14 Ligand-Binding Assays

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14.1 SUMMARY

Ligand-binding assays (LBAs) or immunoassays are the analytical method of choice for pharmacokinetic (PK) and immunogenicity assessment for biopharmaceuticals. As pipelines for this class of drugs are expanding, LBAs are revisited to address current scientific questions with this classic technology. Although immunoassays come in a vast variety, they share some similar concepts. Typically, the analyte of interest is captured out of a mostly unpurified solution using specific antibodies or receptors. Bound analyte is then detected with a labeled reagent either in a competitive or a sandwich format.

Since their earliest inception for the measurement of insulin, immunoassays rely on antibodies specific to the analyte of interest. These days, different capture and detection reagents are often used, for example, two different antibodies or a combination of target and antibody. In any case, reagent specificity, selectivity, and quality are keys to a successful LBA. Monoclonal antibodies and other recombinant technologies allow the generation of custom reagents to meet the requirements of most LBA methods.

Some advantages of immunoassays include their limited cost per sample, sensitivity, and lack of need for extensive sample purification. Disadvantages are the indirect readout that is inherent in the method, long lead times for optimal reagent generation, and the potential for interferences by the sample matrix, in particular, body fluids.

While the basic immunoassay schematic remained the same, recent technologic advancements addressed some of the downsides. Meso Scale Discovery (MSD)-based assays are now widely accepted for immunogenicity assessment and also make their

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way into PK assessment. They potentially have lower background and less matrix interferences than traditional enzyme-linked immunosorbent assays (ELISAs). Gyrolab also has potentially lower matrix interference, while in addition, it is fully automated to reduce the need for extensive operator training. Furthermore, mass-spectrometry-based methods have undergone significant improvement in the recent years. Although no clinical application of mass spectrometry to clinical biopharmaceutical PK/PD (pharmacodynamic) analysis has yet been published, numerous feasibility studies indicate a potential future direction of the field.

Caution should be applied when comparing or extrapolating immunoassays on the basis of different formats or technologies. As they will give different results, a thorough understanding of the bioanalytical background is necessary. Bioassays focus on a functional drug, while mass-spectrometry-based assays only measure a short signature peptide. Antibody therapeutics, for example, can be measured as free, total, or partially target-engaged drug and possibly a combination thereof. The assay itself might influence the balance of these forms as well. Soluble targets are particularly challenging since their serum levels are fluctuating and they remain a part of the sample matrix in the assay.

Toxicology and clinical applications require thorough method validation. So far, no FDA guidance specific to large molecules has been published. However, the general bioanalytical guidance in combination with a series of white papers should be applied to LBAs. Many immunoassay validation parameters are similar to mass spectrometry. Some key differences include the appreciation of less accuracy and greater variability, as well as the need for more extensive selectivity testing. Applicable clinical patient matrices should be kept in mind rather than an exclusive focus on a healthy population during validation. Also, reagent lot qualification and bridging is essential in maintaining a bioanalytical.

PK and immunogenicity of biopharmaceuticals are assessed using LBAs. PK assay formats are dictated by scientific requirements as well as compound biology and program phase. “Generic” human IgG assays relying solely on commercial reagents may be deployed during a basic research stage, while drug-specific assays may be needed for toxicology and clinical applications. Immunogenicity screening assays, on the other hand, typically use a common bridge format with labeled drug, although confirmation assays can display a greater variety.

Despite or because of its age and low technology, immunoassays in their vast variety continue to shape the development of modern biotherapeutics into the foreseeable future. During recent years, advances in mass spectrometry and recombinant technologies demonstrated the feasibility for these methods to replace immunoassays in the long run. However, more method development work and general scientific acceptance are needed. Until then, LBAs will be inseparable from biopharmaceutical development.

14.2 INTRODUCTION—NEW LIGHT THROUGH OLD WINDOWS

During the last three decades, the advances in molecular biology, immunology, and protein biochemistry have led to the rise of a new class of drugs, namely, biopharmaceuticals, often referred to as *biologics* [1]. While traditional pharmaceuticals are of low molecular weight (100–500 Da) and chemically synthesized, biopharmaceuticals are high molecular weight proteins or peptides that are produced in living cells. Owing

to their structural complexity and size, these molecules cannot be readily analyzed by mass spectrometry methods. Up to now, immunoassays have been the method of choice to study the PK and immunogenicity of biopharmaceuticals.

The application of immunoassays to peptide bioanalysis has a long history that goes back to the first uses of animal-derived insulin [2]. Yalow and Berson [3] developed a competitive insulin radioimmunoassay (RIA) method in the 1960s, which was awarded the Nobel Prize in 1977. Their breakthrough demonstrated the feasibility of endogenous hormone quantification at very low concentrations [4]. The first immunoassays used polyclonal antibodies purified from human or animal serum, resulting in limited assay flexibility and reagent availability [2,3]. The monoclonal antibody technology developed by Köhler and Milstein [5] (Nobel prize in 1984) enabled the generation of highly specific, custom-made antibodies with virtually unlimited supply. As a result, antibody use proliferated in research and diagnostics immunoassays, as well as in development as therapeutic agents.

PK measurements of biopharmaceuticals rely on LBAs with high sensitivity and specificity. Antibodies specific to the drug enable quantification down to pg/mL levels in crude serum or other matrices without prior enrichment, thereby potentially reducing technical artifacts. Despite some limitations that are discussed in the following chapters, LBAs still enjoy widespread scientific and regulatory acceptance. Potential future trends including coupling to liquid chromatography-mass spectrometry (LC-MS) will be discussed.

A schematic applicable to most immunoassays is depicted in Fig. 14.1. Although competitive RIAs were the first format developed (Section 14.3.1), today dual-detection assays are the most common. They employ either two antibodies for capture and detection or a biological target in combination with a detection antibody [6]. As there are numerous variations on these themes, we have not discussed all possible scenarios. A common feature of all immunoassays is the need for a detection label. Initially, radiolabels were used allowing for unparalleled sensitivity. Later, enzymes such as horseradish peroxidase replaced the radiolabels [7–9], and most recently, acridinium, ruthenium, and lanthanide labels became available [10,11]. Further details on these methods are discussed later.

14.3 FROM HISTORY TO FUTURE—TECHNOLOGIES

As outlined above, a variety of LBA formats have been developed. Beyond their obvious differences, they share the same principle: an analyte-specific capture (antibody, target, or complementary peptide) is used to immobilize the drug of interest from a solution (matrix); for drug metabolism studies, typically plasma/serum or similar biological fluids are used as applicable. The immobilized drug is then detected with a second labeled analyte-specific reagent, which is either the labeled drug itself or, more often, another antibody. Depending on the assay format, wash steps are used to remove unbound substances.

