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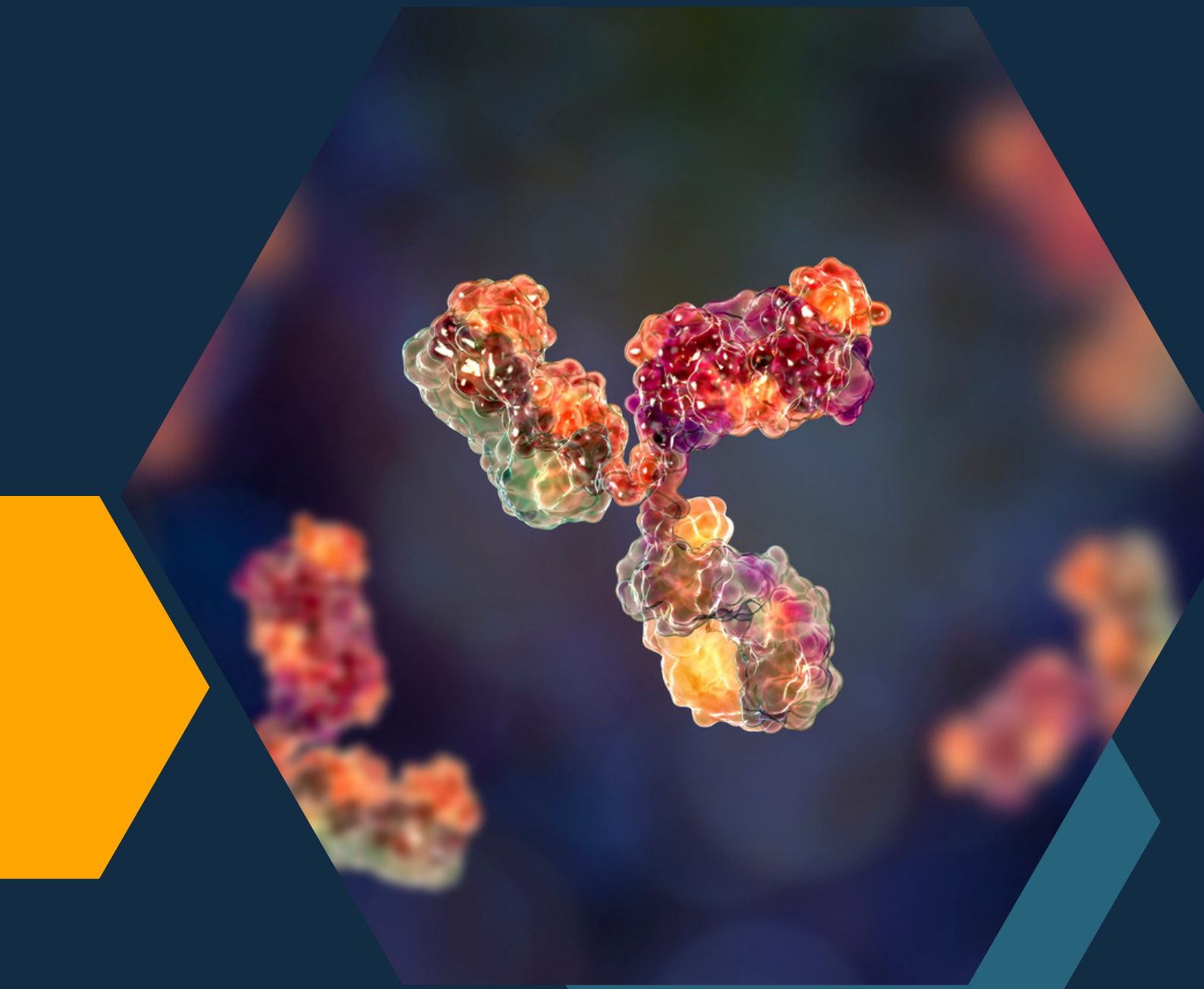

Free versus total analyte quantification using ligand-binding assays

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Contents

Free versus total analyte quantification using ligand-binding assays

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Keywords: Biotherapeutic drug development; ELISA; Ligand-binding assays (LBA); Pharmacodynamics (PD); Pharmacokinetics (PK); Toxicity; Free analyte; Total analyte; Monoclonal antibody.

Free versus total analyte: why is it important to measure?

Drug development is fundamentally supported by preclinical and clinical study data, which measure accurate quantitative biotherapeutic drug concentrations. The data generated by the bioanalytical laboratory demonstrates the relationship between drug exposure and circulating drug concentrations enabling pharmacokinetics (PK), pharmacodynamics (PD) and dosing to be characterized [1]. A detailed report of the PK and PD effects facilitates drug development decision-making and the drug concentration data reveals the relationship between the efficacy and safety of a drug. Therefore, gathering this data is vital for understanding the effect a drug will have on an individual [1–3].

Measurement of the free (unbound) concentration of an antibody-based biotherapeutic is of importance to understand its pharmacologic effects such as PK, PD, efficacy, toxicity and the dose-response relationship [1,3]. However, measurement of the free analyte concentration is complicated due to the multiple forms of free analyte and ligand-bound analyte that can exist, with an equilibrium between the different forms (Figure 1) [4]. As a biotherapeutic may have a soluble circulating target, gathering information regarding the circulating concentrations of soluble drug targets is critical for understanding the PK/PD relationship fully [1].

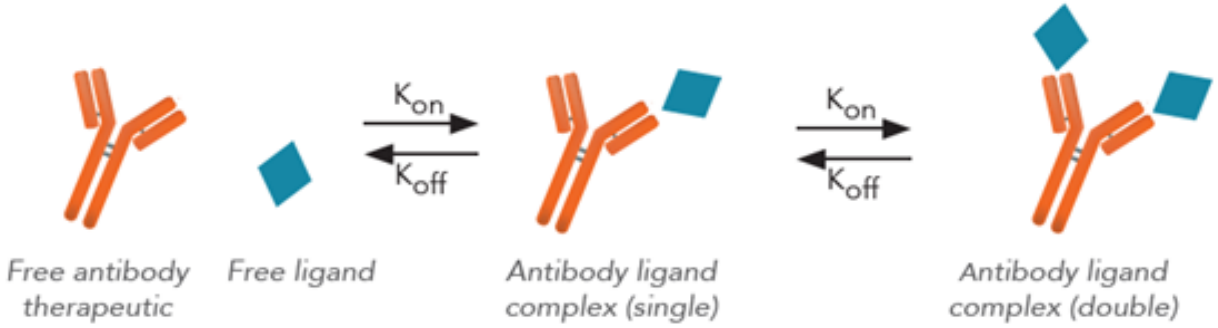

Figure 1: The complex dynamic binding equilibrium that exists between free and bound analytes [4].

Bioanalytical methods used to quantitate free versus total analyte

There are various techniques used to measure target and drug concentrations within a biological matrix but ligand-binding assays (LBAs), such as ELISA, are most common [1]. Historically, LBAs have been used to analyze protein biotherapeutics and target ligands, particularly for monoclonal antibody (mAb) drugs, which non-covalently bind to the ligand. Consequently, multiple forms can exist in vivo including free mAbs, free ligands, monovalent complexes (single) and bivalent complexes (double).

An understanding of this complex dynamic binding equilibrium that occurs in the body after dosing is paramount to selecting the most appropriate approach for method development. LBAs have been designed to measure the free and total analyte forms, but verification of the forms being measured can be technically challenging [3]. A key bioanalytical challenge is to measure free drug concentrations within a mixture of drug-ligand complexes without compromising the data due to sample preparation and assay-dependent equilibrium changes, which can cause measurement uncertainty. Influencing factors on correct free drug measurement that need to be considered include assay format, calibration concept, sample preparation and sample storage. Also, during incubation it is important to avoid 'on plate' complex dissociation due to dilution-induced equilibrium changes or interference of capturing reagents [2].

Typically, most LBAs overestimate the free therapeutic due to disruptions in the equilibrium between free and bound (monovalent or bivalent) analytes, which can shift in response to incubation times where the free therapeutic binds to the capture reagent [5]. Therefore, in PK/PD and safety evaluations, it may not be practical to have a 'one-size-fits-all' bioanalytical strategy as it is technically challenging to design and develop assays that measure only one molecular species (for example free, bound or total) [1].

LBAs enable the determination of free and total analyte concentrations with ELISA immunoassays, often being the 'go-to' technology due to their convenience and approachability [2,5]. The ELISA incubation times (typically 1–2 hours) are often sufficiently long to disrupt the free/bound equilibria leading to overestimation, depending on the affinity and kinetic dissociation rates of the antibody-ligand complexes. To ensure measurement of true free analyte in samples, an available flow-through microfluidic immunoassay technology, utilizing affinity capture immunoassay formats that has very short contact times (~6 seconds) is the optimal approach. This technology, the Gyrolab® immunoassay platform, has been shown to minimize equilibria disruption and accurately measure free analyte. As data accumulates for the microfluidic flow-through approach to measuring free analyte, the adoption of this technique may become more widespread [5].

The Gyrolab platform compared to conventional LBAs

Compared to conventional LBAs, the Gyrolab platform has short binding times, typically in the range of seconds, meaning there is limited time for any dissociation of complex. Plate-based immunoassays can overestimate free analyte concentrations due to longer incubation times, but overestimation is not seen with the Gyrolab platform due to the flow-through affinity column, which enables short contact times as the sample is 'spun' over the 15nL column. The flow-through sample delivery in the Gyrolab compact discs (CDs) with microfluidic channels ensures accurate free analyte measurement that does not cause equilibrium shifts and high matrix tolerance allowing samples to be run at low dilutions or even as neat samples [6]. The Gyrolab CDs differ primarily in the sample volume processed and inherent matrix tolerance, which determines the high sensitivity and wide dynamic range of the assay compared to ELISA. Running immunoassays at a nanoliter scale reduces sample requirements and reagent consumption [7]. Gyrolab xPlore and Gyrolab xPand use the same technology and software, allowing assay transfer between platforms. Miniaturized ELISA technology enables productivity to be increased and the generation of reproducible data through automation of the assays, allowing data-driven decisions to be made more quickly [8]. Additionally, the Gyrolab platform is designed with 21-CFR Part 11 compliance in mind and exhibits the high precision required for regulatory guidelines [6].

