or further preferred embodiments of the computational steps as disclosed herein.

In one embodiment the computer program may be configured for carrying out step e) of claim 2 that is performing the computational steps of determining the dissociations constants k_{d1} and k_{d2} of said two analyte-specific binding component using said signal detected in the sample and in the one or more dilutions as a constraining input for a mathematical fit for said dissociation constant
25 relationship at given binding components and analyte concentrations or further preferred embodiments of the computational steps as disclosed herein.

In one embodiment a concentration reference curve for the bi-component detection method or a dissociation constant relationship for the bi-component detection method may be provided in form of reference data.

30 Herein, reference data for a concentration reference curve for a bi-component detection method shall preferably relate to any data that allows for providing a mathematical function reflecting the dependence of the signal reflecting the formation bi-component/analyte complexes formed in said solution on the concentration of analyte.

35 Herein, reference data for a dissociation constant relationship for a bi-component detection method shall preferably relate to any data that allows for providing a mathematical function for the

relationship between the dissociation constants of the analyte-specific binding components (K_{d1} and K_{d2} , respectively) with the analyte in dependence of the signal reflecting the concentration of bi-component/analyte complexes and the concentration of analytes and/or analyte specific binding components-

5 Typically, the reference data may be stored on a computer-usable or computer-readable medium on the control unit. Any format may be suitable which is used in the industry. The reference data may be stored in a separate file and/or integrated in the computer code or software (e.g. in the source code) for performing the computational steps for determining the concentration of the analyte in the sample or the dissociations constants K_{d1} and K_{d2} , as described above.

10 The computer program product or kit comprising the computer program of the present invention therefore also encompasses and directly relates to the features as described for the method provided herein. Further details on preferred computer-based approaches are provided in the examples and relevant references as described herein.

15 DETAILED DESCRIPTION OF THE INVENTION

Before the present invention is described with regards to the examples, it is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention.

20 The term "sample" or "sample solution" as used herein refers to a solution comprising analytes, preferably of an unknown concentration. Examples of samples include biological fluids such as serum, plasma, urine, tear, cells, cell mixtures, cell culture supernatants, or cell lysates containing one or more biological target molecules. Furthermore, samples can also comprise any conditioning reagents (e.g., permeabilising reagents) needed to render analytes soluble and accessible to detection and quantification. Such conditioning reagents may be added to the sample at any time
25 before or after conduction the methods described herein.

The term "analyte" refers to a substance to be detected, quantified or otherwise assayed by the method of the present invention. Typical analytes may include, but are not limited to proteins, peptides, nucleic acid segments, carbohydrates, lipids, antibodies (monoclonal or polyclonal), antigens, oligonucleotides, specific receptor proteins, ligands, molecules, cells, microorganisms,
30 fragments, products and combinations thereof, or any substance for which attachment sites, binding members or receptors (such as antibodies) can be developed. Analytes may refer also to complexes from said entities. For instance, an analyte may refer to an aggregate or complex formed of multiple entities or molecules, e.g. a protein-protein complex, wherein it is the interaction of the entities/molecules is of interest.

By "protein", a sequence of amino acids is meant for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. "Peptides" preferably refer to smaller molecular weight proteins.

5 The term "nucleic acid" refers to any nucleic acid molecule, including, without limitation, DNA, RNA and hybrids or modified variants and polymers ("polynucleotides") thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid molecule/polynucleotide also implicitly encompasses
10 conservatively modified variants thereof (e.g. degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., *Nucleic Acid Res.* 19: 5081 (1991); Ohtsuka et al., *J. Biol. Chem.* 260: 2605-2608 (1985); Rossolini et al., *Mol. Cell. Probes* 8: 91-98 (1994)). Nucleotides are indicated by their bases by the following standard abbreviations: adenine (A), cytosine (C), thymine (T), and guanine (G).
15

As used herein, the term "nucleic acid amplification" refers to a process by which a limited quantity of nucleic acid undergoes a biochemical reaction in which a larger quantity of nucleic acid is generated. Nucleic acid amplification thus relates to the generation of additional copies of a nucleic
20 acid sequence and is generally carried out using polymerase chain reaction (PCR) or ligase chain reaction (LCR) or other technologies well known in the art (see, for example, Dieffenbach, C. W. and G. S. Dveksler (1995) *PCR Primer, a Laboratory Manual*, Cold Spring Harbor Press, Plainview, N.Y.).

As used, herein the term "bi-component detection method" or "bi-molecular detection method" refers
25 to a method using two analyte-specific binding components and determining a signal reflecting the formation of bi-component/analyte complexes, when said two analyte-specific binding components are brought into contact with a solution containing the analyte. The term "bi-component detection system" or "bi-molecular detection system", preferably refer to components or reagents necessary to carry out a bi-component detection method. This encompasses preferably two analyte-specific
30 binding components, preferably provided in a single or separate solution at a known concentration as well as optional a sample solution containing the analyte to be analyzed.

Preferably the two analyte-specific binding components as well as the analyte are provided non-immobilized, i.e. in solution, such that the of bi-component/analyte complexes likewise form in solution. The term bi-component detection method as used herein, thus preferably address a liquid
35 phase formation of ternary complexes and is distinct from common sandwich immunoassays involving as a solid phase immobilized (primary) capture binding agents as well (secondary) detection binding agents.

The term “non-immobilized” thus preferably refers to components, such as the analyte-specific binding components, that may freely diffuse within a (liquid) solution, such that the binding kinetics to the analyte, equally freely diffusing in said liquid solution, are governed by equations for law of conservation of mass and law-of-mass action in solution as described herein.

5 The term “analyte-specific binding components” or “analyte specific-binding partners” preferably refers to one member of a binding pair, wherein the second member is the analyte and wherein the term “binding pair” includes any of the class of immune-type binding pairs, such as antigen/antibody or hapten/anti-hapten systems; and also any of the class of nonimmune-type binding pairs, such as
10 biotin/avidin; biotin/streptavidin; folic acid/folate binding protein; complementary nucleic acid segments such as complementary DNA strands or complementary RNA strands; protein A or G/immunoglobulins; and binding pairs which form covalent bonds, such as sulfhydryl reactive groups including maleimides and haloacetyl derivatives, and amine reactive groups such as isotriocyanates, succinimidyl esters and sulfonyl halides.

15 The binding components of the bi-component detection method preferably may refer to any component having a binding potential to the analyte. In preferred embodiments the analyte-specific binding components may be nucleic acids, preferably RNA and/or DNA oligonucleotides, antibodies, peptides, proteins, aptamers, molecularly-imprinted polymers, cells or combinations of thereof.

