⟨1103⟩ IMMUNOLOGICAL TEST METHODS—ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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INTRODUCTION

Immunological test methods (ITMs) utilize bindings between an antigen (Ag) and antibody (Ab). (See *Appendix 1: Abbreviations* for a complete list of acronyms used in this chapter.) Enzyme-linked immunosorbent assay (ELISA) is one of the most widely used ITMs for characterization, release, and stability testing of biotechnology products to help ensure the quality of biological drug substances and drug products. The term "ELISA" is used here in a broader sense and includes enzyme immunoassays (EIAs), as well as alternative detection methods, e.g., chemiluminescence and fluorescence.

This chapter provides analysts with general information about principles, procedures, experimental configurations, assay development, and validation for solid-phase ITMs like ELISA and can be used for the other immunoassay variations mentioned above. The chapter also covers reference standards and controls used for immunoassays. The information can be adapted to the specific procedures of a monograph. This chapter does not cover immunoassays for the measurement of immune responses to product in animals or humans (e.g., serological or cellular assays), non-immunoassays (e.g., receptor-ligand interactions), or other related approaches.

The chapter is part of a group of general information chapters for ITMs (*Immunological Test Methods—General Considerations* ⟨1102⟩, *Immunological Test Methods—Immunoblot Analysis* ⟨1104⟩, and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩), and also is related to the general information chapters for bioassays (*Design and Development of Biological Assays* ⟨1032⟩, *Biological Assay Validation* ⟨1033⟩, and *Analysis of Biological Assays* ⟨1034⟩).

Definition

ELISA can be defined as a qualitative or quantitative solid-phase immunological method to measure an analyte following its binding to an immunosorbent surface and its subsequent detection by the use of enzymatic hydrolysis of a reporter substrate, either directly (as with an analyte that has enzymatic properties or is directly labeled with an enzyme) or indirectly (by means of an enzyme-linked antibody that binds to the immunosorbed analyte). Qualitative results provide a simple positive or negative result for a sample. Converting quantitative to qualitative results based on a cutoff value that separates positive and negative results is common practice. Because the performance properties of the assay depend heavily on the cutoff value, the process used to determine the cutoff should be evidence-based and well documented. Quantitative assays determine the quantity of the analyte based on the interpolation of a standard calibration curve with known analyte concentration, run simultaneously in the same assay. This standard should be an appropriate, preferably homologous, reference or calibration material that is representative of the analyte(s) of interest. The power of immunoassays has been demonstrated by the variety of procedures that have evolved, including alternative solid surfaces such as beads of different sorts, various plastics in plates of different configurations, and alternative detection methods, e.g., chemiluminescence and fluorescence. ELISA assays are widely used in the biopharmaceutical industry for various applications such as identity, purity, potency, detection or quantitation of antibody or antigen, and other purposes.

Basic Principles

The essential steps of an ELISA can be broken down as follows (see *Figure 1*):