Since biological matrices are not purified before application in the assay, the selection of appropriate reagents is key to method performance [12,13]. In particular, capture reagents must specifically recognize the analyte of interest among many endogenous proteins. Especially, closely related proteins can cause nonspecific signals that cannot be distinguished from specific binding [14]. If wash steps are used to remove unbound

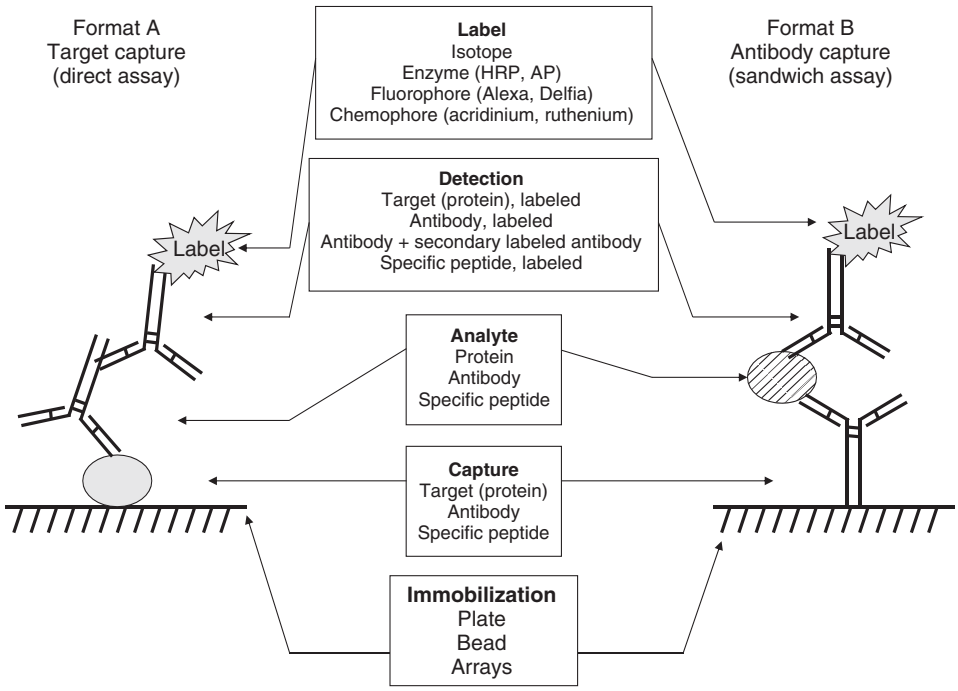

Figure 14.1 Schematic representation of ligand-binding assays. Format A: target-mediated capture with antibody detection. Format B: antibody-mediated capture and detection. Alternative reagents that can be used are listed for each step. The use of secondary anti-species antibodies or labeled streptavidin detection systems has been omitted. HRP, horseradish peroxidase; AP, alkaline phosphatase.

substances, detection can be less specific. For example, anti-species antibodies are often used to detect bound antibodies. When working with samples from the same species as the antibody of interest (e.g., when detecting human therapeutic antibodies in human serum), other antibodies in the serum sample may become nonspecifically immobilized and not completely wash away. The nonspecifically immobilized antibody will be detected and will result in higher background.

As a general rule, decreasing the background improves assay sensitivity if signal/background ratios increase. Close attention should be paid to reagent purity, as contaminating proteins can result in higher assay background or increased variability. Unpurified antisera often contain antibodies that can bind "nonspecifically" in the assay. As greater specificity and affinity for both capture and detection will result in superior assay sensitivity, immunoaffinity purification is recommended for polycloncal antibodies when high background is encountered. In addition, optimization of blocking and assay buffer composition can provide reduced background and improved signal to noise ratios.

The selection of antibodies is dependent on the method's objectives. Polyclonal antibodies tend to offer high affinity and are less affected by slight changes in the analyte [15]. Monoclonal antibodies bind to the molecule only in a single epitope and may not bind if the epitope is altered. This can also be used as an advantage to measure full-length molecules with antibodies binding at either end (alternatively,

polyclonal antibodies generated against subunits of the analyte of interest could be used). Monoclonal antibodies can be produced without changes for decades, making them ideal for long-running methods. When analyzing antibody therapeutics, anti-idiotype antibodies are often used for highest specificity and improved assay sensitivity [16,17]. These antibodies bind to the antigen-binding site of the therapeutic antibody of interest. The downside of using anti-idiotype-specific antibodies and other custom antibodies is the long lead time for immunization and production.

Besides full-length, "natural" antibody reagents, numerous alternatives have been deployed for either capture or detection of analytes of interest. Advances in antibody engineering allowed for molecular design of recombinant antibodies and for phage display-derived antibodies [18,19]. Receptors have successfully been used in immunoassays, mostly for capture but sometimes also for detection [10]. Lately, aptamers have been tested in LBAs [20]. These short oligonucleotide or peptide sequences are generated to bind to a target molecule of interest similar to antibodies. They can be selected and produced relatively quickly, but their applications to the bioanalysis of therapeutics are very limited [21].

14.3.1 Radioimmunoassay (RIA)

Although immunoradiometric assays (IRMAs) employing sandwich formats have been developed [22–25], classical RIAs are competitive assays (Fig. 14.2) [26,27]. The analyte of interest is captured from biological fluids by a specific antibody. Radioactively (^{125}I) labeled analyte is added to compete with the unlabeled drug for binding to the capture antibody. The immune complexes are then precipitated, typically by the addition of protein A or antiantibody-coated beads, although other methods such as ammonium sulfate precipitation have been used. After pelleting the immune complexes by centrifugation, excess liquid, including unbound tracer, can be removed and retained radioactivity can be measured in a scintillation counter. The amount of radioactivity is inversely proportional to the analyte concentration in the sample.

Nowadays, most RIAs have made way for nonradioactive methods described in the following paragraphs. However, they still have their place when high sensitivity is required, for example, to quantify peptides and hormones [28,29]. For historic reasons and in order to achieve greatest sensitivity, RIAs are still used for PK bioanalysis of insulin compounds such as glargine [30] and in clinical chemistry laboratories. Besides traditional RIAs, IRMAs employing two antibodies were published as well for bioanalysis of insulin and insulin analogs [23].