20 The term “antibody” as used herein covers monoclonal antibodies (including full-length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

“Antibody fragments” comprise a portion of a full-length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv
25 fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site.
30 Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular
35 method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The

"monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al, *Nature* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

Complementarity determining regions (CDRs) are part of the variable chains in immunoglobulins (antibodies), generated by B-cells, where these molecules bind to their specific antigen. As the most variable parts of the molecules, CDRs are crucial to the diversity of antigen specificities generated by immunoglobulins. There are three CDRs (CDR1, CDR2 and CDR3), arranged nonconsecutively, on the amino acid sequence of a variable domain of an immunoglobulin. Since the immunoglobulins are typically composed of two variable domains (on two different polypeptide chains, heavy and light chain), there are six CDRs for each antigen receptor that can collectively come into contact with the antigen.

In addition, fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. *et al*, *Nature* 341 :544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')₂ fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird *et al* , *Science* 242:423-426 (1988); Huston *et al* , *PNAS USA* 85:5879-5883 (1988)); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/1 3804; P. Hollinger *et al*, *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993)).

An "antigen binding domain" is the part of an antibody which comprises the area that specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antibody may only bind to a particular part of the antigen, which part is termed an epitope. An antigen-binding domain may be provided by one or more antibody variable domains. An antigen-binding domain may comprise an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

In some embodiments, the binding components may be based on engineered protein scaffolds. Protein scaffolds are derived from stable, soluble, natural protein structures that have been modified to provide a binding site for a target molecule, i.e. an analyte, of interest. Examples of engineered protein scaffolds include, but are not limited to, affibodies, which are based on the Z-domain of staphylococcal protein A that provides a binding interface on two of its α -helices (Nygren, P. A. (2008). FEBS J 275(1 1): 2668- 76); anticalins, derived from lipocalins, that incorporate binding sites for small ligands at the open end of a beta-barrel fold (Skerra, A. (2008) FEBS J 275(1 1) : 2677-83), nanobodies, and DARPinS. Engineered protein scaffolds are typically targeted to bind the same antigenic proteins as antibodies. Short peptides may also be used to bind a target protein. Phylomers are natural structured peptides derived from bacterial genomes. Such peptides represent a diverse array of protein structural folds and can be used to inhibit/disrupt protein-protein interactions *in vivo* (Watt, P. M. (2006). Nat Biotechnol 24(2): 177-83)].

In the bi-component detection methods, preferably two analyte-specific binding components are used that both exhibit a binding potential for the analyte. Preferably, the two analyte-specific binding components do not compete with each other for the binding to the analyte, but are chosen and designed to allow for a simultaneous binding of the components to form bi-component/analyte complexes. As stated previously, the analyte may refer to an aggregate or complex formed of multiple entities, e.g. a protein-protein complex. One of the two analyte-specific binding components may bind to one entity of the analyte complex e.g. a first protein, and the other to a second entity of the analyte, e.g. a second protein. Thereby a bi-component/analyte complex is only form in case of interaction of both proteins, which in turn are labelled with the binding components.

In some embodiments, the binding components may be aptamers. Aptamers are synthetic oligonucleotides (DNA or RNA) that recognize target molecules with high affinity and specificity through a combination of shape complementarity and non-covalent chemical bonds (*Blank & Blind, Current Opin. Chem. Biol., 2005, 9:336-342*). These artificial ligands are obtained *in vitro* and can be developed to recognize a large variety of different molecule classes that range from mere ions (e.g. Pb²⁺, *Liu & Lu, 2003. J Am Chem Soc, 125, 6642-6643*) to nucleotides, small molecules, proteins, viruses, and cells up to whole organisms (*Menger et al., 2006. Handbook of Experimental Pharmacology, 359-373*). High binding affinity aptamers have been selected through the well-known SELEX method (*Ellington & Szostak, 1990. Nature, 346, 818-822*) for the detection of low molecular weight molecules like theophyllin (*Jenison et al., 1994. Science, 263, 1425-1429*), L-arginine (*Geiger et al., 1996. Nucl. Acids Res., 24, 1029-1036*), moenomycin (*Schuerer et al., 2001. Bioorg. Med. Chem., 92, 2557-2563*), 17 β -estradiol (*Kim et al., 2007. Biosens. Bioelectron., 22, 2525-2531*) but also for larger molecules like thrombin (thrombin-binding aptamer:5'-GGTTGGTGTGGTTGG-3') (*Baldrich et al., Anal Chem. 2004, 76, 23, 7053-63*), cholera toxin or HIV-1 tat protein, among others (for review see *Tombelli et al., 2007, Biomolec Eng., 24, 191-200*).

Some of the above-mentioned aptamers have been used in ELISA-like assays on microplates or on the surface of biosensor transducers (QCM, SPR). An aptamer-modified AuNP colorimetric

system has also been developed for the determination of the protein PDGF in a sandwich-based assay (Huang *et al.*, 2005, 77, 5735-5741).

As used herein the term “analyte/bi-component complexes” or “bi-component/analyte complexes” refer to a ternary complex containing the analyte and both analyte-specific binding components. Such bi-component/analyte complexes are formed when applying a bi-component detection method to a solution containing an analyte. More specifically, the formation of bi-component/analyte complexes in a bi-component detection reflects directly upon the presence (and concentration) of an analyte in a given solution. In order to use the formation of bi-component/analyte complexes in order to determine the actual concentration of an analyte a signal reflecting the ternary complex formation is to be acquired.

As used herein the expression “signal that depends on the concentration of bi-component/analyte complexes” or “signal reflecting the formation of bi-component/analyte complexes” refers to any quantifiable information that allows for deduce the quantity of ternary bi-component/analyte complexes formed.

Naturally, the signal will depend on the applied bi-component detection method. For instance, if a Förster resonance energy transfer (FRET) assay is employed the signal may refer to the fluorescence signal that a FRET acceptor will emit when a FRET donor absorbs an excitation beam in close proximity. In the bi-component system, the FRET-acceptor or FRET-donor are preferably coupled the two analyte-specific binding components. Since a fluorescence signal from the FRET-donor-acceptor thus only when the respective binding components are in close proximity, the signal directly reflects upon the formation of ternary bi-component/analyte complexes.

Similarly, Alphascreen (Amplified Luminescent Proximity Homogeneous Assay) developed by PerkinElmer and commercially widely available is a further prototype bi-component detection method, where the signal generation relies on the proximity of “donor” and “acceptor” beads coated with a layer of hydrogel providing functional groups for bioconjugation. When a biological interaction between molecules brings the beads into proximity, a cascade of chemical reactions is initiated to produce a greatly amplified signal. Upon laser excitation, a photosensitizer in the “donor” bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a chemiluminescer in the “acceptor” bead that further activates fluorophores contained within the same bead. The fluorophores subsequently emit light at 520–620 nm. Functional groups on the beads may represent the two analyte-specific binding components, e.g. in form of specific antibodies. Only when ternary complex containing the analyte and the two analyte-specific components are for formed the donor and acceptor beads are in close proximity resulting in a detectable fluorescent signal.

Due to the dependency of the FRET-assay or the Alphascreen assay on the proximity of the donor and acceptor and thus the analyte-specific binding components such assays are termed proximity

based detection assays. Many different proximity based assays in the context of bi-component detections methods are known in the art and may be employed in the methods described herein.