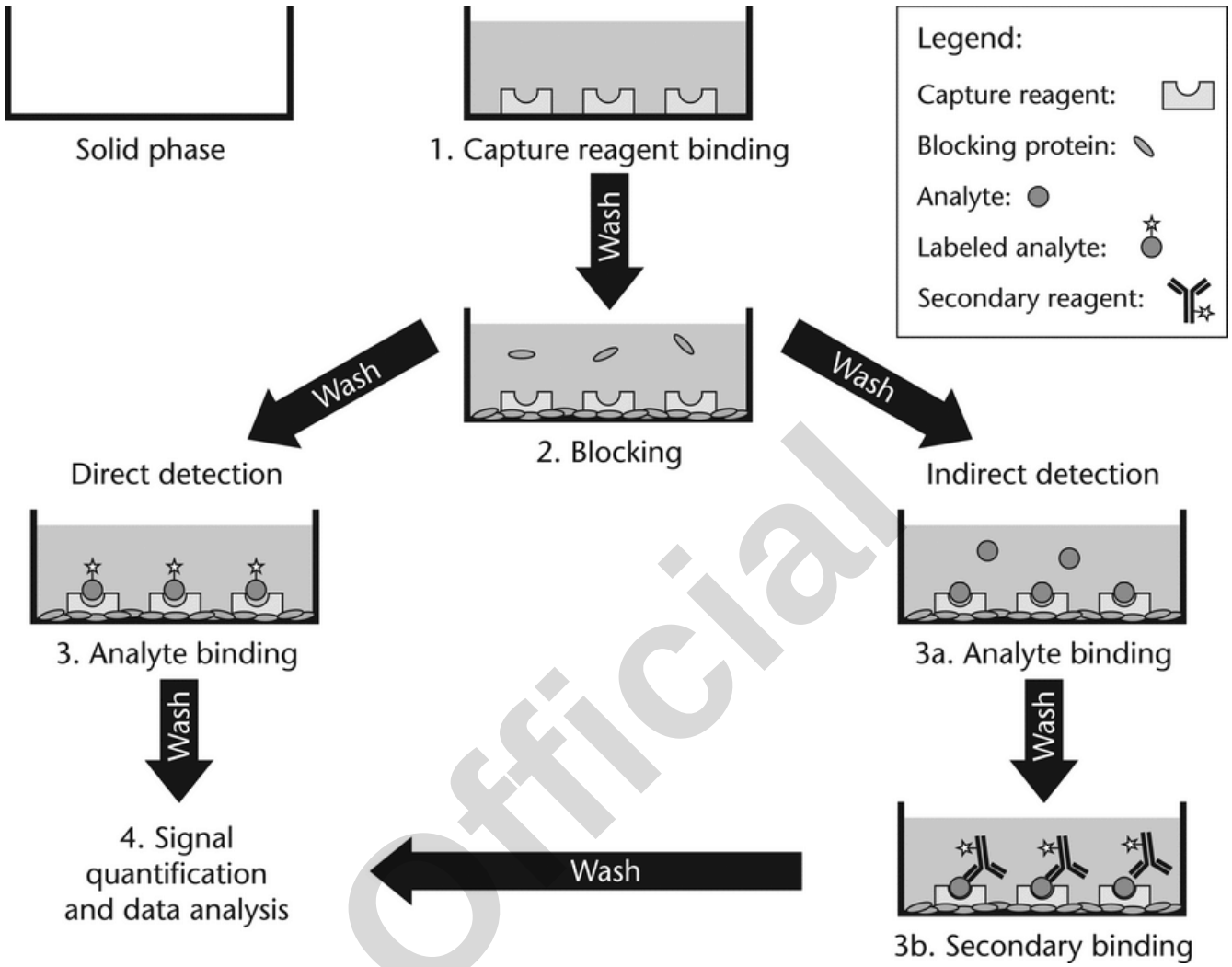

Figure 1. Essential steps for performing an ELISA.[1]

1. Binding of the capture reagent (generally an antibody or antigen), which functions as an immunosorbent for capture of the analyte, to a solid surface;

2. Removal of excess, unbound capture reagent followed by blocking of unoccupied binding sites with a blocking protein such as albumin, gelatin, casein, or other suitable material;

▲3 or▲ (USP 1-Dec-2019) 3a. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and detection of the analyte.

▲3.▲ (USP 1-Dec-2019) Direct detection occurs when the analyte has enzymatic activity or has been linked to a detector molecule (e.g., enzyme); or

3b. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and subsequent detection of the analyte (*Figure 1*, step 3a). Indirect detection occurs when the analyte is detected by the addition of a secondary enzyme-labeled reagent (*Figure 1*, step 3b); and

4. Quantification of the analyte by addition of a substrate suitable for the detector used [e.g., 3,3′, 5,5′-tetramethylbenzidine (TMB)], followed by comparison of the test sample to the reference standard.

[1] Capture reagent binding, blocking, analyte binding, detector antibody binding, and analysis are the five basic steps in an ELISA. Capture reagent binding, blocking, and analyte binding steps are each followed by a washing step to remove unbound reagents before the addition of the next reagent. Before analysis an appropriate substrate is added, followed by measurement of the substrate by appropriate equipment for detection. Quantitation of unknowns takes place by comparison with a standard curve.

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ASSAY DESIGN

▲Four▲ (USP 1-Dec-2019) general categories of ELISA are described in *Table 1* and in the sections that follow. The assay designs are flexible and, depending on specific needs, can be modified from these procedures. The choice of format depends primarily on the amounts and purity of reagents and equipment available. On some occasions the analyte being characterized actually is an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, anti-idiotypic or other antibodies specific for the antibody are used to develop the assays.

Table 1. Representative ELISA Types

ELISA Type	Required Reagents	Attributes	Disadvantages
Direct detection	• Capture analyte[a] • Labeled primary antibody specific for antigen	• Rapid because only one antibody is used • Uses less reagent • Analyte is immobilized	• May modify the conformation of the analyte • Sensitive to matrix and adjuvant components • Not commonly used • Poor sensitivity
Indirect detection	• Capture analyte[a] • Primary antibody specific for antigen • Labeled secondary detector antibody that binds to the primary antibody	• Versatile because a variety of primary antibodies can be used with the same secondary detector • Improved sensitivity because of signal amplification • Analyte is immobilized	• Longer because of more incubation and washing steps
Competitive	• Analyte[a] can be used as a capture reagent or can be labeled with a detection label • Antibody specific for analyte can be used for capture or labeled for detection • Labeled secondary antibodies to bind to primary antibody if an indirect format is used	• Good for assessing antigenic cross-reactivity • Appropriate for smaller proteins with single epitopes • Requires only a single antibody • Analyte in solution competes for binding to primary antibody	• Format difficult to troubleshoot • Limited dynamic range
Sandwich	• Primary capture antibody specific for analyte • Sample solution containing analyte[a] • A different primary enzyme-antibody conjugate specific for analyte	• Improved sensitivity • Good for quantitative assays for larger multi-epitope molecules • Analyte measured in solution	• Requires relatively large amounts of pure or semipure specific antibody • Not suited for smaller proteins that may have only a single epitope or a few closely spaced epitopes