14.3.2 Enzyme Immunoassay (EIA)

Although the nomenclature is used interchangeably throughout the literature, EIAs (enzyme immunoassays) and ELISAs are slightly different: EIAs are a direct homogenous format translation of RIAs into a nonradioactive setting. The radiolabel is replaced by an enzyme such as alkaline phosphatase or horseradish peroxidase, followed by a signal-producing chemical reaction (colorimetric, chemiluminescent, or fluorescent). ELISAs are heterogeneous with an immobilized capture phase (Fig. 14.2). After the analytes of interest are bound, the remaining sample is washed off and a second analyte-specific antibody is used for detection. The enzyme label can be conjugated directly to the detection antibody, to an anti-species antibody, or to streptavidin if a biotin label

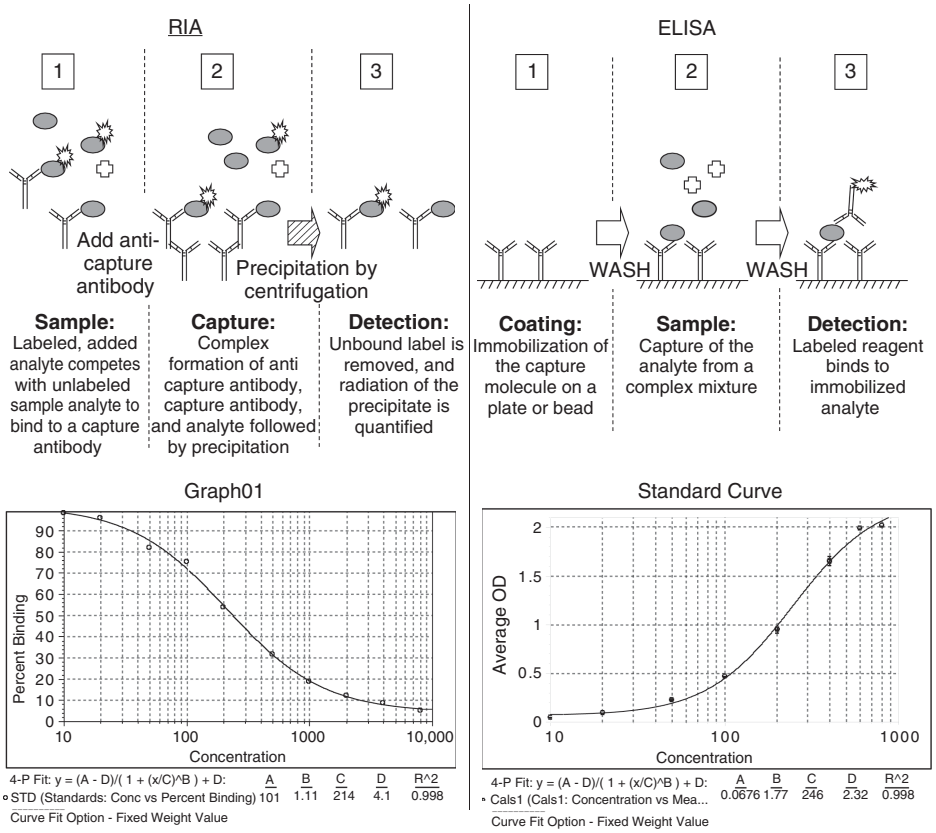

Figure 14.2 Schematic comparison of RIA and ELISA methods with example calibration curves: RIAs are competition assays. A single analyte-specific reagent (antibody) is used to capture the compound of interest. Labeled compound at a fixed concentration is added to compete with the analyte. The immune complexes are precipitated, and radiation is quantified in the pellet. ELISAs use an immobilized capture phase. Analyte is captured from the sample and detected with a second, labeled reagent. Each step is separated by washes.

is used. There are numerous variations on this basic theme, which are beyond this chapter's limits. Even though the strict use of the name ELISA implies the use of an enzyme, alternative labels can be applied, including nonenzymatic fluorophores and chemophores. Often these are referred to as *ELISAs,* although the proper nomenclature is "fluorescent immunoassay" (FIA) and "chemiluminescent immunoassay" (CLIA), respectively.

ELISAs are the most common format for large-molecule PK applications [31], with wide acceptance by regulatory agencies. Its overall simplicity and flexibility with respect to labels and procedures allow implementation in virtually any laboratory environment. However, some shortcomings exist: signal development is dependent on enzyme kinetics that is influenced, among other factors, by temperature, mixing, and substrate feedback inhibition. These are partially compensated for by the use of calibrator curves included on each assay plate, but higher data variability still is higher than that of small-molecule mass spectrometry methods. Also, the dynamic range is

rather limited even with excellent reagents. Therefore, PK study samples often have to be extensively diluted to be measurable within the assay range, introducing additional potential for analytical error. Newer technologies circumvent some of these limitations and are discussed below.

14.3.3 Other Assays

A number of immunoassay-based technologies became available in recent years. These include the following techniques.

14.3.3.1 Electrochemiluminescence. Often abbreviated as "ECL" (not to be confused with the enzyme substrate), electrochemiluminescence was commercially developed by Igen and incorporated into Roche's line of diagnostic immunochemistry analyzers. MSD made the technology more broadly available for research laboratories. The basic format is similar to ELISA. However, the enzyme is replaced with a ruthenium label that is used to generate light on application of electric current. Special plates are required to facilitate the electric charge. Advantages of MSD assays include lower background and larger dynamic range than ELISA, thus potentially allowing for improved assay sensitivity. Enzyme-related variability is reduced as well. Since the signal reaction takes place only at the immediate proximity of the electrode, homogenous assays often can be developed without the nonspecific background issues of conventional ELISA [32,33]. The major disadvantage is the limited commercial choice, as MSD is the only supplier. However, MSD-based assays have found widespread acceptance for immunogenicity assays and increasing applications for PK assays [34].

14.3.3.2 Gyrolab. The Gyrolab (Gyros AB, Sweden) nanoscale assay platform transferred a standard fluorescence-tag immunoassay into a compact disk (CD). The entire assay only requires 5–10 μL of sample or reagents and is fully automated. Briefly, biotinylated capture molecules are immobilized in flow-through streptavidin columns, followed by sample and a fluorophore-labeled detection antibody. Each column corresponds to a well in traditional plate-based assays. The run time per CD is ~50–60 min, but shorter runs can be accomplished using partial CDs. Advantages and disadvantages are similar to MSD assays. The small sample and reagent volume requirements as well as the automation are additional benefits of Gyrolab. We have used Gyrolab-based assays extensively in the lead identification and optimization space for PK measurements for over 14 drug discovery programs. The small sample volumes allowed for serial bleeds from transgenic mice, which are often too costly and limited to use in terminal bleed PK studies. Optimization of assay conditions was routinely finished within one to two days, and troubleshooting of any issues could be carried out with multiple runs in a single day.