As used herein the term “proximity based bi-component detection method” shall refer to any assay wherein the signal reflecting the formation of bi-component/analyte complexes is based upon the proximity of the two analyte-specific binding components. Statistically, the two analyte-specific binding components will likely be in close proximity in a solution to yield a detectable signal, if they form part of the same ternary complex.

Non-limiting examples of proximity based bi-component methods comprise methods employing a resonance energy transfer assay, preferably a Förster resonance energy transfer (FRET) assay or a bioluminescence resonance energy transfer (BRET) assay, a protein complementation assay (PCA), Alphascreen or a DNA labeled proximity assay, preferably a proximity ligation assay (PLA) or a proximity extension assay (PEA).

These proximity assay technologies are well known in the art. For further reference on FRET or BRET see e.g. (Pfleger, Seeber, & Eidne, 2006), for a protein complementation assays Morell, Ventura, & Avilés, 2009, for Alphascreen Taouji, Dahan, Bosse, & Chevet, 2009 and for DNA labeled proximity methods such as PLA (proximity ligation assay) or PEA (proximity extension assay) Söderberg et al., 2006.

Proximity based assays are particularly preferred for “non-compartmentalized bi-component detection methods”, which has used herein shall refer to bi-component detection methods, wherein no compartmentalization of the sample solution is conducted upon applying the bi-component detection method. Some non-compartmentalized assays may also be referred to as homogenous assays. In other words, the non-compartmentalized bi-component method preferably comprises the step of bringing the analyte and the bi-components in contact in a homogenous reaction solution and the detection step in order to acquire the signal reflecting the formation of ternary bi-component/analyte complexes can be performed upon the homogeneous sample solution without the separation of unbound components or analyte.

In particular preferred embodiments the methods as described herein uses compartmentalized bi-component detection methods. As used herein the term “compartmentalized bi-component detection methods” shall refer to bi-component detection methods that compartmentalize the sample after bringing the analyte specific binding components in contact with the analyte to quantify the formation of ternary analyte/binding-components.

In general, the compartmentalized bi-component detection methods are based upon compartmentalizing a solution to small compartments such that without the formation of ternary analyte/binding-component methods the presence of both binding components in the compartment is unlikely and follows a Poisson distribution.

For the compartmentalization, different assays can be envisioned. For instance, an emulsion droplet method may be used to form droplets in an emulsion (e.g. water-in-oil), wherein each droplet represents a separate compartment. Compartmentalization can involve locations or physical compartments, or diffusion limited environment.

- 5 The term of a “droplet” as used herein preferably refers to an isolated portion of a first fluid surrounded by a second fluid. The first fluid comprises preferably a hydrophilic fluid such as water, an aqueous media, or a buffer, and preferably comprises the sample solution or the one or more dilutions thereof to which the bi-component detections system or other reagents are added. The second fluid preferably a hydrophobic fluid, such as hydrocarbons, silicone oils, mineral oils, organic solvent. Emulsions techniques to compartmentalize sample solutions are well known in the art.
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In particularly preferred embodiments the compartmentalized bi-component detection method is emulsion coupling, which refers to is a digital assay concept based on the detection of double-labelled (bi-component), individual ternary molecular complexes in emulsion, which may be identified, for example, by droplet digital PCR (ddPCR) or next generation sequencing (NGS).

- 15 Droplet Digital PCR (ddPCR) preferably refers to a method for performing digital PCR that is based on water-oil emulsion droplet technology. The oil droplets may be made using a droplet generator that applies a vacuum to each of the wells (see e.g. Pinheiro et al. Analytical Chemistry 84 (2):1003-11)). A typically sample may be fractionated into 20,000 or more droplets, and PCR amplification of the template molecules occurs in each individual droplet. Advantageously, ddPCR technology uses reagents and workflows similar to those used for most standard TaqMan probe-based assays.
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- “Next generation sequencing (NGS)” as used herein shall encompass recently developed technologies for the sequencing of nucleic acids that typically allow much higher throughput than the traditional Sanger approach (see Schuster, Next-generation sequencing transforms today's biology, Nature Methods 5:16-18 (2008); Metzker, Sequencing technologies the next generation. Nat Rev Genet. 2010 January; 11(1):31-46. These platforms can allow sequencing of clonally expanded or non-amplified single molecules of nucleic acid fragments. Certain platforms involve, for example, sequencing by ligation of dye-modified probes (including cyclic ligation and cleavage), pyrosequencing, and single-molecule sequencing. Nucleotide sequence species, amplification nucleic acid species and detectable products generated there from can be analyzed by such sequence analysis platforms. Next-generation sequencing can be used in the methods of the invention, e.g. to quantify unique PCR amplifiable DNA labels in order to assess the formation of bi-component/analyte complexes as described below.
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The details of emulsion coupling as a preferred compartmentalized bi-component detection method are described in EP 3224360, which is incorporated by reference herein.

For a ddPCR detection in an emulsion coupling assay the analyte-specific binding components (such as an antibody pair) are labelled by unique PCR amplifiable DNA labels and the thus labeled binding components are added to the sample (or dilutions thereof see e.g. Example 3).

Before an emulsification of the samples, the reaction is highly diluted (~100,000 times) and PCR reagents are added to achieve single-complex separation and PCR amplification per water-in-oil emulsion droplet. ddPCR may be carried out using the standard ddPCR protocol.

The evaluation of the reaction may be based on the partitioning of the labels in a ddPCR reaction using fluorescently tagged PCR products (e.g. using FAM- or VIC-labelled real-time PCR probes). According to the ddPCR, standard evaluation the cluster of droplets may be determined according to the fluorescent signals of the droplets. Herein, the number of labelled binding components (e.g. antibodies) is determined in each reaction (counting all label-positive droplets for a given label, and using the same definition of cluster of droplets for all reactions).

Additionally, the number of the double-colored (having two different binding component labels) is also determined. Without ternary complexes, the partitioning of the labelled antibodies follows Poisson distribution, and results in a calculable number of double-colored droplets (having two binding components in one compartment based upon pure chance). In the case of ternary complexes present in the reaction, the number of the detected double-colored droplets (having additional ternary complexes) is larger than would be expected by Poisson distribution.

On the basis of this measurements the number of complexes can be calculated (see e.g. EP 3224360 or Karakus et al., 2019 for further reference). This results in an absolute (the count of molecules) quantitation of the ternary analyte complexes.

For a next generation sequencing detection in an emulsion coupling assay the binding components may preferably be labelled by unique PCR amplifiable DNA labels, comprising a specific label for binding component (e.g. antibody) and preferably also an individual label for the molecule, i.e. a unique molecular barcode or unique molecular identifier - UMI (see Parekh et al., 2017). The labeled binding components may be added to the samples or dilutions thereof (see Example 3).

After binding of the binding components, e.g. the antibodies, and before the emulsification of samples, the reaction may be highly diluted (e.g. ~100,000 times) and PCR reagents may be added to achieve single-complex separation. PCR amplification can be performed per water-in-oil emulsion droplet. ddPCR may be carried out using ddPCR protocol.