[a] This reagent can be either purified or partially purified. The terms “analyte” and “antigen” are used interchangeably when describing ELISAs.

Direct ELISA

DIRECTLY LABELED ANTIBODY

In this assay an antigen is coated onto a solid surface and the remaining unbound reactive sites are blocked [*Figure 2* (A)]. Then a solution containing a specific antibody labeled with a detector is added. After incubation, the unbound antibody is washed away, followed by the addition of an appropriate substrate for the detector used.

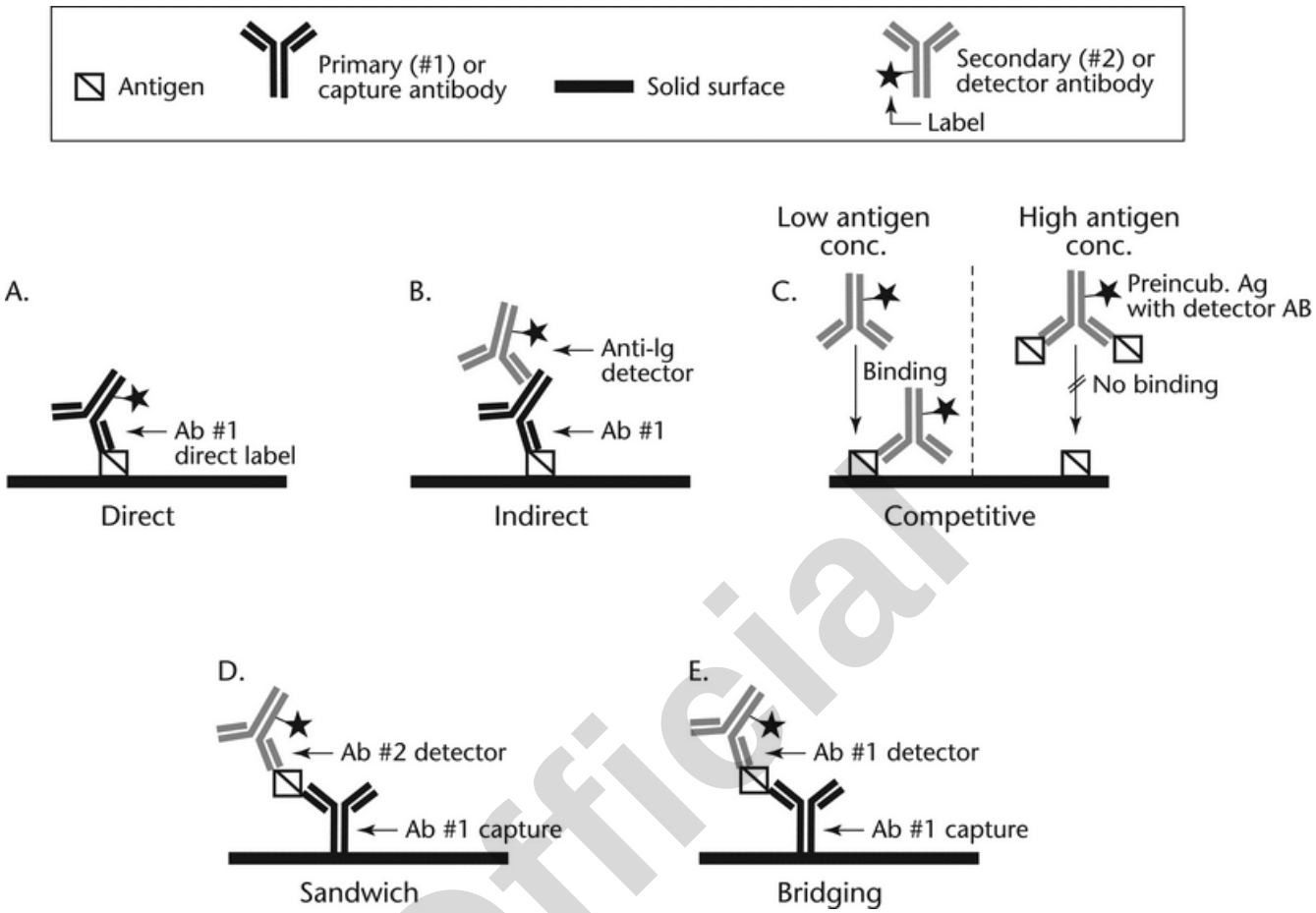

Figure 2. Schematic representations of direct, indirect, competitive, sandwich, and bridging ELISAs.[2] [Ab = antibody; Anti-Ig = anti-immunoglobulin; Ag = antigen (or analyte); Conc = concentration; Preincub = preincubation]