Alma Pihlblad, Application Scientist at Gyros Protein Technologies (Uppsala, Sweden), previously completed her Masters at Uppsala University (Sweden) on the development and comparison of bioanalytical methods to measure free analyte. She commented on the advantages of the Gyrolab technology:

> *"Gyrolab technology, with short contact time between the sample and assay reagents, serves as an excellent platform to quantify free analyte. This can be useful in different applications including measurement of the free drug molecule or target for PK and PD purposes, as well as affinity determination of an interaction in solution. For both of these applications, it is essential not to disturb the equilibrium conditions in the samples and only measure the free molecule excluding the molecular complex. The short contact time between the sample and the capture column, of just a few seconds, enables undisturbed measurement of the sample. This also reduces matrix effects with the opportunity to reduce dilutions of the sample, which otherwise disturbs the equilibrium as well."*

Summary

It is essential for bioanalytical scientists to fully understand the pharmacological effects of a therapeutic and the interactions between the biological drug and its target. Knowledge of the complex dynamic binding equilibrium between free and bound analyte is paramount to selecting the most appropriate approach for method development. LBAs, particularly ELISA immunoassays, have been commonly used to measure the free and total analyte forms, but confirmation of the forms being measured can be technically challenging. Some conventional LBAs can cause bias, for example, longer incubation times can cause dissociation or dilution of samples can shift the equilibrium between free and bound analytes [3,6]. To overcome this, PK scientists, toxicologists and bioanalytical scientists need to collaborate and share technical feasibility and assay development challenges in order to form a sound bioanalytical strategy [1]. Miniaturized ELISA technology is a powerful tool used to generate reproducible data and make data-driven decisions more quickly, thus enabling bioanalysts to overcome challenges and improve the development of drugs that benefit society [8].

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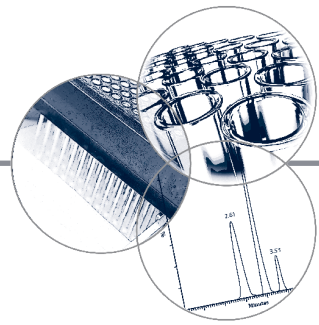

Free analyte QC concept: a novel approach to prove correct quantification of free therapeutic protein drug/biomarker concentrations

Quantification of free drug concentrations is highly challenging due to the dynamic drug–ligand equilibrium, which may result in incorrect results. Current QC concepts do not adequately cover all of the important influencing factors: the assay itself (format and procedure); the calibration concept; the sample preparation; and the sample storage. Here, we propose a 'free analyte QC concept' that enables quantitative testing of these four factors and, thus, provides best possible proof of correct free drug quantification. The principle of the free analyte QC concept and an example of its application for a free drug assay is described. A comparison of this novel approach with current approaches and how the new concept fits (or does not fit) with current regulatory guidelines is discussed.

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A detailed characterization of the PK/TK properties of a drug and the establishment of an understanding of the relationship between PK and PD effects are essential parts of the drug-development process. Thus, an appropriate bioanalytical support is highly important for detailed characterization of these drug properties. The goal of every bioanalytical strategy is the quantification of the most relevant form(s) of the drug. When wanting to quantify therapeutic proteins, the question at hand is usually whether to determine free and/or total drug concentrations. The decision to quantify total, free or both drug concentrations is certainly quite product specific and depends on the target biology; for example, concentration of soluble ligand or shed target/receptor, and on the affinity of the drug to the ligand. A differentiation between free and total drug concentrations is certainly not required in all drug-development programs [1,2]. The use of mathematical simulations that take into consideration the ligand and drug concentrations and the affinity have proved to be very helpful in choosing the appropriate bioanalytical strategy [1].

Besides the ligand, antidrug antibodies can bind to the drug and, thus, compromise the assay result [3,4]. Any effect of antidrug antibodies on an assay intended to determine total drug concentrations could be interpreted as an unwanted interference. Drug neutralization by antidrug antibodies and, thus, a decrease of free drug concentrations, should however be detected by an appropriate free drug assay.

If knowledge of free drug PKs is important for a project, correct free drug quantification is required, but is difficult to achieve [2,5,6]. The question of whether free or total concentration is needed is equally important for the quantification of soluble ligands/biomarkers [7,8]. Here, we discuss the necessity and the challenges by using an example of a free drug (therapeutic protein) assay development; however, the principle would also apply to the development of a free ligand assay [9].

The standard technology for bioanalysis of therapeutic proteins is the ligand-binding assay (LBA), which allows for the determination of target-binding competent/free drug concentrations [2,10]. The bioanalytical challenge to correctly determine free drug concentrations within a mixture of drug–ligand complexes, without compromising the result due to sample preparation and/or assay-dependent equilibrium changes, is well-recognized and discussed within the scientific community [1,2,11]. From a practical assay development point of view, the bioanalyst should consider four important influencing factors: assay format and procedure; calibration concept; sample preparation; and sample storage.

The fundamental prerequisite for free drug quantification is the selection of appropriate assay reagents and of the most appropriate assay format, which is already addressed and discussed in the scientific literature [2,7,10,12–16]. A target/ligand-capture format is the most commonly used assay format. It allows to

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Key Term

Free analyte QC sample: Sample containing a defined concentration of free analyte in equilibrium with its respective ligand/binding partner, prepared by mixing defined amounts of both binding partners into an appropriate matrix. The free analyte concentration at equilibrium is calculated based on the affinity (KD), which describes the interaction in solution.

specifically select the free, ligand-binding competent drug molecules. The selection of an appropriate assay format alone is not sufficient to assure accurate quantification of free drug concentrations. An appropriate assay procedure, including an optimized capture step to minimize or even exclude result falsification, is equally important. It should avoid 'on plate' complex dissociation during incubation as a result of dilution-induced equilibrium changes or equilibrium changes due to interference of the capturing reagent [1,9]. Using a ligand as the capturing reagent is the best possible mimicry of the drug–ligand interaction. This is particularly true for monovalent binding drugs; for multivalent binding drugs avidity effects need to be considered, for example, when selecting the coating density on the plate. Since the interaction between the ligand in the sample and the ligand used for capturing is similar with regard to the epitope that is recognized, there is no real competition and the equilibrium change is mainly driven by the dissociation rate, which is only time-dependent (unit [1/s]). A possible alternative is to use an anti-idiotypic antibody as the capturing reagent in the LBA in the case where the ligand is not available or if the drug-target/ligand interaction has unfavorable binding kinetics (e.g., slow association rate). If used, a careful assessment of whether the anti-idiotypic antibody is really a valid target/ligand surrogate is required. Points to consider are whether the anti-idotypic antibody really only detects free drug molecules without binding drug–ligand complexes, or whether it competes the ligand out of the drug–ligand complex and, thus, impacts the drug–ligand equilibrium.

The importance of an appropriate calibration concept and the potential influence of sample preparation on correct free drug quantification have been described based on theoretical considerations [1]. The importance of the influence of sample storage on correct free drug quantification is also clear. Nevertheless, no detailed procedures on how to address this issue in the context of free drug quantification are described in the scientific literature.

To date, only very limited approaches of how to test whether an assay correctly determines the free analyte concentration can be found in the literature [2,9]. All currently described approaches only check the theoretical suitability of the assay format. These procedures aim for gathering data to support the hypothesis that the free drug concentration is correctly determined, but have limitations. We propose a free analyte QC concept, which offers the possibility to bring the assay characterization to a quantitative level by applying mathematical considerations and models. Therefore, this concept uses a broad basis of data to prove that a putative free drug assay is indeed able to correctly quantify free drug concentrations in *ex vivo* samples. To show proof-of-concept, both the development and qualification of a free drug assay using the free analyte QC concept is described.

The free analyte QC concept

Based on a quantitative readout, the free analyte QC concept considers yet unaddressed challenges of free drug quantification such as: appropriateness of calibration and QC sample preparation in the target-containing matrix; impact of sample dilution; and drug–target complex stability during sample storage.

As an example, a free drug assay was developed, characterized and validated/qualified using this concept. All critical steps are discussed, a comparison to the currently applied/described approaches is done, and how the new concept fits (or does not fit) within the currently available regulatory guidelines is discussed.

ELISA was used to quantify free drug concentrations of a monovalently binding therapeutic protein in plasma samples containing shed target. Analysis of free drug concentrations in preclinical studies in non-human primates (NHP) and in clinical studies was envisaged.

A target capture format using the biotin–digoxigenin system [12] was developed, including an optimized 'short' capturing step of only 10 min (Figure 1) [1,9]. This incubation time was selected after evaluation of the theoretically possible error due to drug–ligand complexes dissociation using mathematical simulations [1]. In addition, routine applicability of the method was considered.

In short, for capturing, biotinylated ligand is coated onto a streptavidine 96-well microtiter plate (1 h at room temperature). After washing, the samples, calibrators or QCs are added and incubated for 10 min, followed by a washing step. Detection of captured drug is performed by a digoxigenylated monoclonal antibody against the drug and anti-digoxigenin–peroxidase Fab-fragments (1 h incubation at room temperature and washing); 3-p-hydroxyphenylpropionic acid was used for the readout.

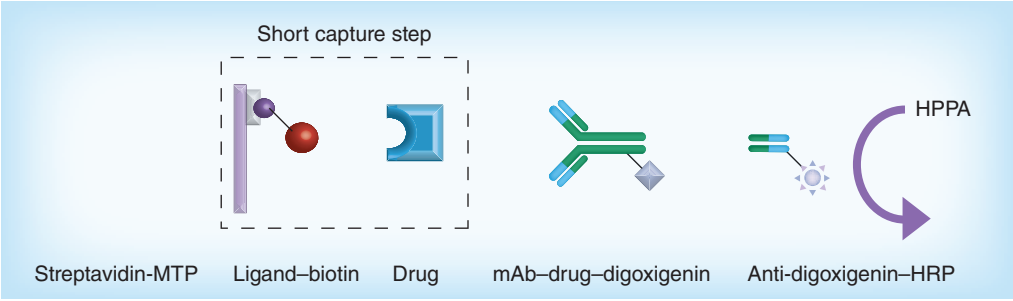

Figure 1. Assay format used to show applicability of the free analyte QC concept. A ligand capture assay format with a short capturing step based on the biotin–digoxigenin system was used to show applicability of the free analyte QC concept.
HPPA: 3-p-hydroxyphenylpropionic acid; HRP: Horseradish peroxidase; mAb: Monoclonal antibody; MTP: Multititer plate; .