The evaluation of the reaction may be based on the NGS reading of the binding component, e.g. antibody, specific dimerized UMI labels generated according to the standard protocol of emulsion coupling. The number of labelled binding components, e.g. antibodies, may be determined in each reaction by counting all unique UMI labels for a given binding component, e.g. antibody (counting restricted to a given binding component). Possible multiple labelling of the same binding component, e.g. antibody, can be eliminated using their preferentially dimerized sequences

(multiple labels per binding component will always result in double UMI label dimers with a given antibody specific label context, as they co-localizing in the same droplet).

Ternary antibody/bi-component complexes are counted based on their dimerized double UMI labeled PCR products (in the context of two different binding component (e.g. antibody) specific labels called heterodimers). A correction for multiple labeling of binding components (e.g. antibodies) may be used following the concept described above, which takes into account double UMI label dimers with a given component (e.g. antibody) specific label.

A further evaluation of the samples can be carried as in case of fluorescently tagged PCR (droplet digital PCR), briefly without ternary complexes, the partitioning of the labelled antibodies follows Poisson distribution, and results in a calculable number of ternary complexes (based on the detection of heterodimers) in droplets (having only two antibodies by chance). In the case of ternary complexes present in the reaction, the number of the detected heterodimers is larger than would be expected by pure Poisson distribution. On the basis of this measurements the number of complexes can be calculated (EP 3224360, (Karakus et al., 2019)). This results in the absolute (the count of molecules) quantitation of the ternary complexes.

The dilutions of the samples can be measured in the same sequencing reaction using samples specific DNA barcodes (e.g. barcoded primers) and as antibodies have distinguishable component (e.g. antibody) specific labels many measurements (using different antibody pairs against different antigens) can be carried out in parallel.

In embodiments of emulsion coupling the binding components may be provided as a library of binding components suitable for the detection of multiple analytes. In these embodiments, each member of the binding component library may be associated with a unique nucleotide sequence, which can be used to identify the binding component.

"Associated" may in the context of emulsion coupling mean that the presence of the binding components in the complex can be detected by the presence of the nucleic acid sequence within the linked sequence generated in the method. The nucleotide sequence may be attached as a label to the binding component, be part of the binding component itself e.g. aptamer, or be present within the binding component e.g. nucleic acid within a phage. For example, each member of the library can be labelled with a unique nucleotide sequence that is a nucleotide sequence is attached to the binding components.

Methods of attaching nucleotides to binding components such as antibody or compounds are known in the art. Alternatively, if the binding component library is a phage display library the unique nucleotide sequence can be the sequence that encodes one or more CDR regions or the displayed binding domain. For example, a display library can be generated by inserting sequences encoding the amino acid sequence to be displayed into a phage at a known location. Universal primers that will amplify the inserted sequences can then be used and thus identify the binding sequence.

Alternatively, if the binding component may be an aptamer and the aptamer itself can be the unique nucleotide sequence.

The nucleotide sequence may be an oligonucleotide and may comprise RNA or DNA, single or double stranded. Nucleotides used to label the binding agent or target can preferably be 5- 150 bases in length, for example, 10-40, or 40 -80 bases in length. The nucleotides that form the nucleic acid can be chemically modified to increase the stability of the molecule, to improve its bioavailability or to confer additional activity on it. For example, the pyrimidine bases may be modified at the 6 or 8 positions, and purine bases at the 5 position with CH₃ or halogens such as I, Br or Cl. Modifications on pyrimidines bases also include 2 NH₃, O -CH₃, N -CH₃ and N²-CH₃. Modifications at the 2' position are sugar modifications and include typically a NH₂, F or OCH₃ group. Modifications can also include 3' and 5' modifications such as capping.

Alternatively, modified nucleotides, such as morpholino nucleotides, locked nucleic acids (LNA) and peptide nucleic acids (PNA) can be used. Morpholino oligonucleotides are assembled from different morpholino subunits, each of which contains one of the four genetic bases (adenine, cytosine, guanine, and thymine) linked to a 6-membered morpholine ring. The subunits are joined by non-ionic phosphorodiamidate intersubunit linkages to give a morpholino oligonucleotide. LNA monomers are characterised in that the furanose ring conformation is restricted by a methylene linker that connects the 2'-O position to the 4'-C position. PNA is an analogue of DNA in which the backbone is a pseudopeptide rather than a sugar.

Each member of a library may be associated with a unique nucleotide sequence, i.e. each member may have a unique detectable, nucleic acid identity labels. Preferably, unique nucleic acid identity labels may be linked. Linked may mean that the linking process has the potential to form random multimer nucleic acid products based on co-localization of these nucleic acid identity labels under suitable assay conditions. Preferably, the multimeric product is a dimer. Suitable assay conditions may include amplifying labels, and specific consensus amplification of the unique sequences produce linkable amplicons. The joining of the linkable amplicons e.g. component specific nucleic acids, form linked identity labels, which encodes the co-localization information of the identity labels. The joining reaction can be amplification based or involve other techniques. Amplification based joining can utilise two or more amplification primer pairs with identical binding abilities, but with complementary 5' tags or dimer linker sequences which result in the formation of polymerase extendable nucleic acid duplexes. The tags or dimer linker sequences mean that the sequence amplified by one primer pair will hybridize to sequences amplified by the second primer pair. The identity labels thereby become linked.

The identity labels i.e. the associated unique nucleotide sequences used in libraries, such as the protein display library and the antibody library, may be different in their biological background, and so the amplification and joining process is based on two different primer pairs, e.g. one primer pair amplifies target sequences such as cDNA based identity labels and the second primer pair amplifies binding agent specific nucleotide sequences used as identity labels. Joining of the different labels

makes it possible to link binding agent specific information to target information e.g. proteins encoded by displayed cDNAs.

The method, kit or other computer implemented aspects of the invention may in some embodiments comprise and/or employ one or more conventional computing devices having a processor, an input device such as a keyboard or mouse, memory such as a hard drive and volatile or nonvolatile memory, and computer code (software) for the functioning of the invention.

The components of the computing devices may be conventional, although the device may be custom-configured for each particular implementation. The computer implemented method steps or system may run on any particular architecture, for example, personal/microcomputer, minicomputer, or mainframe systems. Exemplary operating systems include Apple Mac OS X and iOS, Microsoft Windows, and UNIX/Linux; SPARC, POWER and Itanium-based systems; and z/Architecture. The computer code to perform steps of the method described herein may be written in any programming language or model-based development environment, such as but not limited to Python, C/C++, C#, Objective-C, Java, Basic/VisualBasic, MATLAB, Simulink, StateFlow, Lab View, or assembler. The computer code may comprise subroutines that are written in a proprietary computer language that is specific to the manufacturer of a circuit board, controller, or other computer hardware component used in conjunction with the invention.