14.3.3.3 Biacore. Surface plasmon resonance (SPR) is coming of age to study molecule interactions. Capture molecules are immobilized on a chip that allows detection of subsequent binding events individually through SPR effects. Wash steps are limited, and no labels are necessary. Biacore is routinely used to screen and characterize antibody reagents but has not found widespread acceptance for routine sample analysis because of limited throughput, high costs, and complex technology. Inherent

to SPR technology is limited sensitivity, particularly when studying high affinity interactions. Recently, Biacore became an important tool for immunogenicity assessment of large molecules, as it can detect low affinity anti-drug IgM that are washed away in traditional ELISA and sometimes even MSD (Section 14.7.2) [35,36].

14.3.3.4 Immuno Polymerase Chain Reaction (Immuno-PCR). This combination of traditional immunoassay and molecular biology attempts to harvest advantages from both worlds: protein-based detection and high sensitivity [37,38]. In this case, the detection antibody is labeled with an oligonucleotide that can be amplified via PCR. The immunosandwich capturing the analyte of interest is assembled following a standard immunoassay protocol. However, visualization and quantitation are accomplished by a subsequent PCR amplification of the oligonucleotide label. A number of variations have been developed on this theme, including commercial technologies [37]. Assay sensitivity, low background, and a large dynamic range are obvious advantages of immuno-PCR. On the other hand, the assay process is still rather complex and requires well-trained staff. Automated technologies are being developed to make daily applications more feasible. While there are a number of diagnostic applications, only a few PK assays are based on immuno-PCR [39].

14.3.4 Homogenous Assays

The adsorption of proteins to solid surfaces changes their tertiary and quaternary structures, potentially affecting interactions with other molecules in the assay [40]. Cancer antigen EpCAM II, for example, contains a discontinuous immunogenic epitope that is disrupted when coating the antigen directly onto microtiter plates (Verch unpublished data). Other limitations of solid-phase assays include wash steps and lengthy incubation times, making complete automation difficult. Some traditional plate-based technologies are amendable to semihomogenous formats, in particular, MSD assays or bead (flow) systems such as Luminex. Most homogenous assays, however, are performed entirely in solution in a single step without any washes. Different technologies are based on similar principles. Capture and detection molecules are labeled with complementary donor and receptor tags. On formation of the immunosandwich, energy transfers between the tags results in the generation of light or fluorescence (Fig. 14.1). Several technologies have been developed to optimize the light generation, including fluorescence resonance energy transfer (FRET), homogeneous time-resolved fluorescence (HTRF) [41], and, lately, AlphaLISA [42]. The close proximity necessary to achieve the energy transfer made widespread FRET and HTRF applications difficult, while AlphaLISA allows more flexible and larger immunosandwiches. The signal is based on chemiluminescence, which results from a cascade of several dyes [43]. Although there have been limited publications on AlphaLISA in the PK field, the technology is closely watched for its promising potential.

14.3.5 LC-MS for Proteins

Owing to their large size, proteins cannot readily analyzed by LC-MS-based methods. Large amounts of other proteins such as albumin that are present in preclinical and clinical matrices and often have high homology to the drug add to the bioanalytical complexity. However, LC-MS methods have evolved to potentially address some of

the disadvantages of ELISA [44,45]. Table 14.1 compares immunoassays to mass spectrometry methods. As a key difference, immunoassays can potentially measure entire functional molecules, while LC-MS focuses on peptide signatures after tryptic digests. If selected appropriately, these peptide signatures are unique to the analyte of interest in contrast to signals in immunoassays in which nonspecific and specific binding events are indistinguishable. While the selection of representative peptides out of a digest pool (billions of possible antibody sequences in a patient population) can be a challenge,

TABLE 14.1 Comparison of Immunoassay and Mass Spectrometry

Parameter	Immunoassay	LC-MS	Comments
Sensitivity	pg/mL range	μg/mL range	High affinity antibody reagent can improve sensitivity for both methods
Specificity	Limited (reagent dependent)	High (inherent)	—
Measured molecule identification	No	Yes	—
Accuracy/precision	Low (<20% bias and CV)	High (<10–15% bias and CV)	Both methods often perform well above expectations in the hands of experienced operators
Throughput	High	Medium to low	—
Cost/sample	Low	High	—
Assay development time	3–6 wk + 6–8 mo reagent lead	2 wk (no reagent lead unless immunocapture is used)	—
Special reagents required	Yes (specific antibodies and target)	No (except for immunocapture) methods)	—
Complex sample matrix compatible	Yes	No (extraction necessary)	—
Matrix interferences	Yes	Potentially reduced	—
Internal standards required	No	Yes	Labeled standards for large molecules can be technically challenging
Free/total compound measurements (typical)	Mixture of free and total most common	Total	Method modifications can accommodate different types of measurements
Regulatory and industry acceptance	Reference method for large molecules	Reference method for small molecules	—

Abbreviations: LC-MS, liquid chromatography-mass spectrometry; CV, coefficient of variance.

in silico prediction has greatly reduced the efforts [46]. Chromatographic resolution and sensitivity are greater issues since a certain mass is necessary for detection. Combination methods of immunoaffinity analyte enrichment and LC-MS or MS-MS detection can address these disadvantages [47,48]. However, this also eliminates some of the advantages of LC-MS, that is, reduced development times and the lack of need for method-specific reagents.

A few assays have been published that apply LC-MS to PK of protein therapeutics [44,49,50], but clinical applications have lagged behind to date. Future improvements of the technology are undoubtedly still to come.

14.4 KINETIC CONSIDERATIONS IN LBAs

Immunoassays are influenced by affinities and kinetics between the different reagents and the matrix components. Binding reactions are reversible and limited by the equilibrium concentrations [51], making the application of kinetic models difficult with many unknown parameters (e.g., how much antibody/target has been immobilized in the capture phase) [52]. Affinity determinations made in buffer are not necessarily transferable to a serum matrix. Therefore, underlying principles between binding and release should be kept in mind for LBA development and data interpretation. The assay protocol is a key to understanding the data generated.

Although the distinction between bound and unbound drug is more applicable to small molecules, discussions evolved during the last few years, in particular, for antibody drugs. The so-called free antibody has at least one site available for antigen binding, whereas the "total" drug includes both forms with and without bound antigen [53]. Although some assay formats are claimed to measure free or total antibody, an LBA inevitably will have a mixture of both forms. The assay reagents used for capture and detection as well as the incubation times and conditions influence the equilibrium between free and total drug that may exist in the original samples. Despite these difficulties, some assumptions can be used to guide PK analysis: if the target is used for capture, mostly free antibody will be measured. Indirect capture systems (Section 14.5.2) or general anti-IgG assays are more likely to measure total antibody (Fig. 14.3). Owing to the complexity of the assay interactions, these assumptions should be treated with caution.