Principle of the free analyte QC concept

Usually, QCs with known concentrations of the analyte are generated by spiking a defined amount of the analyte into the respective biomatrix. The principle of the proposed free analyte QC concept is identical insofar as QC samples with known and defined free drug concentrations are used for assay qualification/validation. The free drug QC samples are prepared by spiking defined amounts of drug and ligand into a representative ligand-free matrix, followed by a sufficient incubation time to assure that the interaction between the two binding partners has reached equilibrium. The free drug concentration is calculated based on the affinity (dissociation constant [KD]) between drug and ligand. By using different drug–ligand ratios, QC samples with calculated free drug concentrations covering the whole calibration range can be prepared, which enables an assay validation similar to classical quantitative QC samples. A schematic overview of the free analyte QC concept is given in Figure 2.

■ Limitations of the classical QC approach

The preparation of samples with exactly defined and known analyte concentrations is mandatory for every quantitative QC. The preparation of the **free analyte QC samples** is more complex compared with the classical approach, where the analyte target concentration is equivalent to the nominal drug concentration added to a defined volume of the matrix. For the classical approach, potential variables that might impact the correctness of the assumed concentration are: incorrect concentration of the stock solution (which is not a problem if the identical stock or dosing solution is used for QC preparation and calibration); active (target-binding competent) concentration of the drug; and pipetting errors. In contrast, correct free drug QCs are much more complex to achieve. Incorrect concentrations of the stock solutions, active concentrations and pipetting errors for both drug and ligand are even more critical, since the calculation of the free drug concentration requires knowledge of the exact amounts of drug and ligand that interact (i.e., are functionally active) with each other. Beyond this, the most critical factor is certainly the quality of the affinity/KD data that is used for calculation of the target free drug concentration. Correct KD values that describe the interaction of the two binding partners in solution are a prerequisite for this approach. Drake *et al.* recently described challenges due to surface matrix effects when surface-based approaches are applied to determine affinity data [17].

Finally, the appropriate incubation time needs to be calculated and strictly followed during QC sample preparation to ensure that the equilibrium has been reached.

As an example to underline the importance of correct KD values, the influence of differing KD values on the calculated free drug concentration was simulated. A variation of the given affinity by a factor of 0.33, 0.5, 2 and 3 would result in calculated free drug concentrations of 0.48, 0.68, 1.96 and 2.56 ng/ml, instead of the calculated target concentration of 1.14 ng/ml (based on the given KD value) resulting in recoveries of 238, 168, 58 and 45%.

Calibration concept

Even the best assay will deliver incorrect results if the calibration is not appropriate. This is particularly true for correct quantification of free drug concentrations. Based on theoretical

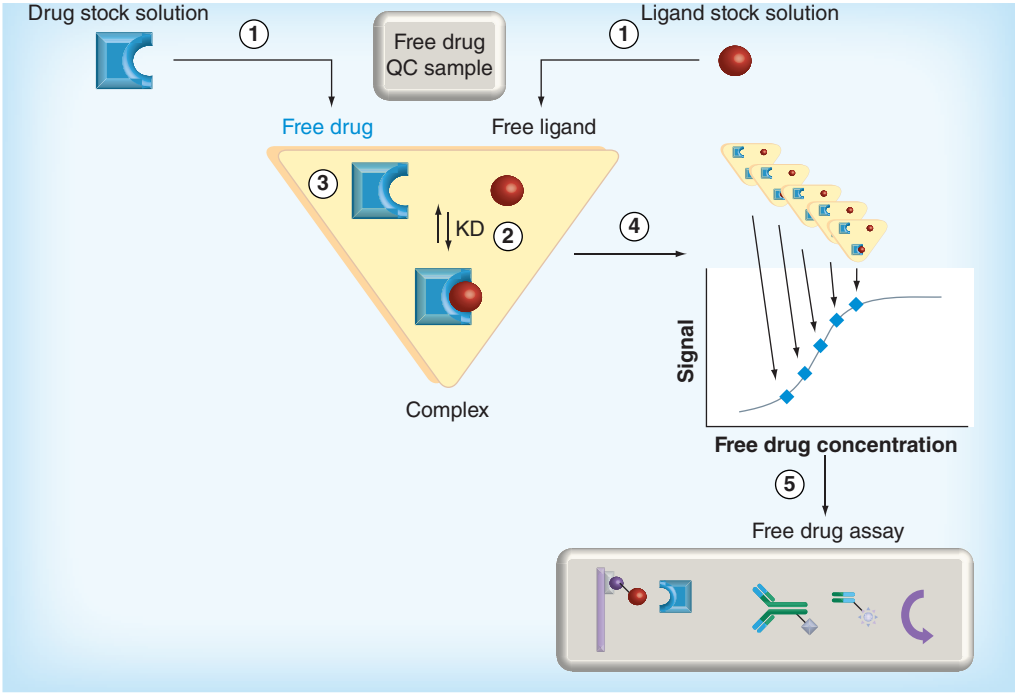

Figure 2. Principle of the free analyte QC concept. The free analyte QC concept is based on the preparation of QC samples containing defined amounts of free drug in equilibrium with the respective ligand and drug–ligand complexes, which enables evaluation of all potential influencing factors on the equilibrium. (1) QCs are prepared by spiking defined amounts of drug and ligand into a target-free matrix. (2) Depending on the affinity, an equilibrium between the drug and the ligand will be reached resulting in (3) a defined free drug concentration, which can be calculated. (4) For assay qualification/validation different concentrations of free drug QCs are prepared, covering the calibration range. (5) These QC samples are used for assay qualification/validation, using the calculated free drug concentrations as the target values for determination of assay performance data, for example, accuracy and precision data.
KD: Dissociation constant.

considerations, it is clear that a ligand-free calibration is necessary for a correct free drug quantification, if relevant amounts of the ligand are present in the naive/blank matrix that is used for calibration and QC preparation [1].

Knowledge of whether the ligand is present in serum/plasma and, if yes, at which concentrations is often scarce at early stages of drug development, even for the human situation. The situation in the animal species used for preclinical studies is usually even more unclear, but equally important, because the toxicological studies are performed in a relevant animal species, which expresses the desired ligand/epitope and demonstrates a similar tissue crossreactivity profile as in humans. In these unclear cases, the suitability of blank plasma for calibration can be achieved by evaluating a potential signal quenching in the respective blank matrix in comparison with buffer. If such differences exist, a potential alternative to the (unavailable) blank matrix of the study species is the use of serum/plasma from nonrelevant animal species or from animals lacking the ligand in relevant amounts, for example, due to absence of target shedding in healthy versus diseased animals. Certainly, the use of such alternatives needs to be tested and validated.

In our example, the corresponding ligand of the NHP plasma was crossreactive to the used therapeutic protein. Comparison of calibration curves in buffer, NHP plasma and rabbit plasma (as a potential alternative matrix) revealed a significant signal quenching in NHP plasma (Figure 3). Based on these calibration curves, the recoveries at 20 and 200 ng/ml of drug in NHP plasma would be 2 and 47%, respectively, compared with the buffer calibration. In contrast to the NHP-based calibration, calibration in buffer and rabbit plasma demonstrated identical calibration curves (Figure 3).

The difference clearly indicated that calibration in the NHP pool matrix was not appropriate

and would affect the correctness of the assay result. However, these data alone do not prove that the observed interference was ligand-dependent. For clarification, an immunoaffinity extraction of the target was performed. For the 'stripping' experiments, two immunocapture beads (streptavidin beads coated with biotinylated reagents) were prepared using the drug or a polyclonal antibody preparation against the ligand as capture reagents.

An aliquot of the NHP plasma sample, which previously showed clear matrix influence, was immunodepleted using streptavidin beads coated with the drug (18 nM drug per sample) and then used for QC sample preparation. Two drug concentrations at which significantly reduced recoveries in untreated NHP plasma samples were observed (20 ng/ml and 200 ng/ml), were spiked into the immunodepleted NHP plasma. In addition, QCs of the same concentrations were prepared in rabbit plasma (ligand-free alternative matrix) and the QCs were quantified against a calibration curve constructed in buffer to assure free drug concentrations without any potential influence from ligand or other matrix components. The recoveries in immunodepleted NHP plasma (107 and 101%, respectively) and in rabbit plasma (105 and 118%, respectively) were within 20% of the nominal concentration. These results indicate that the observed matrix effect is probably due to the endogenous ligand present in the sample, but certainly due to matrix components that specifically bind to the drug. Furthermore, they indicate that in this case rabbit plasma is a suitable biological matrix for calibration and QC preparation, since no significant matrix interference could be observed.

Immunodepletion of the ligand using the drug can easily be performed due to the availability of sufficient amounts; this approach could, however, be questioned, since not only the endogenous ligand as a potential influencing factor, but all matrix components that bind to the drug and influence the target binding or interfere with the detection reagent (e.g. rheumatoid factors, binding proteins or anti-drug antibodies) would be removed from the sample. Therefore, in order to further prove that the endogenous ligand is responsible for the observed interference, a polyclonal antibody preparation derived from a different species against the ligand was used to minimize potential interferences and, thus, enable a more specific ligand immunodepletion. Eight individual NHP plasma samples were spiked with the drug (51.5 ng/ml) and the recovery was determined using a rabbit plasma calibration curve. As shown in Table 1, the mean recovery of the spiked sample was significantly reduced (mean recovery: 12%). Ligand immunoaffinity extraction using two different concentrations of the polyclonal antibody-based immunocapture probe resulted in a concentration-dependent increase of the recovery, up to approximately 100%. Taken together, this proves that the observed signal quenching, that is, reduction of the free drug concentration, is indeed mediated by the endogenous ligand. However, a free drug assay should describe this 'interference' that results in a reduction of free drug concentration on a quantitative basis.

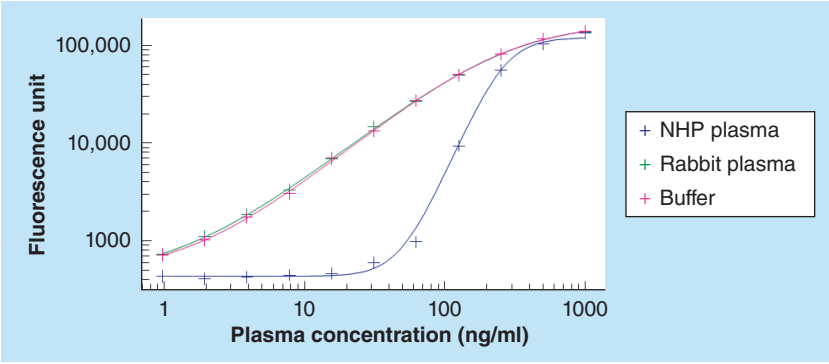

Figure 3. Selection of a suitable calibration matrix. Selection of a suitable calibration matrix is performed by comparison of calibration curves prepared in matrix of an alternative, nonrelevant animal species and buffer, which is the ligand-free benchmark. A clear signal quenching of the calibration prepared in NHP plasma in contrast to rabbit plasma and buffer, which were comparable, can be observed. The results indicate that rabbit plasma is a suitable ligand-free matrix that could be used for ligand-free calibration.
NHP: Non-human primate.