DIRECTLY LABELED ANTIGEN

This assay is similar to that using a directly labeled antibody, except that the antibody is coated onto the solid surface and a labeled antigen is used as the detector.

Indirect ELISA

In this assay an antigen is coated onto a solid surface and then, after blocking, a solution containing a specific antibody is added [*Figure 2* (B)]. After incubation, the unbound antibody is washed away, followed by the addition of an anti-immunoglobulin (anti-Ig) detector antibody. Anti-Ig detectors are available commercially for specific Ig classes and subclasses from a variety of species, which makes this assay format useful for isotyping of antibodies. In addition, the use of a labeled anti-Ig detector amplifies the signal compared to a *Direct ELISA*, thereby increasing assay sensitivity.

Competitive ELISA

DIRECT ANTIBODY COMPETITIVE ELISA

This assay is used to detect or quantitate soluble antigens [*Figure 2* (C)]. It requires an antigen-specific antibody that has been conjugated to an appropriate detector, e.g., horseradish peroxidase (HRP), alkaline phosphatase (AP), ruthenium, or fluorescein. It also requires a purified or partially purified antigen for coating. The antigen is coated onto a solid surface, followed by a blocking step. The antibody–conjugate is incubated with the test solution containing soluble antigen. The mixture is then added to the immobilized antigen, incubated, and unbound antigen–antibody complex is washed away. Substrate is added, and the inhibition of the reaction (e.g., colorimetric, electrochemiluminescence, fluorescence, or chemiluminescence) is

[2] The type of ELISA format depends on the availability of reagents, the intended purpose of the assay, and the physicochemical characteristics of the analyte of interest. For a *Bridging ELISA*, the capture and detector antibodies recognize the same epitope, and therefore the target antigen must have at least two epitopes available for binding.

measured relative to the reaction when no competitor antigen is added. The amount of inhibition is inversely proportional to the amount of antigen in the test sample. Competitive assays can also measure small molecules by coating an antibody to the plate that is specific to the small molecule. The small molecule is often biotinylated with a long linker that does not interfere with binding between the capture antibody on the plate and the small molecule. Antigen (the small molecule) in the sample then competes with the labeled small molecule for binding to the capture antibody. After washing, a detection reagent (e.g., streptavidin labeled with HRP) is added to detect the binding complex.

DIRECT ANTIGEN COMPETITIVE ELISA

This assay is similar to the *Direct Antibody Competitive ELISA*, except that it is used to detect soluble antibodies. The antigen is conjugated to the detector and the antibody is coated onto the solid surface.

INDIRECT ANTIBODY COMPETITIVE ELISA

This assay is similar to the *Direct Antibody Competitive ELISA*, except that instead of directly labeling the antibody, the test uses a labeled anti-Ig reagent for detection.

INDIRECT ANTIGEN COMPETITIVE ELISA

This assay is similar to the *Direct Antigen Competitive ELISA*, except that instead of directly labeling the antigen, the test uses a labeled secondary antibody for detection.

Sandwich ELISA

DIRECT SANDWICH ELISA

In this assay an antibody is immobilized onto a solid surface and blocked, and then a solution containing a specific antigen is added [*Figure 2* (D)]. After an incubation step, the unbound material is washed away, and a labeled detector antibody is added. This assay format requires two antibodies, each of which binds to different epitopes on the surface of the large and complex molecule. The two antibodies are specific for the antigen, and the antigen should be sufficiently large and complex to accommodate the binding of two antibodies.

INDIRECT SANDWICH ELISA

Alternatively, instead of directly labeling the detector antibody, an anti-Ig antibody detector can be used. Indirect sandwich immunoassay formats can be considered only if each binding reagent is from a unique species (e.g., a sandwich assay using two mouse monoclonal antibodies for capture and detection could not be detected indirectly because the resulting signal may become independent of the antigen concentration).