The information processed and/or produced by the method, i.e. a digital representations of signals reflecting the formation of ternary bi-component/analyte complexes can employ any kind of file format that is used in the industry. Any suitable computer readable medium may be utilized. The computer-usable or computer-readable medium may be, for example but not limited to, an electronic, magnetic, optical, electromagnetic, infrared, or semiconductor system, apparatus, device, or propagation medium. More specific examples (a non-exhaustive list) of the computer-readable medium would include the following: an electrical connection having one or more wires, a portable computer diskette, a hard disk, a random access memory (RAM), a read-only memory (ROM), an erasable programmable read-only memory (EPROM or Flash memory), an optical fiber, a portable compact disc read-only memory (CD-ROM), an optical storage device, a transmission media such as those supporting the Internet or an intranet, cloud storage or a magnetic storage device.

All cited documents of the patent and non-patent literature are hereby incorporated by reference in their entirety.

As used herein the term "comprising" or "comprises" is used in reference to expression cassettes, reporter vectors, and respective component(s) thereof that are open to the inclusion of unspecified elements.

The term "consisting of" refers to expression cassettes, reporter vectors, and respective component(s) thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

FIGURES

The invention will be explained more in detail by referring to the enclosed drawing figures, in which:

FIG. 1 shows a curve and the basic principle of the mathematical background of the extended dynamic range of the measurement based upon a simulation of a compartmentalized saturable bi-component process. The following parameters have been used: number of compartments = 20 000, input volume = 20 microL, dilution factor before emulsification = 192000, concentration of target nM — varied, concentration of component A, nM = 1, concentration of component B, nM = 0.3, dissociation constant of component A = 0.1 nM, dissociation constant of component B = 0.1 nM; (1) number of analyte-bi-component, ternary complexes (molecules), (2) — number of double-positive compartments without analyte (Poisson background), (3) - number of double-positive compartments with target (Poisson background), and (4) of sum of (1) and (3).

FIG. 2 show the determination of the dissociation constants of components. Graphical representation of the simulation results. The intersection of the Eq 3 at different antigen (Ag) concentrations (indicated) and at components concentration 1 and 5 nM respectively. Number of droplets: 10000, dilution factor 10000 and the reaction volume is 20 microL. Using these input parameters the chemical balances, the Poisson process is simulated, and number of ternary complexes formed has been determined. The values, using Eq. 3, of the calculated Kds are identical with the values used for the simulation as inputs (see Example 3).

BEST MODE FOR CARRYING OUT THE INVENTION

EXAMPLES

Example 1.

According to the law-of-mass action in solution and law of conservation of mass, the saturable bi-component reactions between two binding components (e.g. antibody) and an analyte/target molecule (e.g. antigen) can be expressed as the following system of equations:

$$kd_1 = \frac{ab_1}{c_1}, kd_2 = \frac{ab_2}{c_2}, kd_1 = \frac{b_1c_2}{c_{12}}, kd_2 = \frac{b_2c_1}{c_{12}},$$

$$b_{10} = b_1 + c_1 + c_{12}, b_{20} = b_2 + c_2 + c_{12}, a_0 = a + c_1 + c_2 + c_{12} \quad \text{Eq. 1.}$$

where b_{10} , b_{20} are the overall concentrations antibodies (components) with corresponding dissociation constants (kd_1, kd_2) and a_0 is the overall antigen concentration, the antibodies and the antigen are either in free a, b_1, b_2 or c_1, c_2, c_{12} bound state, where c_{12} is the bi-component (ternary) complex (e.g. both antibodies bound) of the antigen (the signal proportional).

Solving the system of Eq 1. for a_0 results in two roots:

$$\begin{aligned}
a_0 = & \frac{1}{2c_{12}(c_{12} - b_{10})(c_{12} - b_{20})} \left[c_{12}^4 - c_{12}^3(b_{10} + b_{20} + kd_1 + kd_2) - \right. \\
& c_{12}b_{10}b_{20}(b_{10} + b_{20} + kd_1 + kd_2) + c_{12}^2 \left(2b_{20}kd_1 + 2b_{10}(b_{20} + kd_2) - \right. \\
& \left. \sqrt{2c_{12}((c_{12} - b_{10})(c_{12} - b_{20}) + c_{12}kd_1)kd_2 + c_{12}^2kd_2^2} \right) + \\
& \left. b_{10}b_{20} \left(b_{10}b_{20} + \sqrt{2c_{12}((c_{12} - b_{10})(c_{12} - b_{20}) + c_{12}kd_1)kd_2 + c_{12}^2kd_2^2} \right) \right] \\
a_0 = & \frac{1}{2c_{12}(c_{12} - b_{10})(c_{12} - b_{20})} \left[c_{12}^4 - c_{12}^3(b_{10} + b_{20} + kd_1 + kd_2) - \right. \\
& c_{12}b_{10}b_{20}(b_{10} + b_{20} + kd_1 + kd_2) + c_{12}^2 \left(2b_{20}kd_1 + 2b_{10}(b_{20} + kd_2) + \right. \\
& \left. \sqrt{2c_{12}((c_{12} - b_{10})(c_{12} - b_{20}) + c_{12}kd_1)kd_2 + c_{12}^2kd_2^2} \right) + \\
& \left. b_{10}b_{20} \left(b_{10}b_{20} - \sqrt{2c_{12}((c_{12} - b_{10})(c_{12} - b_{20}) + c_{12}kd_1)kd_2 + c_{12}^2kd_2^2} \right) \right]
\end{aligned}$$

Eq 2.

Both of them are real positive roots according to the non-bijective reference curve, i.e. it does not allow for a one-on-one correspondence between the detected signal (c_{12}) and analyte (a_0) concentration. Ratios of active/inactive (regarding the ability of binding) antigen and antibodies also can be added to these equations, considering that the overall concentrations of these analytes are not available for the reactions, but only a fraction of them. These ratios can be determined using a concept similar what is described in Example 2.

Example 2.

Simulation of determination of Kds using emulsion coupling (EC) technology

- 10 Antigen (Ag) and two different Ag-reactive antibodies reaction ($Ab_1 = 5 \text{ nM}$ and $Ab_2 = 1 \text{ nM}$) was simulated (according to chemical equilibrium and mass conservation equations (Eq. 1.) with $Kd_1 = 0.2 \text{ nM}$ and $Kd_2 = 0.1 \text{ nM}$ set) in 20 microliter volume. The simulated reaction volume was diluted

(1e+5 times) and by Poisson distribution based simulation was used to sort the reactants into 100000 droplets (total volume: 20 microliter). All droplets are classified and counted according to their encapsulated reactants. The kd_1 - kd_2 functions (Eq. 3) was calculated at three different Ag concentration [a0] with the following inputs: total concentration of Ag [a0], Ab1 [b10], Ab2 [b20] and the simulation determined Ag-Ab1-Ab2 [c12] complex was substituted in equations and the intersection point of functions is determined (see Figure 2).