■ Agreement of the calibration concept with current bioanalytical guidelines

Unfortunately, the current guidelines (US FDA/European Medicines Agency [EMA]) focus only on total drug assays and do not consider the requirements for correct free drug quantification. The FDA guidance for industry on bioanalytical method validation states, *"a calibration curve should be prepared in the same biological matrix as the sample in the intended study by spiking the matrix with known concentration of the analyte"*. In the section on method development of LBAs, subsection "Matrix effects unrelated to the analyte", it is recommended to compare the standard curve in biological fluids with standard in buffer to detect matrix effects, implying that ideally the curves should be comparable [18]. This

Table 1. Characterization of specific matrix interference.

ID	Recovery (%)		
	No IAE	IAE 96 nM pAb<ligand>	IAE 640 nM pAb<ligand>
1	23	80	109
2	7	51	111
3	8	52	108
4	11	57	108
5	7	51	112
6	7	51	108
7	19	77	108
8	16	56	103
Average	**12**	**59**	**108**

For specific ligand depletion, IAE were performed on eight individual non-human primate (NHP) plasma samples using two different concentrations of the extraction reagent (pAb against the ligand, coated onto beads). Aliquots of the treated and untreated NHP plasma samples were spiked with the drug at 51.5 ng/ml, and the recoveries were determined using a calibration prepared in rabbit plasma as an alternative ligand-free matrix. A significantly decreased recovery could be detected in the samples without IAE as well as an increase of up to approximately 100% depending on the amount of the used IAE reagent.
IAE: Immunoaffinity extractions; pAb: Polyclonal antibody.

is, however, only valid for total drug assays. As shown in Figure 3, calibration curves of a free drug assay prepared in buffer (ligand-free) and the matrix that contains the ligand must be different.

The EMA guideline addresses in the "Matrix selection" section potential issues due to structurally related endogenous compounds for replacement therapies, but does not discuss the potential influence of endogenous target on calibration and, as a result, also on the quantification result. However, in the same section, the EMA guideline states: *"the use of extracted matrix (e.g., charcoal, immuno-affinity) or alternative matrix (e.g., protein buffers, dialyzed serum) is not recommended, the use of such matrices may be necessary when there is no other strategy to quantify the analyte of interest"* [19]; thus, in our opinion, provides the possibility to use matrix alternatives. The described signal quenching clearly confirmed the theoretical considerations and can be considered as sufficient justification of the necessity to use one of the described alternative approaches. In our opinion, the use of a nonrelevant species matrix or a relevant species that does not have the ligand present in serum/plasma seems to be favorable over the proposed alternatives, such as charcoal or immune-affinity purified matrices, protein buffers or dialyzed serum, since it represents a complete, untreated plasma or serum and, thus, is more comparable to the matrix of the relevant animal species or human samples. Besides these reasons, reagent availability, the costs for the preparation of immunodepleted matrix samples, the long-term reagent supply, as well as the additional potential of handling errors during preparation, have to be considered. In our example, a rather high amount of the antibody for immunocapturing was necessary to obtain a quantitative immunodepletion, possibly due to unfavorable affinity properties of the available polyclonal antibody preparation. If such an approach is considered for calibration or QC sample preparation in routine analysis, significant amounts of reagent would be required, which might not be feasible due to reagent limitations and might also result in significant costs. It is also questionable whether it is possible to generate a large batch of immunodepleted matrix due to issues in upscaling the bead-based immunodepletion process. In addition, reproducibility of batches of comparable quality to ensure long-term supply might be challenging, and as a result appropriate blank matrix would become a critical reagent. Therefore, we would recommend to use a target-free neat matrix of an alternative species for calibration.

With respect to QC samples, the EMA guideline states, *"QC samples should be prepared in the actual sample matrix and the accuracy should be calculated to demonstrate the absence of matrix effect"*. In the immunoassay field, matrix effect is often referred to as a calibration curve constructed in buffer or a surrogate matrix resulting in a different assay response compared with a calibration curve constructed in biological samples [20,21]. When discussing free versus total analyte determination, the definition of matrix effects must be questioned. The goal of the classical QC approach is to ideally show 100% recovery of the nominal spiked drug amount. However, a free drug assay should exactly describe this matrix interference. In our opinion, **specific matrix effects**, such as the interference of the ligand, should be differentiated from unspecific matrix effects, which compromise the assay performance, for example, due to unspecific binding to assay reagents.

If the target concentration of the matrix that is used for QC preparation is known, it is theoretically possible to prepare QCs in the actual matrix. Addition of a defined amount of drug should result in the expected free drug concentration. The use of ligand-containing matrix samples with known concentrations is, however, not practicable. In most cases, ligand concentrations are not available, the result is not appropriately defined (free or total ligand concentrations)

Key Term

Specific matrix effect: Response reduction of an analyte in a biological matrix compared with a ligand-free matrix (e.g., buffer or alternative matrix) in a 'free' ligand binding assay, which is due to specific interference of the analyte–ligand interaction, for example, soluble ligand.

or is subject to a certain inaccuracy, which in turn influences the calculation of the free drug concentration. Moreover, preparation of QC samples with different free drug concentrations covering the whole calibration range is very difficult. Only a limited variety of (endogenous) ligand concentrations are available, and therefore the flexibility to mix drug and ligand resulting in the required free drug concentrations is missing.

If the required information is available, it is highly recommended to use real target-containing samples to get a better understanding of whether the recombinantly expressed protein (ligand) is a suitable surrogate for the endogenous counterpart. Correct quantification of the calculated free drug concentration in such 'real' samples demonstrates that the recombinantly expressed protein and the endogenous protein interact similarly with the drug. In addition, it proves that the binding characteristics, which are typically determined in buffer, reflect the drug–ligand interaction in the biological matrix.

Certainly, if the calibration curve and QCs are prepared in a different matrix than the matrix that is envisaged to be analyzed, it is even more important to show the absence of unspecific matrix effects. To minimize unspecific matrix effects, it is advisable to minimize the amount of matrix in the assay as much as possible. A balance must be found between maximal matrix content, sufficient sensitivity and no impact of the sample dilution on the free drug result.

In our example, 1% matrix was chosen. Analysis of eight individual blank NHP plasma and human plasma samples revealed signals (mean signals are NHP: 100 fluorescence unit [FU]; human: 104 FU) that were comparable to the blank signal of the rabbit plasma (118 FU) and even to buffer (106 FU). All samples showed signals below the lowest calibrator (218 FU), which proves the absence of unspecific matrix effects and confirms the applicability of the rabbit plasma-based calibration for quantification of free drug concentrations in NHP and human plasma samples.

■ Appropriateness of assay format & procedure

As mentioned in the background section, procedures for clear-cut proof of whether the selected assay format and procedure is really able to accurately determine the free drug concentration in an *ex vivo* sample are still missing. Commonly, accuracy and precision of an assay are determined by the classical QC concept, using the nominal spiked, that is, total drug concentration as the target value, which is set to 100%. In contrast to this approach, the target concentration of the free analyte QC concept is the free drug concentration, which is calculated based on known concentrations of the drug, the ligand and the binding affinity/KD.

In accordance with the guidelines, QC samples with free drug concentrations covering the whole calibration range were prepared by using different drug–ligand ratios, (calibration range: 1–1000 ng/ml). As determined by 14 runs on nine different days by two operators, accuracy and precision of all QCs at five different concentrations covering the whole calibration range were 87–110% and 7–21% (at LLOQ) and, thus, were well within acceptance criteria. This proves the applicability of the free drug QC concept, as well as the fact that the assay really detects the expected free drug concentration. In addition, it shows that the assay procedure does not impact the drug–ligand equilibrium.

Accuracy and precision data were derived from the analysis of one batch of each of the differently concentrated QC samples. To demonstrate reproducibility of the QC preparation, QCs were prepared independently (five-times) using three different rabbit pool plasma preparations. Accuracy (95–109%) and precision (10–11%) of these QCs were well within the acceptance range. Despite the much higher degree of complexity and variability of the free drug QC preparation, these data demonstrate the possibility to reproducibly prepare QC sets even in different matrix lots, which would allow this assay concept to be used in a routine setting.

■ Influence of sample preparation

The sample preparation, especially the sample dilution and selection of the appropriate dilution buffer, is a critical step. Dilution with buffer alone or with ligand-free matrix might result in complex dissociation, leading to an artificial increase of the free drug concentration, whereas dilution with a target-containing matrix results in the formation of new complexes leading to a decrease of the free drug concentration [1].

Evaluation of accuracy and precision provides information on the assay performance. In the case of a free drug assay, the free drug QC accuracy and precision data ideally show the absence of any assay-dependent perturbations of the drug–ligand equilibrium, including the minimal required dilution. Typically, due to the limited

assay range of LBAs, the minimal required dilution is not sufficient, and higher dilutions of samples are required to bring the concentrations into the assay range. If a ligand-free dilution buffer is used, the free drug concentration might be increased as a result of drug–ligand complex dissociation; on the other hand, if a ligand-containing dilution buffer (e.g., matrix of the relevant species) is used, the free drug concentration might be decreased as a result of new drug–ligand complexes forming because of the addition of new ligands.

Testing dilution parallelism is a very important and useful approach to test the influence of the dilution procedure on the free drug result. Dilution nonparallelism indicates an influence. However, dilution parallelism, tested with classically prepared QC samples with only known total drug concentrations, is not necessarily proof of the absence of an influence of sample dilution on the assay results. Furthermore, dilution parallelism of results of real study samples with unknown concentrations does not prove that the assay result is correct, that is, that the determined free drug concentration detected in the assay corresponds to the concentration in the sample [1]. Dilution parallelism testing using the proposed free drug QC concept offers the possibility to account for the two major dilution factors: dissociation of drug–ligand complexes results in an increase of the free drug concentration, and formation of new drug–ligand complexes due to addition of ligand results in a decrease of the free drug concentration.

Typically, therapeutic proteins show high affinity for their target with low dissociation rates. Consequently, a ligand-free dilution procedure is advantageous and highly advisable. Complex dissociation can be considered as the major influencing factor. Ideally, the complexes are stable enough and the dissociation is so low that the dilution-induced new equilibrium is not reached within the timeframe of the critical assay steps, and therefore the result is not confounded. An observed influence may necessitate a reduction of the assay incubation time of the capture step, as well as the time required for sample dilution.