BRIDGING ELISA

This subset of *Sandwich ELISA* assays often uses a single antibody for both capture and detection [*Figure 2* (E)]. If a monoclonal antibody is used, it requires that the target antigen have at least two identical epitopes that are adequately spaced to prevent steric hindrance so that one epitope binds to the capture antibody and the other epitope binds to the detector antibody. Alternatively, a polyclonal antibody can be used but still requires that the target antigen be large enough to accommodate the binding of two antibody molecules. With respect to specificity and sensitivity, bridging assays usually are suitable for most large molecules.

CHOICE OF ASSAY

Deciding which ELISA procedure or format to use often depends on individual choice and availability of reagents, instruments, and other equipment. For example, sometimes a laboratory repeatedly engineers a particular epitope into multiple fusion proteins. In this case, the laboratory can use certain common qualified reagents (e.g., an antibody to a glutathione S-transferase region in multiple fusion proteins), facilitating rapid sandwich immunoassay development. Small antigens with a limited number of epitopes available for antibody binding restrict ELISA format choices. If there is only one binding epitope, then ELISA methods that use the sandwich/two-site binding or other bridging formats cannot be used because they require at least two available epitopes for antibody binding. In addition, small molecules are not usually used as a capture reagent on a plate because the process may interfere with binding to the detection reagent. Examples of such small molecules are some peptides, oligosaccharides, nucleotides, and antibacterials. Analysts usually adopt a competitive assay format for such small analytes.

Different assays and formats may demonstrate different properties and characteristics, e.g., specificity, precision, accuracy, sensitivity, dynamic range, dose-response ratio, sample throughput, sensitivity to interference, and simplicity or efficiency for automation. Ease of validation also may vary between different assay protocols and formats. Assay designs with replicates in adjacent wells could be biased if there are location effects; hence, in this case, replicates should not be in adjacent wells. Assay designs that are convenient to perform on 96-well plates, using relatively few single-channel pipet actions and more multi-channel pipet actions, are usually easier to adapt to automation. Assays with steep dose-response curves are generally better able to deliver high precision estimates; however, some assays with steep dose-response curves are imprecise in the EC_{50} and require a wider dose range.

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PROCEDURES

Solid Phase

Solid phases are available in a variety of forms (e.g., membrane, plate, or bead) and chemistries [e.g., nylon, nitrocellulose, polyvinylidene fluoride (PVDF), polyvinyl, polystyrene, or a chemically derivatized surface]. The selection of the solid phase determines the most likely binding mechanism, i.e., hydrophobic, hydrophilic, or covalent interactions. In general, compared to plates, beads offer higher capacity and are more commonly used in clinical assays whereas plates are more commonly used to test biotechnology products. Additional information on plates is provided below.

COATING THE SOLID PHASE—IMMOBILIZATION OF CAPTURE REAGENT

Capture reagents are coated onto a solid phase by adding a solution containing the capture reagent to the surface. The most commonly used solid-phase materials for capture reagent immobilization are plastic 96-well microtiter plates. Those with flat-bottom wells are recommended for spectrophotometric readings, and round-bottom well plates are useful for visual assessment of a dye's color development. The degree of coating is influenced by the concentration of capture reagent, temperature during coating, duration of capture reagent adsorption, the surface properties of the solid-phase material, and the nature of the buffer of the capture reagent solution. Although the optimum coating concentration must be determined for each capture reagent, concentrations of 1–10 µg/mL are most commonly used. The volume of capture reagent added to each well usually corresponds to the sample volume that will be analyzed, i.e., 50–100 µL. Coating duration, temperature, and buffers are discussed separately below. During the coating procedure analysts should avoid introducing bubbles. Proteins that bind to plastic can be denatured, which alters antigenicity. In such cases, a capture antibody or an intermediary protein such as Protein A or Protein G can be used. In addition, streptavidin can be used if the reagent is biotinylated. The pH of the coating buffer should be optimized based on the isoelectric point of the capture reagent and the surface properties of the assay plate chosen.

MICROTITER PLATES

The composition and commercial source of the microtiter plate can influence binding of the capture reagent during coating. Several microtiter plates from different suppliers should be compared using a single coating procedure to select those that provide high specificity for the capture reagent of interest and low nonspecific background. Comparisons of different grades of plates from a single supplier also may be needed. Clear plates typically are used for colorimetric ELISA, and opaque plates often are used for chemiluminescent and fluorometric ELISA. Acidic capture reagents may require a lower pH solution to neutralize repulsive forces between the protein and solid phase. Peptides often require optimization of buffer pH based on their charge for optimal coating conditions during assay development. Polysaccharides, lipopolysaccharides, or glycoproteins may be difficult to coat directly to the plate and may require a capture antibody or a buffer that contains lysine or glutaraldehyde. Coating with an antibody can be enhanced by precoating the microtiter plate with Protein A or Protein G or a combination of the two, which allows binding to the Fc region so that the Fab portion can bind to the analyte of interest. However, care must be taken to ensure that subsequent secondary antibodies do not react with the Protein A- or Protein G-coated wells. In this case, for example, chicken IgY or another appropriate antibody class could be used. Microtiter plate formats other than the 96-well variety, such as half-volume 96-well or 384-well plates, can be used to increase throughput and/or conserve reagents.