$$\begin{aligned}
 kd_1 = & -\frac{-a_0c_{12}^2 + 2a_0c_{12}b_{20} - c_{12}^2b_{20} - a_0b_{10}b_{20}}{2c_{12}b_{20}} + \\
 & \frac{2c_{12}b_{10}b_{20} - b_{10}b_{20}^2 - c_{12}^2kd_2 - b_{10}b_{20}kd_2}{2c_{12}b_{20}} - \\
 & \frac{(c_{12}^2 - b_{10}b_{20})\sqrt{a_0^2 - 2a_0b_{20} + b_{20}^2 + 2a_0kd_2 + 2b_{20}kd_2 + kd_2^2}}{2c_{12}b_{20}} \\
 kd_1 = & -\frac{-a_0c_{12}^2 + 2a_0c_{12}b_{20} - c_{12}^2b_{20} - a_0b_{10}b_{20}}{2c_{12}b_{20}} + \\
 & \frac{2c_{12}b_{10}b_{20} - b_{10}b_{20}^2 - c_{12}^2kd_2 - b_{10}b_{20}kd_2}{2c_{12}b_{20}} + \\
 & \frac{(c_{12}^2 - b_{10}b_{20})\sqrt{a_0^2 - 2a_0b_{20} + b_{20}^2 + 2a_0kd_2 + 2b_{20}kd_2 + kd_2^2}}{2c_{12}b_{20}}
 \end{aligned}$$

Eq. 3

Eq 3. Ag [a0], Ab1 [b10], Ab2 [b20] and Ag-Ab1-Ab2 [c12] are substituted in equations. In the case of [c12] its value is determined by simulation, the others are input values of the simulation.

Fig. 2 depicts the intersection of the Eq 3 (using equation above) curves at different Ag concentrations. The values of the calculated Kds are identical with the values used for the simulation as inputs (see above).

According to the simulation results, both Kds can be calculated from the initial amount of antigen, and antibody concentrations and the measured concentration of the analyte-bi-component (ternary) complex, by the determination of the intersections of Eq 3. In experiments, it is beneficial if pairwise intersections are calculated as an approximation of the experimental Kds and the given values are treated by statistical approaches as for instance mixed Gauss distributions or others.

It should be noted, that not all combination of input parameters result in a solvable system, but adequate conditions could always be found, by not only changing the antigen concentration, but changing the concentrations of the antibodies as well. Also only one of the equations (Eq. 3) will result in two positive roots, which in an individual case need to be determined.

Ratios of active/inactive (regarding the ability of binding) antigen and antibodies also can be added to these equations, considering that the overall concentrations of these analytes are not available for the reactions, but only a fraction of them. These ratios can be determined using more dilutions extending the concept described in Example 2.

5 Example 3.

An exemplary measurement of an analyte concentration

A sample containing an unknown concentration of a known analyte is provided. As an exemplary case the analyte is a protein, also as an example it is HER2, an EGFR (epidermal growth factor receptor) receptor variant. To detect the analyte using a bi-component method, Alphascreen is applied. Alphascreen is a prototype bi-component detection method, where the signal generation relies on the proximity of “donor” and “acceptor” beads coated with a layer of hydrogel providing functional groups for bioconjugation. When a biological interaction between molecules brings the beads into proximity, a cascade of chemical reactions is initiated to produce a greatly amplified signal. Upon laser excitation, a photosensitizer in the “donor” bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a chemiluminescer in the “acceptor” bead that further activates fluorophores contained within the same bead. The fluorophores subsequently emit light at 520–620 nm. The functional groups, which are involved in the biological interaction is provided by two antibodies, as an exemplary figure ErbB2/Her2 Antibody Pair (Novus Biologicals DP0061), the antibodies are conjugated to the surface of the donor and acceptor beads, respectively. Upon contact of the donor and acceptor beads to the sample, the antibodies will bind to the HER2 protein forming a ternary complex, which holds the donor and acceptor beads in close proximity resulting in a detectable fluorescent signal according to the principle described above.

To measure the unknown concentration of a HER2 protein analyte a comparison can be made to predetermined signals of a series of different known concentrations of HER2 proteins. Such a reference curve for a bi-component assay is characterized by a certain signal value that has a correspondence to two analyte concentration values in most cases, e.g. a non-bijective reference curve. Hence, the curve does not allow for a one-on-one correspondence between the analyte concentration and the detected signal. To determine the concentration of HER2 one or more defined factor dilutions are made from the sample of unknown amount of HER2 and the signals are determined. Each signal of the dilutions has two corresponding concentrations if compared to the reference curve. As the ratio of the deduced concentrations and their defined dilution factor must be equal, the concentration of the HER2 sample can be determined. Mathematically $f(x) = s_1$ where $f(x)$ the reference curve function, which a function of concentration x and s_1 is the measured signal. $f(x) = s_1$ can be satisfied by two concentrations x_1 or x_2 . However x can be determined if $f(x/c) = s_2$ is determined, where c a defined dilution factor and s_2 the measured signal; similarly, as above, $f(x/c) = s_2$ can be satisfied by x_3 or x_4 . As x_1/x_3 or x_1/x_4 or x_2/x_3 or x_2/x_4 must be c and it can be proved that only one or two of them can be equal to c and if two of them are equal they can be

satisfied at the same x only, thus the ambiguity can be solved. For example if $s_1=1000$ fluorescent unit (FU), it corresponds in hypothetical reference curve to 100 nM (x_1) and 5 nM (x_2) HER2 protein, diluting the original HER2 sample by a factor of 10 results in 2000 (FU) signal, which corresponds in a hypothetical reference curve to 90 nM (x_3) and 10 nM (x_4) HER2 proteins. $x_1/x_3=100/90$ or $x_1/x_4=100/10$ or $x_2/x_3=5/90$ or $x_2/x_4=5/10$ as only $x_1/x_4=c$ the results is $x = x_1 = 100$ nM.

Example 4.

An exemplary measurement of an analyte concentration using a compartmentalized bi-component method

A sample containing an unknown concentration of a known analyte is provided. As an exemplary case, the analyte is a protein, also as an example it is HER2, an EGFR receptor variant, the ErbB2/Her2 Antibody Pair (Novus Biologicals DP0061) is used as detection reagents, i.e. as analyte-specific binding components. The compartmentalized bi-component method is emulsion coupling (EP 3224360, Karakus et al.). Emulsion coupling is a digital assay concept based on the detection of double-labelled (bi-component), individual ternary molecular complexes in emulsion, which are identified, for example, by ddPCR. For ddPCR detection, ErbB2/Her2 Antibody Pair (Novus Biologicals DP0061) are labelled by unique PCR amplifiable DNA labels. The sample is diluted by defined factors and the labeled antibodies are added to the samples, as in Example 3. Before the emulsification of samples, the reaction is highly diluted (~100,000 times) and PCR reagents are added to achieve single-complex separation and PCR amplification per water-in-oil emulsion droplet. ddPCR is carried out using the standard ddPCR protocol (Biorad QX200 ddPCR).