Ligand-containing dilution is much more complex. Both dilution-dependent dissociation of the complexes and complex formation due to the addition of new ligand have to be considered. Since the association rates between drug and ligand are typically high, complex formation is critical. To avoid this, the assay dilution and capture step would have to be executed in a very short timeframe, which is either simply not manageable or makes the assay unsuitable for routine use.

In addition, the fact that the ligand concentration in the matrix is often not known or is subject to batch-to-batch variation is another reason to avoid ligand-containing dilution procedures. We, therefore, applied a target-free dilution process. Dilution linearity testing was performed by preparing a highly concentrated plasma sample, which contained a defined free drug concentration and a significant amount of drug–ligand complexes (67%). This allows monitoring of potential dilution-dependent complex dissociation. Dilution linearity could be shown up to a dilution factor of 729 without significant influence on the expected free drug results. In our example, these dilutions were sufficient to dilute even the samples with the highest expected drug concentrations into the assay range. Actually, it is only necessary to show that samples that are expected to contain a significant amount of drug–ligand complexes can be diluted without affecting the results. At higher drug concentrations, the drug is in excess compared with the ligand, and thus the free drug equals total drug. As previously described, mathematical simulations enable this assessment [1].

In the given example, dilutions of the NHP samples were performed using a buffer containing 1% rabbit plasma. Comparability of blank signals of rabbit and NHP blank samples (see section titled 'Calibration concept') was demonstrated and is an argument to underline the assumption that the different matrix mixtures did not impact the result.

■ Complex stability during storage

Only very scarce data about the impact of sample storage and freeze–thaw cycles on the drug–ligand equilibrium can be found in the literature. However, evaluation of analyte stability in the matrix is an integral part of assay validation [18,19].

Drug instability or loss of drug during sample storage might result from biochemical modifications, for example, proteolysis or simple adsorption of the analyte to surfaces to which it is exposed. In the case of drug–ligand complexes, storage instabilities are even more fragile since two components are prone to these processes. More critical are all effects that perturb the noncovalent drug–ligand interaction. The importance of sample *ex vivo* conditions for potential equilibrium changes of the drug–ligand interaction has been

described on a theoretical basis [2]. However, no possibilities of how to address this challenge have been described in the literature so far. To date, complex stability was presumed without any scientific data backing this. The free drug QC concept enables detailed stability studies. Every effect on the drug–ligand complex stability results in a change of the free drug concentration, which can be monitored by QCs with defined free drug and drug–ligand complex concentrations. The opportunity to assess the influence of storage conditions enables the bioanalyst to react and optimize the conditions in case of observed instabilities. This approach covers testing of physicochemical complex stability. If the complex stability is altered by biochemical influences to the drug–ligand equilibrium (e.g., proteolytic processes), comparability of the matrix of the alternative, nonrelevant species and the study species has to be unfortunately presumed. Our approach is the only possibility for early evaluation of storage stability, as it allows for evaluation of the potential impact of the freeze–thaw cycle between sample collection and sample analysis on the complex stability and, thus, the free drug result. Storage stability data in the study species, which cannot easily be prepared, could be gathered retrospectively by reanalysis of real study samples. However, these data give limited information on long-term storage stability, since the first freeze–thaw cycle point is missing.

In our example, storage stability of more than 5 months could be shown (recoveries of HQC, MQC and LQC were 87, 90 and 111% at day 1 and 87, 85 and 83% at day 161), strengthening the assumption that no equilibrium perturbation occurred during the selected storage conditions.

Conclusion

Correct quantification of free drug concentration, and consequently knowledge of the free drug PKs, can play an important role for establishment of a PK/PD correlation. Development of appropriate bioanalytical methods is a challenging task. Selection of an appropriate assay (format and procedure), the calibration concept, the sample preparation and the sample storage are the four major influencing factors on correct free drug quantification that need to be considered.

Assay characterization using the classical validation approach does not unequivocally prove that all of the major influencing factors for correct free drug quantification have been accounted for based on a scientifically sound database.

Undefined bioanalytical data that cannot be unequivocally interpreted hamper the development process. This is a well-recognized issue in the scientific community, and the importance of clear communication of assay limitations is highlighted in the literature [2,22].

The proposed free analyte QC concept offers the possibility to test the actual influence of all critical steps. Application of this concept at early stages during assay development enables the bioanalyst to identify and solve potential issues. At later stages, it allows for a comprehensive characterization of the free drug assay and a thorough qualification/validation that can prove that the assay provides correct free drug concentrations. Hence, the free analyte QC concept enables optimal bioanalytical support by providing clearly defined assay results.

QC samples, which contain defined concentrations of free drug in equilibrium with the drug–ligand complexes, are prepared by spiking defined amounts of drug and ligand into target-free matrices. The actual free drug concentration is calculated based on the KD between the drug and ligand. This approach certainly requires a profound knowledge of drug and ligand quality, and a deep understanding of the drug–ligand interaction. The most critical factor is the quality of the KD data. Correct KD values that accurately describe the interaction of drug and ligand in solution are mandatory in order to prepare accurate QCs with defined free drug concentrations suitable for assay validation.

Ideally, free drug QC samples would also be used during sample analysis. Nevertheless, it could be discussed whether it is sufficient to use the free drug QC samples for assay development, characterization and qualification/validation only to ensure that the assay provides correct free drug concentrations. A potential QC sample alternative during sample analysis, for example, if the ligand is only available in limited amounts, is to prepare free drug QC samples by spiking defined amounts of drug in a ligand-free matrix. These QC samples could confirm that there was no technical problem during analysis. This could be deemed sufficient, if a proper assay characterization and validation using the free analyte QC concept was previously performed, showing that the assay procedure does not induce any equilibrium changes.

For a ligand-free calibration and QC preparation, we used plasma derived from an alternative, nonrelevant animal species. The use of

matrix-based QC samples is certainly favorable for storage stability studies over buffer-based QC samples. For correct free drug quantification, a simple buffer-based calibration could be sufficient (as shown in FIGURE 3, calibration constructed in buffer and in rabbit matrix are overlaying). Use of buffer calibration during sample analysis might be an alternative after proper assay characterization. This might even be the only possibility if no alternative, non-relevant animal species can be identified or if the availability of the alternative matrix is limited.

Although the concept is demanding, particularly with regard to the required quality of the affinity data, our example showed that application of this concept is possible and can be implemented into bioanalytical assay development. Assay qualification/validation data show acceptable assay performance, in terms of accuracy and precision.

Future perspective

In a previous publication, we described how mathematical simulations can be used to assess potential influences during assay execution on the assay result, and how this can be used to guide assay development [1]. The free analyte QC concept is the next step, since it provides a scientifically sound database for best possible proof that a given assay generates correct free drug concentrations. The given example shows that this concept is not only theoretically possible but provides proof of applicability.

Development of free drug assays requires additional effort compared with standard assay developments. Selection of an appropriate ligand-free alternative matrix for calibration and QC preparation, and experiments to show that the observed influence is indeed ligand-dependent are two important additional working packages. The benefit of the additional effort is the availability of a characterized free drug assay for meaningful interpretation of preclinical or clinical PK, PD or other data.

Establishment of such a free drug assay is highly desirable in cases where free drug concentrations are relevant, since it is sensitive to all potential factors influencing the drug–ligand equilibrium, such as further binding partners, for example, shed receptor if the drug target is a soluble ligand [23], endogenous binding proteins [24] or antidrug antibodies [25–28]. Implementation of a characterized free drug assay into the bioanalytical strategy improves the quality of the data compared with a total drug–total ligand approach that only considers the ligand as an influencing factor, neglecting other binding partners, especially antidrug antibodies.

The most critical prerequisite for the free analyte QC concept is the correctness of the KD value. From a theoretical perspective, KD is a constant that describes the drug–ligand interaction. However, it is well-known that affinity characterization is a highly challenging task and might be dependent on how it was determined [17]. Often, so-called apparent KDs are reported, which might be sufficient for relative comparisons, for example, of different antibody drug candidates, but for the free analyte QC approach, as well as for the use of KD values in PK/PD models, correct affinity data that truly describe the drug–ligand interaction in solution are required.

Another essential component for correct free drug quantification is target-free calibration [1]. As previously discussed, the current regulatory guidelines do not cover this aspect and are mostly focused on the validation of total drug assays. From an industry and quality perspective, it would be highly desirable if the guidelines would provide a clearer statement on the appropriate calibration and QC preparation for free drug assays, as we have shown here the value of free drug assays in therapeutic protein development.

The applied procedure enables a better interpretation of PK/PD effects during PK, TK or PD studies in the preclinical and clinical setting, and as such is regarded as a powerful tool in therapeutic protein development.

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Executive summary

Principle of the free analyte QC concept

- QC samples containing defined concentrations of free drug in equilibrium with drug–ligand complexes are used for assay development and qualification/validation.
- Free analyte QC samples are prepared by mixing defined amounts of drug and ligand, and then incubating until equilibrium is reached.
- Mathematical models are used to determine the required incubation times and calculation of the final free drug concentration based on the affinity between the drug and ligand.

Challenges of the free analyte QC concept

- Availability of high-quality affinity data that correctly describes the interaction of the drug and ligand in solution is required.
- Matrix selection for calibration/QC preparation: current regulatory guidelines are focused on total drug assays and do not fully cover the requirements for correct free drug quantification, particularly the need for target-free calibration.

Benefits of the free analyte QC concept

- The free analyte QC concept provides the best possible proof of correct free drug quantification.
- The concept enables better interpretation of preclinical and clinical data (e.g., PK, PD and TK) by providing clearly defined and reliable bioanalytical results.

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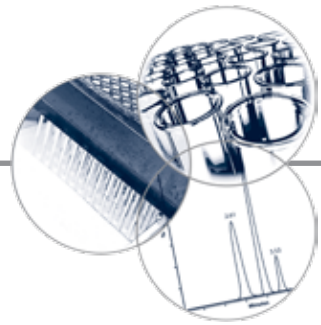

Free versus total ligand-binding assays: points to consider in biotherapeutic drug development

"It is therefore very helpful for end users of bioanalytical data such as pharmacokinetic scientists and toxicologists to work together with bioanalytical scientists to assess the needs, technical feasibility and challenges of developing suitable bioanalytical assays in order to form a sound bioanalytical strategy."