14.5 ASSAY FORMATS FOR LBAs

Immunoassays are very flexible and offer the possibility of numerous formats [10]. We have reviewed only a selected few that appear to be common and are of specific interest. Some general points should be considered for any immunoassay. Depending on the question, different assay formats may lead to different conclusions. There are also technical considerations of methods details that often are not widely appreciated. As discussed in Section 14.5.3, the sample matrix is not readily exchangeable: even seemingly small differences such as EDTA plasma versus heparin plasma or one rat strain versus an alternative strain can significantly change assay performance.

The use of commercial kits to analyze proprietary biopharmaceuticals is not always straightforward either. At minimum, the assay calibrators and quality controls (QCs)

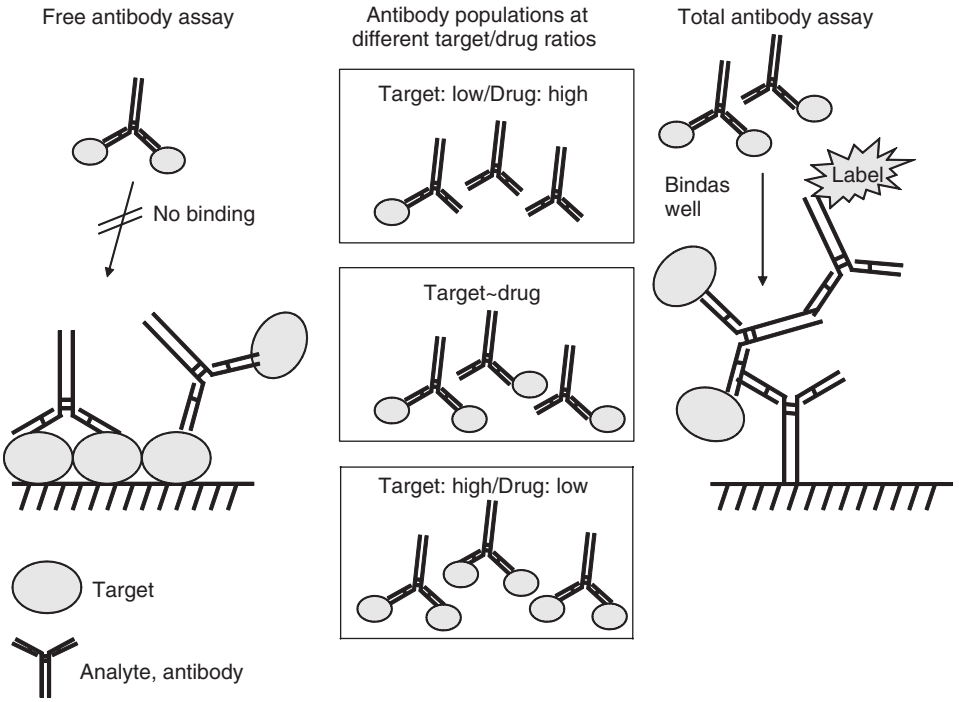

Figure 14.3 Comparison of free versus total antibody immunoassays: Free antibody assays typically use the target or anti-idiotype antibodies for capture. If target is bound to the antibody analyte, binding sites will not be available for binding in the assay. Total antibody assays capture and detect the antibody analyte independently from target engagement. Different populations of free, partially target-engaged, and bound antibodies are present depending on the ratios of target to drug (i.e., antibody analyte).

will need to be changed to reflect the new analyte. Typically, additional optimization for the new analyte is required, although options are limited due to the kit's preset reagent titrations. When using *in vitro* diagnostics (IVDs), some manufacturers may ask for a written assurance that data are not used to make patient treatment decisions outside the IVD label.

Assay sensitivity is tightly linked to reagents. Although assay platforms (Section 14.3) have an influence, sensitivity gains usually are modest if the reagents are not optimized. A switch from anti-species-specific to anti-idiotype-specific detection can result in up to 100-fold greater sensitivity. More details are discussed in the following sections.

In the following sections, we present more details of assay formats. Since many biopharmaceuticals under development are antibody based, we focus on these to a greater extent. However, with some extrapolation, many of the lessons may be transferable to other analytes of interest.

14.5.1 Direct Assays

Formats that capture the analyte of interest directly out of the matrix are the most common applications. Typically, the target is immobilized on the solid phase and an

anti-species antibody may be used for detection. In the case of antibody compounds, a bridge assay may be employed, with immobilized target for capture and labeled target for detection. Many assays for biopharmaceuticals employ compound-specific custom reagents, in particular, anti-idiotype-specific antibodies against antibody therapeutics. The key to a successful method is a combination of a suitable format and the quality of the reagents. Specificity and selectivity make or break LBAs.

Using anti-species antibodies can be problematic when operating with a similar matrix (anti-human antibodies and a human serum sample). Other endogenous antibodies beyond the therapeutic are present in 1000-fold excess so that even 1% nonspecific binding can result in significant background noise. These problems are reduced when clinical antibodies are tested in preclinical species. If cross-reactivity is limited, the so-called generic anti-human antibody assays can be conducted, and they have been published [54]. Anti-human antibodies are used for capture and detection to support various animal studies.

14.5.2 Soluble Target Assays

Soluble targets represent a specific challenge: at low drug-target ratios, very few binding sites of the drug may be available to engage immobilized target in the immunoassay, whereas at high drug-target ratios the reverse effect can be observed (Fig. 14.3). These effects can impact the measured PK profile depending on the assay format. Assays measuring Remicade, for example, were reported to exhibit TNF-α interference [55]. Other soluble targets that are subject to antibody therapy include various interleukins, Dickkopf-related protein 1, and proprotein convertase subtilisin/kexin type 9. Soluble ligands may also arise from shedding of membrane-associated molecules such as in the case of CD20 [56] and erb2/Her2 [57].

It may be necessary to evaluate whether the assay measures free or total compound and evaluation in two assays may help to establish the PK picture. Different approaches may be used to develop a total compound immunoassay with minimal soluble target interference.

An indirect capture assay to address potential interference of shed Her2/neu receptor in a Herceptin PK assay has been reported [58]. As the plate is coated with a mixture of target-engaged and free anti-receptor antibody, both compound forms (bound and free) can theoretically be captured in the assay. Dissociation steps, for example, with acid, are not commonly used for PK assays but could be applied to resolve anti-drug antibody interference and soluble target interference [54,59–62]. Very high affinity anti-idiotype-specific antibodies can potentially elevate target interference as well, if they can outcompete the target engagement.

Recently, Doucet and Avrameas [63] used enzymatic digestion to degrade interfering targets. Through careful optimization of pepsin digestion conditions, they were able to digest soluble target in samples while retaining the activity of the antibody of interest.