The evaluation of the reaction is based on the partitioning of the labels in the ddPCR reactions using fluorescently tagged PCR products (using FAM- or VIC-labelled real-time PCR probes). According to the ddPCR standard evaluation the cluster of droplets are determined according to the fluorescent signals of the droplets, the number of labelled antibodies are determined in each reaction (counting all label-positive droplets for a given label, and using the same definition of cluster of droplets for all reactions). Additionally the number of the double-colored (having two different antibody labels) is also determined. Without ternary complexes (HER2 protein with two antibodies bound), the partitioning of the labelled antibodies follows a Poisson distribution, and results in a calculable number of double-colored droplets (having two antibodies based upon pure chance). In the case of ternary complexes present in the reaction, the number of the detected double-colored droplets (having additional ternary complexes) is larger than would be expected by Poisson distribution. On the basis of this measurements the number of complexes can be calculated (see EP 3224360, Karakus et al., 2019 for further reference). This results in the absolute (the count of molecules) quantitation of the ternary analyte complexes. According to Eq. 1. with known dissociation constants and active fraction of the applied antibodies the original absolute concentration of the analyte (HER2) also can be calculated. However Eq 1. has two roots, only one of them is the concentration. Following a similar calculation as in Example 3 given, the correct

concentration of the unknown HER2 sample can be determined (see Example 3 for a numerical example).

Example 5.

An exemplary measurement of dissociation constants of antibodies in a non-compartmentalized bi-component assay

A sample containing a known concentration of a known analyte is provided. As an exemplary case, the analyte is a protein, also as an example it is HER2, an EGFR receptor variant, ErbB2/Her2 Antibody Pair (Novus Biologicals DP0061) is used as an exemplary reagent. The bi-component method is Alphascreen as in Example 3 using dilutions and reference curve described in Example 3. To determine the relationship of the Alphascreen signal and the corresponding concentration of the ternary complexes, one can use a method described in Example 4 parallel to the Alphascreen reference curve measurements. This results in the absolute (the count of molecules) quantitation of the ternary complexes related to the Alphascreen signals. To calculate the dissociation constants (Kd) of the antibodies Eq 3. are applied, having more than one analyte dilutions with known amount of analyte and the determined amount of ternary complex – the latter is determined using the determined correspondence between the concentration of the ternary complexes and the Alphascreen signal. The two Kds can be calculated according to the Example 2. Mathematically $f(kd1, c12, a0) = kd2$ where $kd1$ and $kd2$ are dissociation constants and $c12$ is the measured concentration of ternary complex of the analyte and a is the concentration of the analyte, is applied. As different dilutions of the analyte corresponds to different concentration of the $c12$, the f function has different forms. However as the Kds are material constants, consequently all forms of the f function must be satisfied by a certain pair of values of Kds, which can be determined, see Example 2.

Example 6.

An exemplary measurement of dissociation constants of antibodies in a compartmentalized bi-component assay

A sample containing a known concentration of a known analyte is provided. As an exemplary case, the analyte is a protein, also as an example it is HER2, an EGFR receptor variant, ErbB2/Her2 Antibody Pair (Novus Biologicals DP0061) is used as exemplary detection reagents, i.e. analyte-specific binding components. The compartmentalized bi-component method is emulsion coupling (EP 3224360, (Karakus et al., 2019)) using Biorad QX200 ddPCR as detection device as in Example 4 using dilutions and the number of ternary complexes can be calculated (EP 3224360, Karakus et al., 2019) as in Example 4. This results in the absolute (the count of molecules) quantitation of the ternary complexes. To calculate the dissociation constants of the antibodies Eq 3. are applied, having more than one analyte dilutions with known amount of analyte, antibodies and determined amount of ternary complex, the two Kd can be calculated according to the Example 2. Mathematically $f(kd1, c12, a0) = kd2$ is applied, where $kd1$ and $kd2$ are dissociation constants and

ac is the measured concentration of ternary complex of the analyte and a is the concentration of the analyte. As different dilutions corresponds to different (concentration) values of the 'ac', the f function has different forms. However, since the Kds are material constants, consequently all forms of the f function must be satisfied by a certain pair of values of Kds, which can be determined, see Example 2.

Example 7.

An exemplary measurement of an analyte concentration using a compartmentalized bi-component method using next-generation sequencing

A sample containing an unknown concentration of a known analyte is provided. As an exemplary case, the analyte is a protein, also as an example it is HER2, an EGFR receptor variant, ErbB2/Her2 Antibody Pair (Novus Biologicals DP0061) are used as detection reagents, i.e. analyte-specific binding components. The compartmentalized bi-component method is emulsion coupling (EP 3224360, Karakus et al., 2019)). Emulsion coupling is a digital assay concept based on the detection of double-labelled (ternary), individual molecular complexes in emulsion, which are identified, for example, by next-generation sequencing (NGS). For NGS detection the ErbB2/Her2 Antibody Pair (Novus Biologicals DP0061) are labelled by unique PCR amplifiable DNA labels (antibody specific label), which also has the individual label molecule unique molecular barcode (UMI) as well (Parekh et al., 2017). Defined analyte dilutions are made and the labeled antibodies are added to the samples, as in Example 3. After antibody binding and before the emulsification of samples, the reaction is highly diluted (~100,000 times) to achieve single-complex separation, PCR reagents are added and PCR amplification per water-in-oil emulsion droplet like in ddPCR is carried out using ddPCR protocol (Biorad QX200 ddPCR).

The evaluation of the reaction is based on the NGS reading of the antibody specific dimerized UMI labels generated according to emulsion coupling concept (EP 3224360). The number of labelled antibodies is determined in each reaction by counting all unique UMI labels for a given antibody (counting restricted to a given antibody specific label context), and the possible multiple labels of the same antibody was eliminated using their preferentially dimerized sequences (multiple labels per antibody always have double UMI label dimers with the same antibody specific label context, as they co-localizing in the same droplet). The ternary complexes are counted on the basis of their dimerized double UMI labeled PCR products (two different antibody specific labels called heterodimers) with correction according to the antibody multiple labels (following the concept presented above to eliminate the effect of the multiple labels per antibody). The further evaluation of the samples is carried out according to Example 4, briefly without ternary complexes, the partitioning of the labelled antibodies follows Poisson distribution, and results in a calculable number of ternary complexes (based on the detection of heterodimers) in droplets (having only two antibodies by chance). In the case of ternary complexes present in the reaction, the number of the detected heterodimers is larger than would be expected by pure Poisson distribution. On the basis of this measurements the number of complexes can be calculated (EP 3224360). This results in the

absolute (the count of molecules) quantitation of the ternary complexes. According to Eq. 1-2 with known dissociation constants and active fraction (latter is optional) of the applied antibodies the original absolute concentration of the analyte (HER2) also can be calculated. However, Eq 1-2 have two roots, only one of them always is real. As described in Example 3, using dilutions the correct concentration of the unknown HER2 sample can be determined (see Example 3 for a numerical example). The dilutions of the samples can be measured in the same sequencing reaction using samples specific DNA barcodes (e.g. barcoded primers).

Example 8.