Keywords: biotherapeutic drug development ■ ELISA ■ ligand-binding assay ■ monoclonal antibody ■ pharmacodynamic ■ pharmacokinetics

Accurate quantitative information on biotherapeutic drug concentrations from both preclinical and clinical studies is critical to support drug development. These data reveal the relationship between drug concentrations in circulation and drug exposure; help characterize pharmacokinetics (PKs) of the drug candidates; facilitate projection of doses for human Phase I–III studies; and provide the foundation of PK/pharmacodynamic (PD) modeling. In addition, drug concentration data are important in revealing potential relationships between drug exposure and efficacy or safety. Information about circulating concentrations of soluble drug targets is also useful in understanding PK/PD relationships in situations where a biotherapeutic has a soluble circulating target. While there are many different techniques that can be used to quantify drug and target concentrations in a biological matrix such as serum or plasma, ligand-binding assays (LBAs) such as ELISA are commonly used. Recently, there has been an increasing interest among bioanalytical and PK scientists in understanding how the design and format of an LBA can impact apparent drug and/or target concentration data, and what the potential impact of this may be on interpretation of PK/PD and safety data. This is especially the case with monoclonal antibody (mAb)-based therapeutics.

Since the introduction of the first mAb-based drug, muromonab-CD3 (orthoclone OKT3) [1], over 20 mAb therapeutics have been approved by the US FDA and over 100 additional mAb-based therapeutics are currently under development [2]. Typically, a therapeutic mAb needs to bind to its target antigen in order to exert its effect. When the mAb and the target both circulate, various molecular species of mAb and target co-exist in a dynamic equilibrium that is based upon the law of mass action, first described by Guldberg and Waage in 1864. These molecular species include: free drug, free circulating target, total drug and total target. In situations where there is more than one target-binding region on the drug (e.g., a full-length bivalent mAb) or more than one drug-binding region on the target (e.g., VEGF and IgE), more molecular species, such as partially complexed drug–target, may also be present. These partially complexed species are often considered to be part of the free fraction of drug or target.

It has long been acknowledged that data on different drug and target species (e.g., free vs total levels of drug target as two possible biomarkers) may satisfy different needs. Recently, there has been a high level of interest in this topic and its potential impact on drug development in the biopharmaceutical industry. This has partially been driven by bioanalytical scientists, who are increasingly involved in helping to create PK, PD and safety strategies during drug development. In addition, advances in analytical technologies have helped bioanalytical scientists to better understand the limits of their own methods, and in some cases, have enabled them to develop more specific assays that enable the measurement of either free or total molecular species.

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A discussion group on free versus total PK/PD assays was formed by the AAPS LBA Bioanalytical Focus Group (LBABFG) following a hot topic session on this subject at the AAPS National Biotechnology Conference in 2008. Since then, multiple scientific presentations on this topic have been given at many different conferences. In addition, a growing number of publications have appeared on this topic in recent years, including detailed case studies and review articles as well as a consensus-based 'AAPS White Paper' that resulted from discussions of the LBABFG [3,4]. However, due to the complexity of the issue, it may not be realistic to have a 'one-size fits-all' bioanalytical strategy for all PK/PD and safety evaluations. This partially results from the technical challenges in designing and developing assays that measure only one molecular species (e.g., free or bound or total therapeutic species). Assay data are easily confounded by sample handling, dilutions, reagent limitations and so on. In addition, the lack of information on what specific data are actually needed to inform PK/PD and safety evaluations for a particular program also poses another challenge in formulating bioanalytical strategies [5]. For example, when only one PK assay (free or total assay) can be developed to measure drug concentrations due to limited resources, data from a free PK assay may be more informative for one project, while data from a total assay may be more informative for another project. Going into a new biotherapeutic development program, we do not typically know *a priori* if there is likely to be a difference between data from a free versus a total biotherapeutic drug assay. This is because the information on the target concentrations, especially after drug treatment, is not available prior to the development of a bioanalytical assay [6]. From the overall bioanalytical assay strategy point of view, it is also important to consider the development stage of a particular program because bioanalytical data usage may differ for preclinical and clinical studies and in support of a second-generation drug. As a result, the development stage of a molecule may affect the overall bioanalytical assay strategy. Given limited resources, it is often challenging to know what assay may be the most appropriate for a particular drug-development program. Information in the literature is scant on such assay comparisons and on the impact of the analyte selected for analysis on PK/PD and safety evaluation conclusions, which makes it hard to generalize from one particular case study.

"Given limited resources, it is often challenging to know what assay may be the most appropriate for a particular drug-development program."

It is therefore very helpful for end users of bioanalytical data such as PK scientists and toxicologists to work together with bioanalytical scientists to assess the needs, technical feasibility and challenges of developing suitable bioanalytical assays in order to form a sound bioanalytical strategy. In addition, such discussions will facilitate data interpretation and help our PK/PD and safety assessment colleagues understand the limitations/caveats that are associated with this type of bioanalytical data. Thus, bioanalytical support of drug development will become more context driven and bioanalytical data should enable more informed decision making. It is worth pointing out that although there have been some significant advances in our knowledge regarding free versus total LBAs, it is still every bioanalytical scientist's dream that one day, sensitive and specific assays that are 'immune' from various interferences (e.g., those caused by sample handling and reagent limitation) could be readily developed. This means that we will need to significantly improve (or disruptively innovate beyond) current bioanalytical technologies. This discussion on free versus total LBAs is also expected to motivate analytical technology vendors to work more closely with their drug-development customers. Finally, we hope that a constructive and robust dialog on this topic will help to foster a collaborative environment for drug-discovery and -development scientists, regulatory agencies and instrument/technology manufacturers. This should help us all to speak the same (bioanalytical) language, understand each others needs and challenges, and, ultimately, develop new, efficacious and safe drugs that benefit the society that we are all a part of.

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Accurate measurement of free analyte using Gyrolab® platform vs ELISA

Application Note D0044111/A

Abstract:

As the free concentration of a biotherapeutic is theoretically responsible for its pharmacological effect, the importance of measuring free analyte in complex matrices during preclinical and clinical studies is undisputed. However, quantification using immunoassays present inherent technical challenges that can result in overestimations. Incubation steps that can disrupt the equilibrium of free and bound analyte are the main source of error and can vary with the assay technology used. In this study, the free concentrations of biotherapeutics Avastin® (bevacizumab) and Lucentis® (ranibizumab) were measured using Gyrolab immunoassays where samples have very short contact time with capture reagent and ELISA with extended incubation time for analyte binding. Overestimations were observed for Avastin using ELISA, where the complex $t_{½}$ is about the same as the incubation time in the assay, but not for Gyrolab® platform, where the capture reagent exposure time is very short.

Introduction

Measurement of free analyte in serum samples during preclinical or clinical studies is an essential step to understanding the pharmacological effects of a drug in the body, the binding to its ligand, and the effective drug concentrations. Measurement of free analyte is complicated by the multiple forms of free analyte and analyte bound to its ligand that can exist, with an equilibrium between the forms (Figure 1). One challenge of ligand-binding assays (LBAs) that are often utilized in free analyte measurement is the potential for an equilibrium shift to occur during the assay, leading to an overestimation of free analyte (Figure 2). Ligand-binding assays consist of a capture ligand to bind the biotherapeutic in solution, followed by addition of a detection reagent to measure the free analyte captured during the assay. To accomplish this, LBAs typically require incubation and during these incubation times, the equilibrium may be shifted, producing additional free analyte. The additional free analyte is also captured, ultimately resulting in an overestimation of the true free analyte in the unperturbed sample.

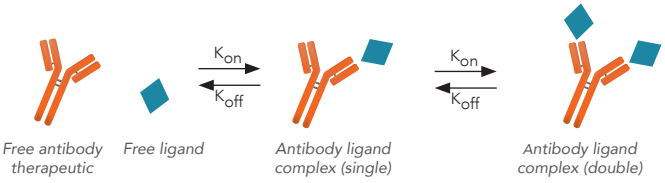

Figure 1. Multiple forms of free analyte and analyte bound to its ligand. Antibody therapeutics are in equilibrium with their ligand in solution, with on and off rates for the binding steps (k_{on} and k_{off}) determining the relative concentrations of each form.

Another factor affecting measurement of free analyte is the affinity of the capture ligand to the antibody therapeutic, and the dissociation time of the complex to produce free interactants, or $t_{½}$. (Figure 3). In short, reactants A and B can form the complex AB, which can also dissociate to release its components. The rate constants for this reaction are k_{on} or the association rate constant and k_{off} or the dissociation rate constant where $t_{½}$ represents the time it takes until 50% of complexes have dissociated. When $k_{off} = k_{on}$, equilibrium has been reached.

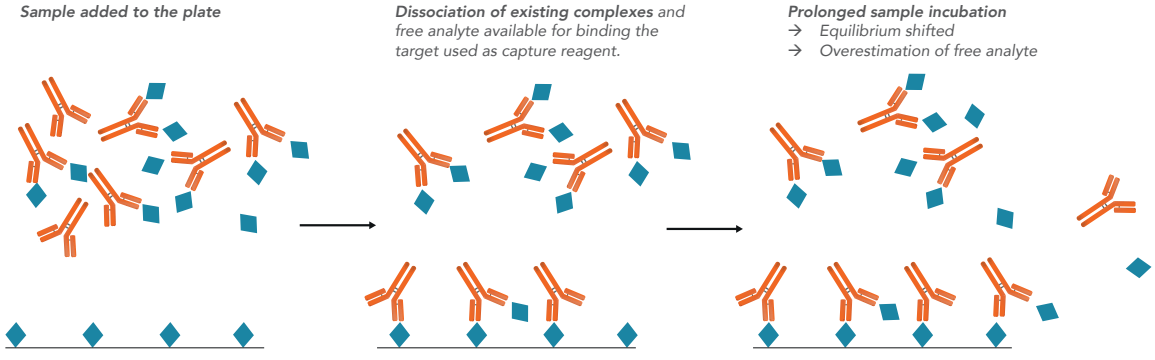

Figure 2. Schematic representation of an equilibrium shift during the sample incubation step of a plate-based ELISA.

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$$A + B \underset{K_{off}}{\overset{K_{on}}{\rightleftharpoons}} AB \qquad t_{\frac{1}{2}} = \frac{In2}{k_{off}}$$

k_{on}	Association rate constant
k_{off}	Dissociation rate constant
$t_{½}$	Time for 50% of complexes to dissociated
$k_{on}=k_{off}$	Equilibrium reacheded

Figure 3. Effect of affinity on measurements of free analyte. Affinity, as determined by k_{on} and k_{off} rates, determines the overall strength of a binding interaction.