COATING TIME

Coating time depends on binding kinetics, stability, concentration of capture reagent, and incubation temperature. Although different combinations of coating times and temperatures often result in the same coating efficiency, the stability of the capture reagent (which should be determined during method development) influences which conditions to select. Analysts must assess the impact of varying the coating time in order to determine the robustness of the assay procedure.

COATING TEMPERATURE

Coating temperature and time are closely related assay parameters. The coating temperature depends on the binding kinetics and stability of the antigen. Higher temperatures can increase the rate of adsorption and may shorten the coating time, but they are likely to affect interaction sites and to reduce antigen-antibody affinity. Typical combinations of time and temperature are 1–4 h at ambient temperature, 15 min to 2 h at 37°, or overnight at 4°. Analysts should determine the effects of variations in temperature in order to assess the robustness of the assay procedure.

BUFFERS

Buffers used for diluents, coating, blocking, and washing plates can affect overall assay performance. Buffer components can interact with the test sample and inhibit binding. They also can cause low antigen sensitivity or high nonspecific background activity.

Diluent: Buffers [e.g., phosphate-buffered saline (PBS) or imidazole-buffered saline] with polysorbate 20 (0.01%–0.1%) are used commonly for different ELISA steps as a diluent and washing buffer.

Coating buffers: Coating buffers should maximize assay consistency and promote binding of the capture reagent to the solid phase. Commonly used coating buffers include 50 mM carbonate, pH 9.6; 20 mM tris-hydrochloride (tris-HCl), pH 8.5; and 10 mM PBS, pH 7.2. The choice of coating buffer depends on the nature of the individual antigens and should be determined empirically.

Blocking agents and buffers: A blocking agent is a compound (e.g., protein or detergent) that should saturate the remaining immunosorbent binding sites following capture reagent (antibody or antigen) binding. This reduces nonspecific binding of analyte and nonanalyte components to the immunosorbent matrix and/or the absorbed reagent. Nonspecific binding occurs when protein in the test sample binds to the plastic of the microtiter plate or absorbed reagent instead of specifically binding to the capture reagent of interest. Nonspecific binding can be reduced by adding blocking reagent to the wells and by the addition of another protein such as bovine serum albumin (BSA) to the dilution buffer. The choice of blocking agent should be governed by the nature of the capture reagent, plate, coating buffer, test sample diluent, and related factors. If any of these parameters changes, a change in blocking agent may be needed. Commonly used blocking agents include BSA, nonfat milk, gelatin, casein, normal horse serum, fetal bovine serum, polysorbate 20, and others. Several grades of BSA are available commercially, and the optimal grade should be empirically determined for each assay. In addition, many commercial blocking and assay diluent reagents are available for ITM.

Adding Samples and Reagents

Samples and reagents generally are pipetted into the ELISA plate wells. Care should be taken to avoid cross-contamination, frothing, or bubbles. ▲A sample loading pattern should be included in the test method procedure. For reproducibility and accuracy of results, consistency between the wells of the ELISA plate is very important. This can be achieved by using replicates; however, as mentioned above, care should be taken to avoid replicates in adjacent wells. A common way of avoiding the edge effect is not to use the edge wells at all. Additionally, plate edge effects can also be avoided by reducing assay time, using a low evaporation lid, or sealing the plate with a clear or breathable sterile tape.▲ (USP 1-Dec-2019) Labor-saving equipment such as electronic pipets, automated liquid handlers, plate washers, and robotic pipets also can be used to improve precision, reduce analyst-to-analyst variability, and increase throughput.

PIPETS

Single, multi-channel, and robotic pipets with set or fixed volumes are available. The type and accuracy of pipets should be evaluated for each application. Regular maintenance and professional calibration of pipets should be performed and documented.

PIPET TIPS

A variety of pipet tips are available, some of which are specific to the type of pipet. The type and accuracy of the pipet tip, particularly related to the viscosity and nonspecific binding of the materials, should be evaluated for each application.