14.5.3 Selectivity Issues

As LBAs provide only an indirect measurement via the assessment of a reporter signal, assay selectivity is essential and numerous articles have been published on that subject. Particularly, diagnostic biomarkers are scrutinized as patient decisions are made on the bais of assay results. Without selectivity and specificity, it is not possible to

determine whether a positive assay signal is actually related to the compound rather than nonspecific binding [64,65]. While it is acceptable to use normal, pooled matrix for calibrators and QCs, selectivity validation (also known as *spike recovery*) should be undertaken with the expected clinical patient population in addition to a normal population. Commercial, diagnostic patient matrices as close to the final population as reasonable can be spiked with the compound and tested as surrogate samples in the assay. Since selectivity issues can occur in unspiked and spiked samples, both require evaluation [66,67]. In particular, samples from patients with underlying autoimmune diseases can be challenging. Human-anti-mouse antibody (HAMA) or human-anti-rabbit antibody (HARA) can potentially bind to assay reagents and result in a false signal. Additives such as heterophilic blocking reagents or animal serum in the assay diluent may address potential selectivity issues [68,69].

14.5.4 Design of Experiments (DOE)

Immunoassays are a classic example of a multifactorial system where different factors influence each other in generating the final result [70]. Traditionally, assays were developed "one factor at a time," that is, first optimal coating, then blocking, then detection, and so on. Since each condition is dependent on the others, checkerboard systems were applied to optimized coating and detection in conjunction.

The Quality by Design (QbD) initiative from the FDA aims at improving pharmaceutical manufacturing processes [71] using design of experiments (DOE) as a central component for optimization. Although not commonly applied yet, DOE is gaining more attention in the development of various types of immunoassays [72,73]. It can be used at three stages: (i) development (screening of factors and influences), (ii) optimization, and (iii) method robustness [74,75]. While a similar result may be achieved by applying good scientific rationale and experience, DOE can speed up the development of complicated assays and better define the design space of any method. This can be advantageous when accommodating changes (reagents, equipment, environment) or when outsourcing methods in later stages of development to other laboratories. Although DOE can help significantly in assay development and the control of critical factors, it does not replace critical scientific evaluation. Experimental conditions and results need to be analyzed not only from a statistical but also, most importantly, from a scientific perspective. Mathematical models cannot replace biological understanding of what is going on in the assay.

14.6 IMMUNOASSAY VALIDATION

14.6.1 Guidelines, White Papers, and Industry Standards

The application of immunoassays in the safety assessment and clinical development space requires validation to FDA and ICH (International Conference on Harmonization) standards. The chapter titled ***Analytical Method Development and Validation in Accordance to the Regulatory Guidelines*** of this encyclopedia focuses on this subject in more detail, while below, we present a few basic concepts as they apply to immunoassays. Existing FDA industry guidance is mostly applicable to LC-MS methods, and only limited sections apply to immunoassays [66,76]. A series of white

papers coming out of industry–FDA workshops have been published to help with setting up suitable validation plans [67,77–79], and some key validation parameters are listed in Table 14.2. Generally, LBAs are associated with greater variability and lower accuracy compared to LC-MS methods. Prevalidation experiments including different reagent lots, if available, may avoid surprises during a critical time line.

Ruggedness is an important aspect of validation since the method might have to be transferred to other laboratories [80]. Incubation times, temperatures, and equipment minimally should be evaluated. For assays carried out on the laboratory bench, a room temperature range should be tested. Caution should be applied to changes in the laboratory environment between summer and winter, as humidity and temperature can shift drastically. Ideally, temperature-controlled incubators are used. Some assays may even be sensitive to regular fluctuations from room air handlers, for example, if low affinity IgM antibodies are used whose binding can be influenced by subtle changes.

Once the method is deployed for study support, incurred sample reanalysis (ISR) is a regulatory requirement [81]. Selected samples are reanalyzed and compared to their original test value. The two results should be within a certain acceptance range: typically at least 66% of the retests should be within 30–40% of the original test value.

14.6.2 Best Practices and Common Pitfalls

The complexity of immunoassays can result in seemingly limited method changes having major effects with—in extreme cases—the method falling apart. Thus, extensive method validation, as described earlier, is a prerequisite to generating solid data.

As reagents are essential for immunoassay performance, lot switches should be avoided or minimized. Any new critical reagent lot, for example, capture and detection reagents, needs to be qualified for assay performance at least with accuracy and precision evaluation. Obviously, the uniqueness of immunoassay reagents prohibits the use of "equivalent" reagents; for example, replacement of an anti-species detection antibody from one supplier with an antibody from a different source. Calibrators and QCs should be bridged to previous lots and trending in preparations should be monitored.

As immunoassays can exhibit significant matrix effects, calibrators and QCs should be prepared in a matrix that is reflective of the samples. Attention needs to be paid to details: for example, plasma comes in different varieties (EDTA, Heparin, LiCl) that are not interchangeable. At minimum, the QCs need to simulate actual samples appropriately even if calibrators are prepared in buffer. It is preferable to have both calibrators and QCs prepared in the appropriate matrix.

Large molecules can also display a nonparallelism phenomenon. In this case, study samples, but not necessarily *ex vivo* spiked samples, do not dilute parallel to the calibrator curve [82]. Thus, the calculated sample concentrations differ depending on what dilution is used and multiple dilutions are necessary for each sample.

14.7 APPLICATIONS

It is important to keep the bioanalytical background of each method in mind. Mass spectrometry methods, LBAs, or cell-based bioassays each measure different aspects of a molecule and, thus, will seldom result in comparable data when applied to the same sample [83,84]. While mass spectrometry focuses on purified, small fragments

TABLE 14.2 Validation Parameters for Immunoassays and Expected Performance as Outlined in White Papers[a]

Validation Parameter	Expected Assay Performance	Comments
	Pharmacokinetic Assays	
Accuracy and precision	20% Bias/CV 25% Bias/CV at the LLOQ	Higher bias/CV acceptance criteria need to be justified with performance data
Dilution linearity	Linear over the applicable sample dilution range	—
Hook (prozone) effect	None	If a hook effect is observed, samples need to be tested at different dilutions
Specificity	No cross-reactivity with other sample components	—
Selectivity	Unspiked: BLOQ Spiked: 20% bias to nominal concentration	—
Robustness	Incubation ranges (time, temperature) do not affect assay	—
Stability, freeze–thaw	20% Bias/CV[b] compared to fresh (unfrozen) baseline	—
Stability, sample handling	20% Bias/CV[b] compared to baseline	Test conditions should cover sample processing during the assay
Stability, sample storage (long term)	20% Bias/CV[c] compared to baseline or nominal	Time should cover the storage time of the samples and beyond for retest options
Stability, reagents	No effects on assay performance as tested by accuracy and precision	—
	Immunogenicity Assays	
Accuracy and precision	CV:<20%	High variability affects the normal cutoff
Negative sample cutoff	5% False-positive rate	False positives are used to demonstrate the method that can detect bridging
Positive control sensitivity	Preclinical: 1 µg/mL Clinical: 250–500 µg/mL	—