The $t_{½}$ values come into play in LBAs and measuring free analyte during sample incubation. If the assay incubation time is greater than, or in the range of $t_{½}$, overestimations of free analyte can occur, since complexes are actively dissociating while free ligand is binding to the capture reagent in the assay.

The goal of this study was to compare two assay technologies, Gyrolab immunoassays and ELISA, for the accurate measurement of free analyte for complexes with short and long dissociation times using two biotherapeutics binding the same ligand, Avastin® (bevacizumab) or Lucentis® (ranibizumab) with vascular endothelial growth factor (VEGF). The VEGF ligand plays an important role in pathological tumor angiogenesis when overexpressed, generating tumor growth and metastatic spread. Avastin inhibits tumor growth by blocking its blood supply, and is FDA approved for use in several different cancers. The VEGF ligand also is thought to play a role in the development of neovascular (wet) age-related macular degeneration (AMD), and Lucentis is approved for this indication. Avastin is a recombinant, humanized, monoclonal antibody, and Lucentis is an affinity-matured Fab region that was derived from Avastin.

Materials and Methods

Description of Gyrolab and ELISA assay systems

Gyrolab immunoassays are fully automated utilizing capillary and centrifugal force for liquid movement through microfluidic compact disks (CDs). The CD consists of structures with hydrophobic barriers, volume defining chambers, overflow channels, and affinity capture columns on nanoliter scale (Figure 4). Use of hydrophobic barriers that "break" when the discs are spun at a certain speed along with volume defining chambers provide highly reproducible volumes that are not affected by the pipetting of samples onto the CD. The immunoassays are built on affinity columns filled with streptavidin-coated particles to which biotinylated capture reagents bind and the assay is automated by the flow of samples and reagents over this column to build the immunoassay, resulting in very short contact times between the sample and the affinity column. Fluorescent-labeled detection antibody binding is automatically read by an internal laser, producing results at the end of the run.

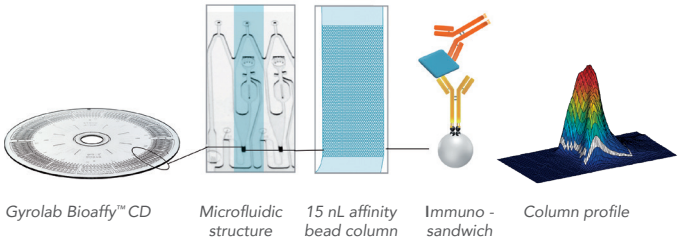

Figure 4. Principle of a Gyrolab assay. Using centrifugal force and capillary action with proprietary microfluidic technologies, samples are guided through a microstructure with a nano-column packed with streptavidin beads. An immunosandwich is built using a biotinylated capture and Alexa Fluor® labeled detection reagent. Each microstructure generates one data point that can be visualized as a fluorescent 3D-binding profile.capture and Alexa Fluor labelled detection reagent. Each microstructure generates one data point, that can be visualised as a 3D-binding profile.

The Gyrolab immunoassay workflow is described below as essentially 3 steps:

Dilute reagents, samples and prepare buffers → Load reagents, samples and buffer onto the microplate → Load microplate onto Gyrolab, start the run

For the Gyrolab immunoassay, biotinylated VEGF was bound to the streptavidin-coated column, and the fluorescent-labeled detecting antibody was bound to Avastin or Lucentis analyte (Figure 5). The assay design was similar for ELISA except that ELISAs are conducted on the solid surface of a microplate, with the capture reagent bound directly to the well bottom. A fixed concentration of Avastin or Lucentis was mixed with different concentrations of VEGF.

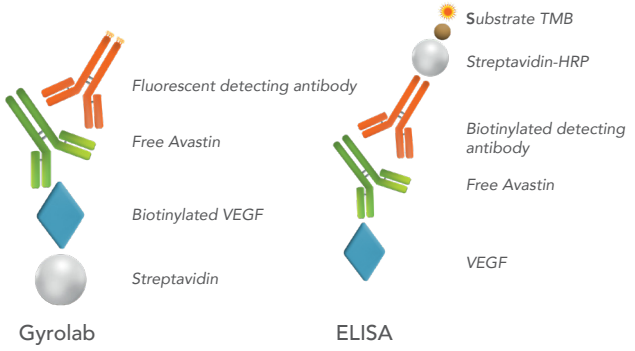

Figure 5. Assay formats used for quantification of free Avastin. The target protein VEGF was used as capture reagent and an anti-hulgG was used as detection reagent.

For ELISAs, the detecting antibody was biotinylated since the enzyme horseradish peroxidase (HRP) was conjugated to streptavidin which binds to the biotinylated detecting antibody. The ELISA substrate, tetramethylbenzidine (TMB), reacts with HRP, generating a specific color at a specific wavelength for detection.

While ELISA assays are commonly used, they are not automated and require a series of reagent additions and washes as in the example shown in Figure 6, compared to the minimal manual steps of an automated Gyrolab assay.

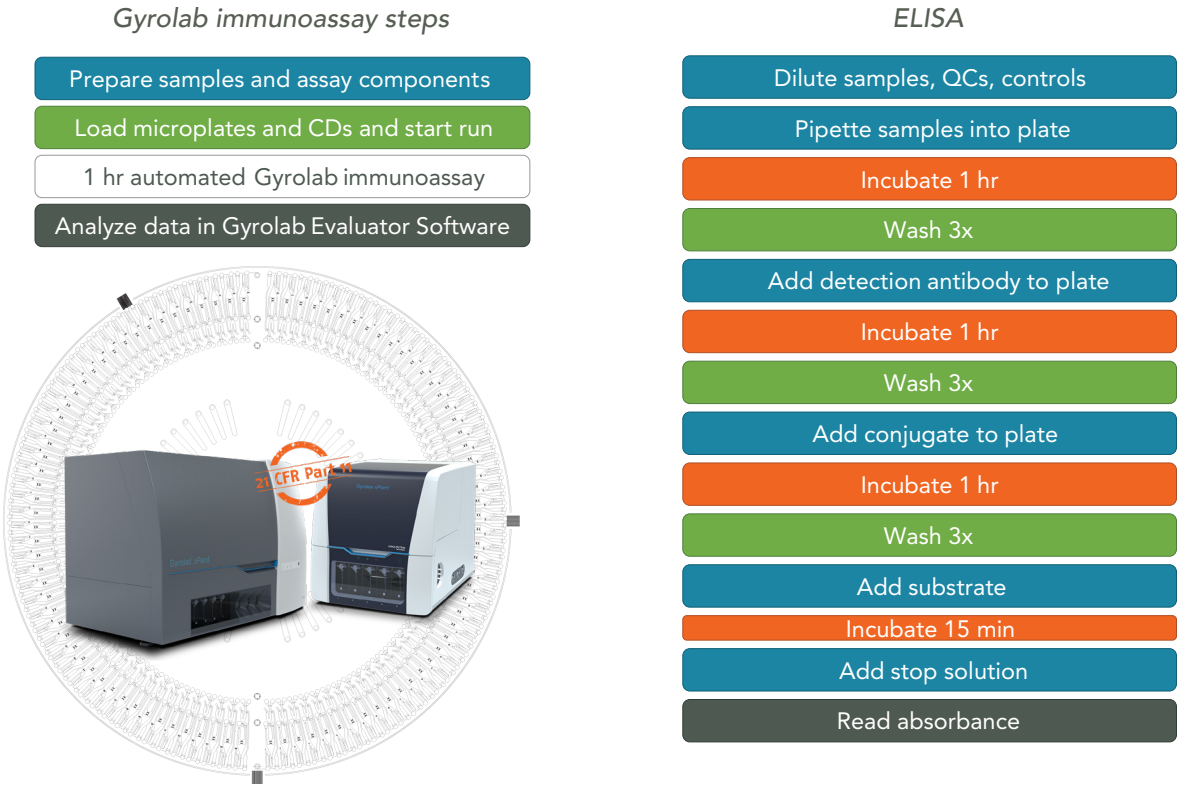

Figure 6. Manual steps and incubation times for ELISA and Gyrolab immunoassays. Manual pipetting and plate manipulation steps are shown in blue and green, respectively, and incubation steps in orange.

Sample preparation

Free analyte was measured by preparing samples with fixed concentrations of the drug (Avastin or Lucentis) and titration of the ligand (VEGF). The samples were incubated offline to reach equilibrium and were then measured, with triplicates, on both Gyrolab and ELISA, in parallel. The preparation and dilution of the samples was done in the same manner with the same dilutions and volumes for Gyrolab and ELISA, to achieve conditions that were as similar as possible.

For Avastin assay optimization, it was determined to use a sample (drug + ligand) pre-incubation time of 24 hours at +4 C˚, since there was no significant difference in the measurement of free analyte after 24 hours. These same sample pre-incubation times were used for Lucentis. The ELISA requirement for incubation of the sample in the assay microplate for analyte binding can vary but are typically 2 hours. For this study, both 2 hour and 4-hour assay incubation times were chosen.

Results

Assay conditions used for the Gyrolab and ELISA assays:

Assay format	Capture	Capture conc.	Analyte	Detect	Detect conc.	CD or dilution*
Gyrolab	bVEGF	6.6 µg/mL	Avastin	H2	20 nM	Bioaffy 1000 HC
Gyrolab	bVEGF	13.3 µg/mL	Lucentis	kLC	10 nM	Bioaffy 1000 HC
ELcISA	VEGF	1 µg/mL	Avastin	bH2	0.13 nM	1:1000 dilution
ELISA	VEGF	2.5 µg/mL	Lucentis	bkLC	6.7 nM	1:1000 dilution

*dilution of streptavidin-HRP detection reagent

H2, α-human IgG Fc monoclonal antibody H2; kLC, α-human IgG Kappa light chain monoclonal antibody SB81a; bH2, biotinylated α-human IgG Fc monoclonal antibody H2; bkLC, biotinylated α-human IgG Kappa light chain monoclonal antibody SB81a

The measurement of free Avastin for different ratios of Avastin:VEGF with Gyrolab and ELISA (2 hr and 4 hr incubation) are shown in Figure 7. Overall, free Avastin measurements using ELISA were greater than with Gyrolab assay measurements. ELISA with four hours of sample incubation measured the highest concentrations of free Avastin.

Free Lucentis measured with ELISA (2 hr and 4 hr of sample incubation) showed almost no difference in free Lucentis compared to the Gyrolab measurement, as can be seen in Figure 8. In addition, there was almost no difference between 2 and 4 hr of sample incubation on the ELISA measurements.