Washing

Wash steps are included throughout the ELISA procedure to remove the unbound coating antigen, sample, and detection reagents. Washing is critical for assay performance, can be a source of assay failure, and is important to evaluate during method development. Multiple approaches can be used for washing. Manual procedures include using a squeeze bottle, dipping the microtiter plate in wash buffer, and adding wash buffer with a multi-channel pipet or hand-held multi-channel (8- or 12-pin) manifolds. Analysts should wash carefully to avoid cross-well contamination. Automatic microplate washers generally provide more washing consistency. Strip-well and multi-well washers are available. Most automatic washers can be programmed for different dispensing volumes and speeds, number of washes, speed of buffer aspiration, and amount of residual buffer left in the well. Incorrectly programmed or maintained, as well as incompletely cleaned, automatic washers can cause assay variation and elevated assay background.

Incubation

ELISAs are incubated following the addition of samples and reagents. The optimal time, conditions, and temperature of each incubation step should be determined during method development. Incubation times vary from minutes to overnight. Commonly used incubation temperatures are ambient temperature, 4°, and 37°. ELISA plates commonly are sealed or placed in a secondary container to avoid evaporation or contamination during incubation. Atmospheric conditions such as dry or humidified incubation should be evaluated during method development. Rocking, shaking, or rotating the microtiter plates may be necessary or desirable depending on the kinetics of binding.

Blocking Conditions and Nonspecific Reactions

After immobilization and removal of the unbound antigen or antibody, unoccupied binding sites are blocked to ensure that the measured analyte in the test article or subsequent (detection) reagents does not bind nonspecifically to the solid surface or to the coated antigen or antibody. If nonspecific binding occurs, any reported signal could bias the measurement and may reduce the sensitivity and dynamic range of the assay. Blocking is critical to ensure the sensitivity and/or specificity of the assay. Sources of nonspecific binding fall into two general categories:

1. Ionic or hydrophobic interactions occur when binding is mediated by nonspecific ionic or hydrophobic interactions between assay reagents and the solid surface or another assay reagent.
2. Immunological interactions occur when binding is mediated by unintended antigen–antibody interactions. This occurs when antibody preparations used in the assay interact with other assay reagents. For example, if an ELISA was designed to test a serum-derived analyte using murine capture and detection antibodies, antibodies in the test article with reactivity to murine Ig (also known as heterophilic antibodies) could be nonspecifically detected in the assay.

The choice of blocking agent (examples are found in *Blocking agents and buffers*) is determined empirically, and the balance between the reduction in nonspecific binding and the impact on assay sensitivity should be assessed during method development. Cross-reactivity with other assay reagents should be considered; for example, endogenous biotin is found in milk and serum, and serum may contain antibody to viral or bacterial proteins. Therefore, screening of serum lots may be necessary. The volume of blocking solution added to the well should be greater than the maximum reaction volume used for later steps so that all of the potential surface area that may interfere with the binding reaction is blocked.

In addition, Ig in the test materials can be removed by using buffers that inhibit antibody conformation or aggregate the heterophilic antibodies, by blocking with nonimmune serum, or by removing Fc regions in critical antibody reagents, thereby reducing or eliminating undesired immunological interactions that cannot be addressed by the blocking reagents described above. Negative control wells can be included to monitor nonspecific reactions. The nature of the negative control wells depends on the assay but can include blocked wells without coating antigen, eliminating the primary or secondary antibody, or using buffer in place of sample. Control wells also can be useful as part of system suitability testing.

Pretreatment of Samples

Although ELISA methods are designed to measure an analyte in complex mixtures, the presence of other materials can prove problematic if they interfere with analyte detection. In order to ensure assay specificity, the specific procedure to treat samples to remove nonspecific interfering substances (e.g., reducing agents or precipitates) can be determined empirically during method development and then can be incorporated into the validated assay. Any sample-processing step should be evaluated against the potential that the treatment will alter the test article's properties and/or introduce further variability that results in biased measurements. Samples, standards, and controls should be prepared and handled in processes as similar to each other as possible. Analysts should verify that sample pretreatments have not damaged the sample so much that it can no longer be measured (e.g., by spiking experiments).

Detector Antibodies

Depending on the ELISA format, detector antibodies labeled with enzyme or other labels can be used as primary or secondary reagents to enable detection of the immobilized analyte. In a direct or competitive ELISA [*Figure 2* (A and C)], after the analyte is bound to the immunosorbent surface, excess analyte is washed away and the immobilized analyte is detected using a detector antibody that is considered to be the primary antibody. In other ELISA formats [*Figure 2* (B, D, and E)], the analyte-specific Ig (nonconjugated primary antibody) is allowed to bind to the immobilized analyte, and any excess antibody is washed away before the addition of a detector antibody, which is termed the secondary antibody.

To facilitate detection, in all ELISA formats that use enzyme-conjugated antibodies, a substrate specific for the conjugated enzyme is introduced into the assay system. An enzymatic reaction ensues, converting a substrate into a soluble product that can be measured using appropriate wavelengths and a suitable reader.