An exemplary measurement of dissociation constants of antibodies in a compartmentalized bi-component assay using next-generation sequencing

A sample containing a known concentration of a known analyte is provided. As an exemplary case, the analyte is a protein, also as an example it is HER2, an EGFR receptor variant, ErbB2/Her2 Antibody Pair (Novus Biologicals DP0061) labeled with antibody specific UMI labels. The compartmentalized bi-component method was emulsion coupling (EP 3224360,) using NGS as the detection method as in Example 7 and using dilutions and Eq1-2. the number of ternary complexes can be calculated (EP 3224360). This results in the absolute (the count of molecules) quantitation of the ternary complexes. To calculate the dissociation constants (Kd) of the antibodies Eq. 3. is applied, having more than one analyte dilutions (these can be partially the same dilutions as above) with known amount of analyte and determined amount of ternary complex the two Kd can be calculated according to the Example 2. Mathematically $f(kd1, c12, a0) = kd2$ where $kd1$ and $kd2$ are dissociation constants and $c12$ is the measured concentration of ternary complex of the analyte and $a0$ is the concentration of the analyte, is applied. As different dilutions corresponds to different (concentration) values of the $c12$, the f function has different forms, however as the Kds are material constants, consequently all forms of the f function must be satisfied by a certain pair of values of Kds, which can be determined, see Example 2. The dilutions of the samples can be measured in the same sequencing reaction using samples specific DNA barcodes (e.g. barcoded primers).

Ratios of active/inactive (regarding the ability of binding) antigen and antibodies also can be added to these equations, considering that the overall concentrations of these analytes are not available for the reactions, but only a fraction of them. These ratios can be determined using more dilutions extending the concept described in Example 8.

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CLAIMS

1. A method for determining the dissociation constants of analyte-specific binding components with an analyte in a sample of known concentration in a bi-component detection method comprising:
 - a. providing two non-immobilized analyte-specific binding components at known concentrations for conducting a bi-component detection method in a solution comprising bringing said two analyte-specific binding components in contact with the analyte to produce a signal that depends on the concentration of bi-component/analyte complexes formed in said solution,
 - b. providing a dissociation constant relationship for said bi-component detection method which is a mathematical function for the relationship between the dissociation constants of the two analyte-specific binding components with the analyte (k_{d1} and k_{d2} , respectively) in dependence of said signal reflecting the concentration of bi-component/analyte complexes and the concentration of analyte and analyte-specific binding components,
 - c. preparing one or more dilutions of said sample and/or said analyte-specific binding components,
 - d. applying the bi-component detection method to the sample and the one or more dilutions,
 - e. determining the dissociations constants k_{d1} and k_{d2} of said two analyte-specific binding components using said signal detected in the sample and in the one or more dilutions as a constraining input for a mathematical fit for said dissociation constant relationship at different analyte concentration and/or analyte-specific binding components concentrations.
2. The method according to claim 1, wherein said dissociation constant relationships are calculated analytically by solving chemical balance, and mass conservation equations.
3. The method according to any of the preceding claims, wherein the analyte is selected from a group consisting of proteins, peptides, nucleic acid segments, carbohydrates, lipids, antibodies (monoclonal or polyclonal), antigens, oligonucleotides, specific receptor proteins, ligands, molecules, cells, microorganisms as well as fragments products or combinations of thereof and/or wherein the sample comprise two or more different types of analytes.
4. The method according to any of the preceding claims, wherein the two analyte-specific binding components are selected from a group consisting of nucleic acids, preferably RNA and/or DNA oligonucleotides, antibodies, peptides, proteins, aptamers, molecularly-imprinted polymers, cells or combinations of thereof and/or wherein two or more pairs of different kind of analyte-specific binding components are used.

5. The method according to any of the preceding claims, wherein the bi-component method comprises employing a proximity-based assay to produce the bi-component/analyte complexes concentration dependent signal, wherein the proximity-based assay uses two analyte-specific binding components that produce a detectable signal in dependence of their proximity.
6. The method according to any of the preceding claims, wherein the bi-component method comprises employing a resonance energy transfer assay, preferably a Förster resonance energy transfer (FRET) assay or a bioluminescence resonance energy transfer (BRET) assay, a protein complementation assay (PCA), Alphascreen or a DNA labeled proximity assay, preferably a proximity ligation assay (PLA) or a proximity extension assay (PEA).
7. The method according to any of the preceding claims, wherein the bi-component detection method comprises employing a compartmentalized assay to produce a bi-component/analyte complexes concentration depended signal, wherein the signal reflects the presence of said two analyte-specific binding components in a single compartment wherein the compartmentalized assay preferably employs an emulsion droplet method, wherein each droplet in the emulsion represents a separate compartment, more preferably wherein the compartmentalized assay is emulsion coupling.
8. The method according to any one of the preceding claims, wherein multiple analytes are determined in parallel.
9. The method according to any one of the preceding claims, wherein the bi-component detection method comprises employing an absolute molecular count based analytical method and/or wherein the bi-component detection method comprises employing a droplet digital PCR assay.
10. The method according to any one of the preceding claims, wherein the bi-component detection method comprises using analyte-specific binding components associated with unique amplifiable nucleic acid labels and employs a compartmentalized assay, wherein a nucleic acid amplification is performed for each compartment using fluorescently tagged amplification products.
11. The method according to any one of the preceding claims, wherein the bi-component detection method comprises using multiple analyte-specific binding components comprising nucleic acid molecule-identifying – unique molecular identifier - barcodes and employs a compartmentalized assay, wherein an nucleic acid amplification is performed for each compartment producing linked molecule-identifying – unique molecular identifier - nucleic acid barcodes, the compartments are reunited in a common pool and a parallel nucleic acid sequencing technique is used to produce the bi-component/analyte complexes concentration depended signal.
12. The method according to any one of the preceding claims 10 or 11, wherein the parallel nucleic acid sequencing technique is a next generation sequencing technique (NGS).

13. A Kit for determining dissociation constants in the bi-component detection method of claim 1, comprising:
- a. reaction buffer for two or more analyte-specific binding components at known concentrations for conducting a bi-component detection method comprising bringing said two analyte-specific binding components in contact with a solution containing an analyte to produce a signal that depends on the concentration of bi-component/analyte complexes formed in said solution,
 - b. reference data on a dissociation constant relationship for said bi-component detection method which is a mathematical function for the relationship between K_{d1} and K_{d2} in dependence of said signal reflecting the concentration of bi-component/analyte complexes and the concentration of analytes and/or analyte-specific binding components,
 - c. optionally instructions for preparing one or more dilutions of said sample and applying the bi-component detection method to the sample and the one or more dilutions using defined dilutions factors,
 - d. a computer program when executed on a computer configured for performing the computational steps referred to in the method of claim 1 e. of determining the dissociations constants K_{d1} and K_{d2} of said two analyte-specific binding component using said signal detected in the sample and in the one or more dilutions as a constraining input for a mathematical fit for said dissociation constant relationship at different analyte and/or analyte-specific binding components concentrations.