These results point to assay equilibrium not shifting with Gyrolab assays due to short contact time with the affinity column (about 6 seconds). For ELISA, the results show shifted equilibrium and overestimation of free analyte since ELISA incubation time (2 hr) > Avastin $t_{1/2}$ (approximately 1 hr). For Lucentis, almost no difference in measurement of free analyte due to stronger affinity and approximately 1 day $t_{1/2}$ for Lucentis. Equilibrium does not shift even after long incubation since incubation time << $t_{1/2}$.

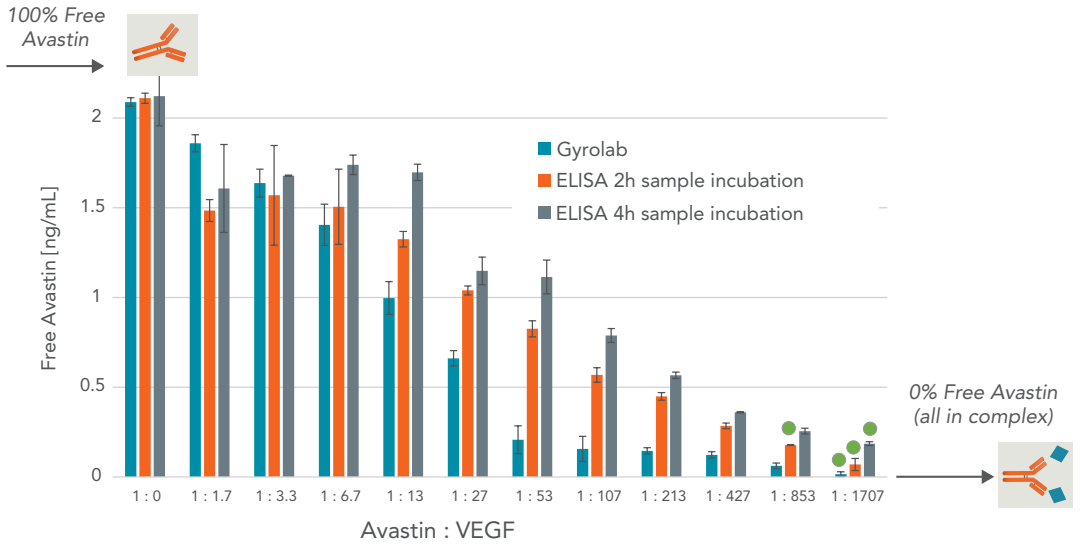

Figure 7. Free Avastin measured at different ratios of Avastin:VEGF, with a standard deviation for each mean value. Gyrolab data in teal, ELISA with two hours of sample incubation in orange, and ELISA with four hours of sample incubation in grey. Uncertain values are marked with a green dot. Gyrolab data in teal, ELISA with two hours of sample incubation in orange, and ELISA with four hours of sample incubation in grey. Uncertain values are marked with a green dot.

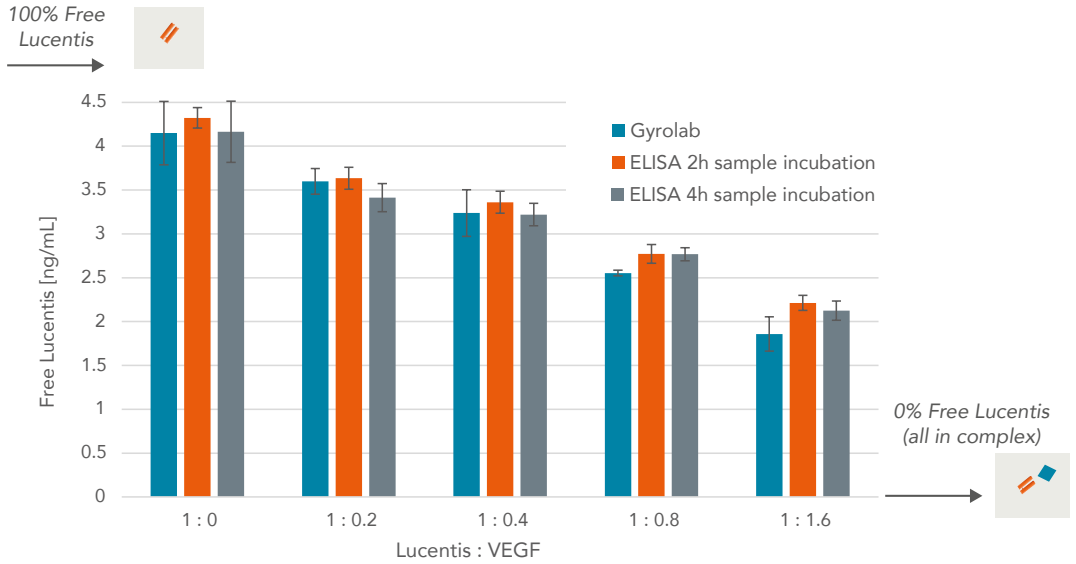

Figure 8. Free Lucentis measured at different ratios of Lucentis:VEGF, with a standard deviation for each mean value.

Discussion and Conclusions

A substantial difference (up to 4-fold) in measured free analyte between Gyrolab immunoassays and ELISA was observed for Avastin. Notably, the Avastin $t_{½}$ was equivalent (approximately 30 – 400 minutes [1-4]) to the ELISA incubation time (2-4 hr), potentially allowing active equilibrium shifts towards free analyte during the assay. The incubation time with the Gyrolab affinity column is negligible (around 6 seconds), minimizing any equilibrium shifts and presumably providing a more accurate quantification of free analyte.

However, for Lucentis, the free analyte measurement differences between ELISA and Gyrolab immunoassay were not significant, likely since the $t_{½}$ of this complex is much longer (~1 day) compared to the binding incubation time for either method. The sample incubation time for ELISA would be shorter than the time to produce significant dissociation of complexes, so the measurement of free analyte is not overestimated or affected by incubation times of 2-4 hrs.

These results taken together show that depending on the affinity of the analyte and capture reagent binding pair, overestimations of free analyte from longer incubation times can occur when measuring free analyte since the equilibrium can be shifted during sample incubation, especially where the $t_{½}$ is less than or equivalent to the incubation time in the assay.

This study reinforces the importance of selecting the right analysis method for free analyte measurement considering the effect of incubation times and complex dissociation times on shifting equilibria in immunoassay incubations. These results support the conclusion that Gyrolab immunoassays, with extremely short (seconds) exposure of sample to the affinity column for binding during the capture step, are likely to provide the best measurement of free analyte without shifting the equilibrium leading to an overestimation. Supporting this advantage, Gyrolab immunoassays measuring free complement component 5 (C5) levels are utilized in pharmacodynamic studies for ravulizumab, a recently approved long-acting C5 inhibitor [5,6].

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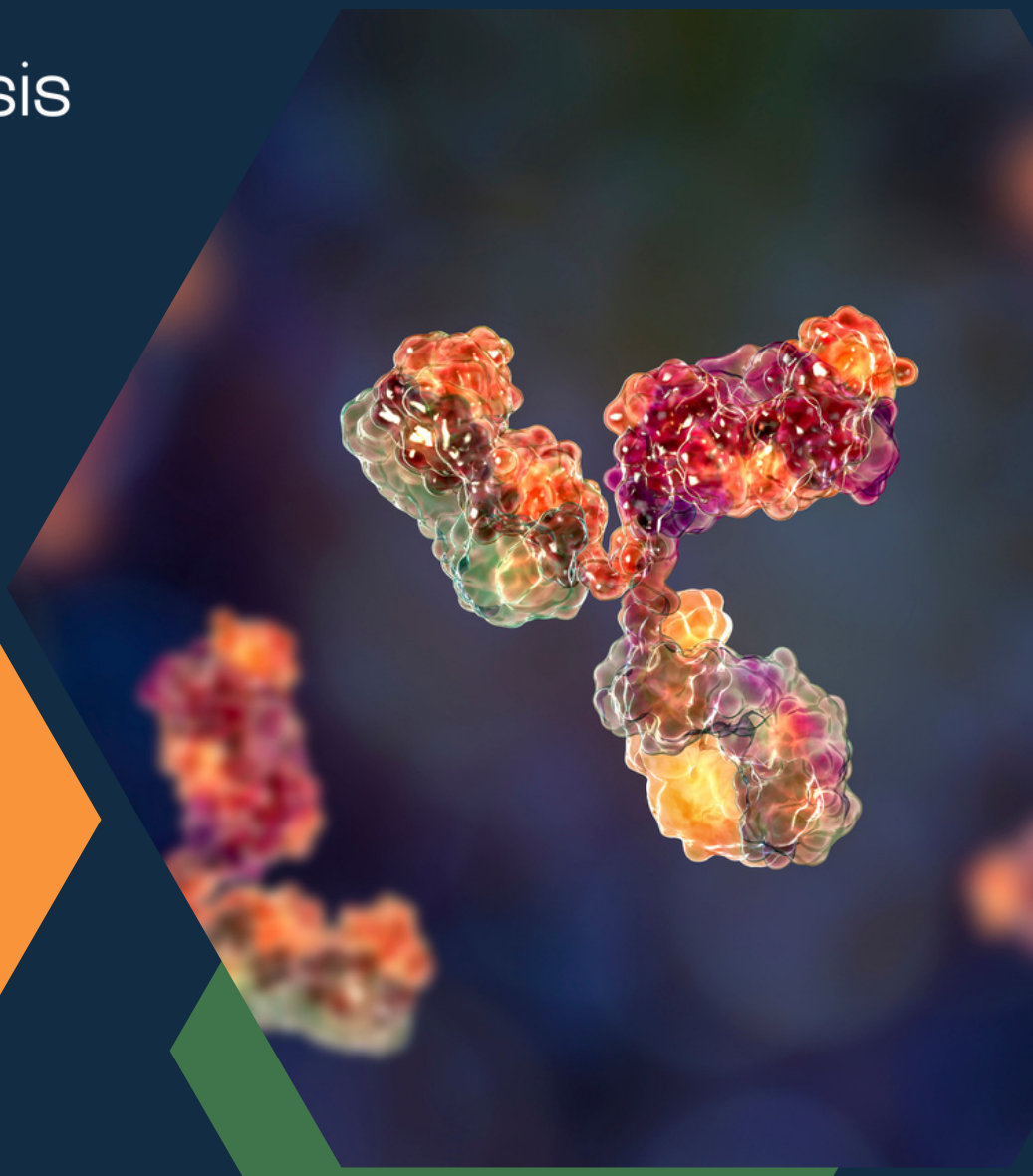

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