(*continued overleaf*)

TABLE 14.2 (*continued*)

Validation Parameter	Expected Assay Performance	Comments
Drug interference	No interference with the expected drug concentrations at the sampling time point	Immunogenicity samples should be taken when little or no drug is on board
Robustness	Loss of sensitivity of samples spiked with positive control[c]: <20% bias	—
Stability, freeze–thaw	Loss of sensitivity of samples spiked with positive control[c]: <20% bias	—
Stability, sample handling	Loss of sensitivity of samples spiked with positive control[c]: <20% bias	—
Stability, sample storage	Loss of sensitivity of samples spiked with positive control[c]: <20% bias	—
Stability, reagents	No effects on assay performance as tested by accuracy and precision	—

Abbreviation: CV, coefficient of variance; LLOQ, lower limit of quantification; BLOQ, below the LLOQ.
[a]Refs 62,63,72–74, and 79.
[b]Alternative: bias/CV within the method acceptance criteria for accuracy/precision.
[c]Actual positive samples may be used if available.

of large-molecule digests, bioassays measure functional aspects that often require a complete protein. Depending on the assay format, LBAs might measure subparts or complete and functional analytes.

Different immunoassay formats do not necessarily give similar results [84,85]. The PK parameters of gentamicin derived from RIA and ELISA displayed significant differences in distribution volume, clearance, and half-life despite good bioanalytical correlation [86]. Similar issues were encountered for a PK rituximab assay [87]. Therefore, a thorough understanding of bioanalytical assay background is essential to analyze PK data for large molecules [88].

Interestingly, to characterize abnormal PK bioanalytical data caused by anti-drug antibody responses interfering with LBAs, another immunoassay is used to assess immunogenicity.

14.7.1 Pharmacokinetics

Most if not all biotherapeutics that are marketed established label PK parameters by immunoassay, although the assays rarely are published in detail [31]. Their applications stretch through the drug development process from lead optimization of optimal PK factors, over toxicology studies to assess the compound's safety profile to determination of final PK for the drug label. Basic information derived from bioanalytical immunoassay data includes area under the curve, half-life, and maximum and trough concentrations, while more complex modeling allows for human dose prediction.

As assay development can be time consuming and format switches can lead to changes in measurements, a suitable PK strategy should be considered early enough to avoid spending resources on molecules that will not see the development space. Well-designed and characterized LBAs can sometimes replace *in vivo* studies for early compound screening. Binding of antibodies to the neonatal Fc-receptor (FcRn) can be assessed *in vitro* in a preliminary fashion [89]. Ideally, the assay format remains the same from discovery to clinical so that bioanalytical data can be easily compared. As this is not realistic under a drug development paradigm, a staged approach can be pursued: a basic assay with minimal qualification can be established with commercial reagents initially to carry the molecule through discovery and candidate selection. Limited toxicology studies potentially could still be supported by an early format if it will be validated. In the case of antibody therapeutics, a "generic" anti-human IgG assay sometimes is used throughout the preclinical space [54,90]. Clinical assays, on the other hand, often require compound-specific assays for regulatory compliance, and the assay should be set up more robustly with tight control over assay reagents. Typical development points where bioanalytical assays can be easily switched are before toxicology and clinical programs. Whenever format changes are involved, extrapolations of PK data should be approached, with close consideration of the bioanalytical background.

Since preclinical studies including toxicology are limited in scope and time frame, novel assay technologies can easily be implemented. On the other hand, clinical studies can last for years and often are outsourced as a commodity. Therefore, clinical assay development needs to take equipment and capabilities of contract research organizations (CROs) into consideration. In addition, clinical assays need to be robust enough to withstand reagent lot, operator, laboratory site, and other changes. If necessary, a qualification strategy should be put in place to address these issues. Abbreviated

accuracy and precision evaluations typically are used. To avoid a need for assay redevelopment and bridging in the middle of a clinical program, novel technologies with limited vendor and CRO support should be avoided.

14.7.2 Immunogenicity

Assessment of anti-drug immune responses is gaining increased attention driven to a large extent by the development of anti-erythropoietin-α antibodies in patients receiving recombinant erythropoietin products. Between 1998 and 2000, ~13 patients with pure red cell aplasia were reported in France compared to 3 cases in the previous 10 years, ultimately accumulating to 191 cases throughout Europe [91]. This sharp increase was linked to anti-drug antibodies (ADAs) against recombinant erythropoietin α therapy leading to an autoimmune reaction against the patients' endogenous protein. The investigation of the case displayed well how different immunoassay formats and technologies can monitor various aspects of an issue. Initial radioimmunoprecipitation assays and ELISAs were able to detect high affinity IgG only. Later investigations with Biacore revealed the presence of temporary IgM against erythropoietin α [36,92]. These low affinity interactions had been overlooked as the stringent conditions required for ELISA/RIA-washed IgM out before detection. The elimination of secondary detection antibodies also made detection of subclasses other than IgG more feasible.

Bridge immunoassays using labeled drug are a standard format to screen sera for potential, with various technologies being deployed. RIAs and ELISAs are widely spread, but in recent years, MSD-based assays have been favored, as they allow for greater sensitivity [93]. They combine plate-based assay advantages such as high throughput and limited costs with Biacore/SPR advantages including the detection of low affinity IgM antibodies [94].

Immunogenicity assessment should not be based on a single assay, but it requires a more comprehensive strategy [95–98]. The initial screening assays take the variability of an undosed patient population into account to statistically set up a false-positive rate at 5%. This serves as a demonstration that the assay can recognize potential immune responses against the drug among a high background of nonresponders. Since factors other than specific ADA has the potential to form a bridge in the screening assay, caution should be applied when interpreting potential positive results [60]. Confirmation assays are necessary, which often include immunoprecipitation and/or titration of the response. Only a positive signal in screening and confirmation assays can be acknowledged as true ADA in order to be characterized further, for example, through isotyping or evaluation of neutralizing ADA activity. In recent years, replacement of cell-based functional assays by LBAs has been discussed. Although used occasionally, regulatory agencies are not quite convinced by this approach.

During drug development, PK and ADA analyses go hand in hand. A sudden decline of drug concentrations in serum beyond the molecule's expected PK is a good indicator of ADA interfering with PK measurements. Since animal immunogenicity is not predictive of human immunogenicity, the PK profile may be sufficient during early compound development. Toxicology studies eventually will require at least a screening assay to identify potential ADA-positive sera. Clinical immunogenicity assessment is a multilayered approach consisting of a screening assay, a confirmatory assay, and characterization assays. The immunogenicity assay strategy will closely define the patient's immune responses that can be detected [98,99].

14.7.3 Pharmacodynamics

While PK and ADA analyze the effects of the body onto the drug, PD measures the effects of the drug on the body. Biomarkers are increasingly used to assess biotherapeutics efficacy and have become a focus of regulatory agencies throughout the world [100]. The chapter titled ***Bioactivation and Reactive Metabolite Assays*** of this encyclopedia discusses the subject in more detail. Here, we focus on some bioanalytical examples where immunoassays play a role again.

Three basic assay categories can be distinguished: (i) standard clinical chemistry such as cholesterol and blood protein assessment; (ii) target monitoring, for example, the Her2/neu assays for Herceptin treatment; and (iii) complex multiplex assays monitoring marker panels, for example, for cancer risk prediction (Fig. 14.4).

14.7.3.1 Clinical Chemistry Assays. Changes in blood proteins often are useful biomarkers that do not require extensive assay development. Diagnostic assays are available for most serum components and routinely deployed in clinical reference laboratories using automated immunoanalyzers. These machines can analyze hundreds of patient samples per hour from beginning to end, requiring only sample and reagent loading by the operator. The assay principles are the same as discussed earlier, with an immunosandwich being assembled on a bead surface followed by detection with a label. However, assays specific for drug development often are difficult to establish due to the closed platform design of immunoanalyzers.

14.7.3.2 Target Monitoring. Biotherapeutics typically have a well-defined target pharmacology that can be used to develop biomarkers or companion diagnostics.

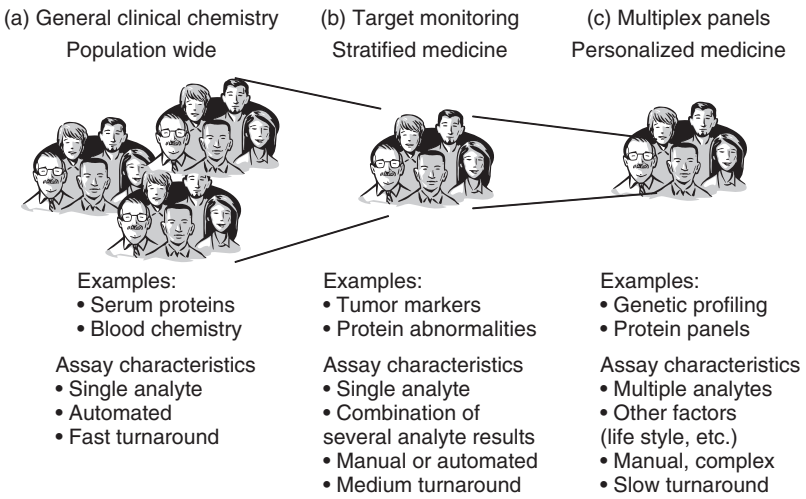

Figure 14.4 Schematic overview of (a) general, (b) stratified, and (c) personalized medicine. Immunoassays measuring standard blood chemistry and components typically are high throughput, automated assays that can be applied to the general population. Assays measuring drug targets can be either manual or automated and are used to identify patient subsets with specific features, for example, potential responders to therapy. Personalized medicine assays tend to be complex and carried out manually to date. Results are different for each person in the ideal case.

Herceptin is a good example for biomarker and assay development. This therapeutic antibody is used to treat Her2/neu-receptor-positive breast cancer [101]. Starting in early clinical trials and all the way to marketing of the drug, the target has been used to select patients who can benefit from the drug [102]. Initially, an immunohistochemistry assay and a fluorescence *in situ* hybridization (FISH) assay were available for patient selection. Recently, ELISAs measuring serum Her2/neu concentrations have become available. It is important to note that the different assay types also differ in their claims: patient selection for drug therapy can be based on FISH and immunohistochemistry results, as these are FDA-approved companion diagnostics [103]. ELISA measures a different aspect of the target pharmacology and is only cleared for cancer monitoring. Similar to the discussion of PK assay formats, Her2/neu biomarker assays are not interchangeable.

14.7.3.3 Multiplex Analysis. The trend to personalized medicine requires more extensive biomarker analysis. Instead of single target measurements, panels of multiple markers are combined to diagnose or predict disease. Most of the research focuses on pharmacogenomics, but some protein panels are being developed as well. When dealing with a small number of protein biomarkers, individual immunoassays can be carried out. But complex panels may be too costly and cumbersome for single assay analysis, and multiplex assays are more efficient. Going beyond single Her2/neu tests, the next generation of assays is on the horizon, combining multiple analytes to a single patient score [104]. The diagnosis of ovarian cancer also exemplifies the development from single biomarker tests over "oligoplex" to multiplex assays. Initially, CA125 serum concentrations were measured by RIA and ELISA to monitor cancer progression [105]. Recently, combinations with HE4 serum concentrations indicated a diagnostic benefit [106], and current trends indicate multiplex immunoassays on the horizon [107–109].

14.7.3.4 LBA Technologies for PD Assays. While some LBA technologies discussed previously can be modified for multiplex analysis, additional options are available to parallely measure several analytes. MSD offers multiarray plates where up to nine different analytes are spot coated onto a single microtiter plate well. Signals from each spot can be analyzed individually.

Time-resolved ELISA and AlphaLISA can accommodate up to four multiple analytes by using different detection labels in the same assay. Thus, the analytes are only resolved by the detection side of the LBA but not at the capture as in MSD's plates.

Bead-based arrays as offered by Luminex and its partners as well as by BD Biosciences have advanced into diagnostic applications already. The immunosandwich is assembled with capture reagents immobilized on dye-coded beads combined with a fluorescently labeled detection antibody. The dual signal of bound detection antibody together with each bead dye is measured individually in a flow-through system. Up to 100 analytes can be distinguished through the bead colors on Luminex systems. A number of other multiplex options are available, which are beyond the scope of this chapter [110,111]. The main obstacles for multiplex immunoassays are reagent selectivity and cross-reactivity, as well as potentially increased variability of multiplex test systems.

If patient treatment decisions and/or drug label claims are made based on biomarker analysis, thorough assay validation is required. While many parameters are similar to

PK assay validation (see above), more stringent standards are applied to diagnostic biomarkers as outlined in guidelines by the ICH [112,113].

Since a detailed discussion of PD markers and assays are beyond the scope of this, the above-listed examples represent only a very limited view of a vast and rapidly expanding field.

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