ELISA sensitivity depends on the quality of the reagents and the detection system, including the label and substrate. If multiple differently conjugated antibodies are available, analysts should select one appropriate for the assay. During this evaluation, the dilution of each conjugate that yields desirable sensitivity and specificity should be determined using appropriate controls.

The most commonly used labeling enzymes for conjugating to antibodies include AP, HRP, and galactosidase. These enzymes are highly specific, sensitive, and stable in catalyzing chromogenic, luminescent, or fluorescent reactions. *para*-Nitrophenyl phosphate (pNPP) is a commonly used substrate for AP. Commonly used substrates for HRP include TMB, *o*-phenylenediamine dihydrochloride (OPD), and [2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS)] (see *Table 2*). The substrates for AP and HRP are chromogenic and result in the formation of a colorimetric product that can be measured using a spectrophotometer. Chemiluminescent and fluorescent substrates for AP and HRP also are available, and in many cases they are available as commercial kits. Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD) is a known chemiluminescent substrate for AP (see *Table 2*). Well-known fluorescent substrates for galactosidase include 4-methylumbelliferyl galactoside (MG) and nitrophenyl galactoside (NG). If a chemiluminescent substrate is used, then a luminometer is required to quantitate the formed product. A fluorometer is needed if a fluorescent substrate is used in the ELISA.

Table 2 also provides a summary of the advantages and disadvantages of different types of ELISA substrates. Colorimetric substrates have been prevalent since the origin of ELISAs and may yield robust assays that generally are more cost efficient than assays that use chemiluminescent and fluorescent substrates. Nevertheless, chemiluminescent and fluorescent ELISA methods may yield more rapid and sensitive assays with a wider dynamic range than assays that use a colorimetric readout. The final choice of readout should be governed by the assay's purpose and the requirements of the assay.

Table 2. Enzyme Conjugates and Substrates

Readout	Principle of the Enzymatic Reaction	Enzyme	Substrate	Reader	Advantages	Disadvantages
Colorimetric	Produces a colored product that yields absorbance values directly proportional to analyte concentration	AP, HRP	pNPP TMB OPD ABTS	Spectrophotometer	• Robust • Economical • Reagent availability	• Less sensitive

Table 2. Enzyme Conjugates and Substrates *(continued)*

Chemiluminescent	Produces a light emission that is directly proportional to analyte concentration	AP	CSPD	Luminometer	• Wide assay dynamic range • Lower coating concentrations • More sensitive • Rapid signal generation	• Requires special plates • Costly
Fluorescent	Produces excitation-induced light emission that is directly proportional to analyte concentration	Galactosidase	MG NG	Fluorometer	• Rapid • Sensitive	• Requires special plates • Costly • Interference by excipients

ASSAY DEVELOPMENT AND VALIDATION PLAN

Critical Reagent Development

Key considerations for critical reagents are source, purity, specificity, and stability. For quality measurements, ITMs use reference standards along with critical reagents for analyte capture and detection. Any changes of critical biological reagents should be evaluated (see, for example, guidance contained in ⟨1032⟩).

SOURCE

The availability and quality of the starting material should be controlled so that manufacturing of the (purified) reagent can be reproducibly and consistently performed, potentially over several decades. Because critical reagents are biological molecules, sources can range from chemical synthesis (e.g., peptides) to complex biological matrices (e.g., antibodies prepared from serum, monoclonal antibody from ascites/cell culture, or fermentation/cell culture products). When appropriate for the intended use of the assay, a single lot of a critical reagent can be manufactured to establish a substantial supply and to prevent lot-to-lot variability. In other instances, it may be appropriate to include in the validation multiple lots or multiple suppliers in order to demonstrate that the assay is sufficiently robust for its intended use.

PURITY

In general, the purity of critical reagents should be assessed to ensure the removal of impurities and manufacturing process residuals that can influence reagent performance and/or stability.

SPECIFICITY

The specificity of a critical reagent refers to its ability to capture or detect only the analyte of interest. The reagent must be specific to the analyte and should show little nonspecific binding or no cross-binding to off-target molecules in complex test materials.

STABILITY

The stability of critical reagents should be empirically determined to ensure assay performance over time (issues include accuracy, precision, reproducibility, and assay drift). Long-term (months to years) stability of critical reagents under required storage conditions (e.g., with defined temperatures and containers) should be determined so that appropriate expiry dating can be assigned. Short-term (minutes to days) stability (and freeze/thaw and room temperature stability for frozen critical reagents) also is required to ensure day-to-day assay accuracy, precision, and reproducibility.

Feasibility/Pilot Studies

The steps of the process by which an ELISA method is developed, validated, and used in routine sample analysis are described below:

1. Generate or purchase critical reagents to measure the analyte. Determine storage conditions and stability.
2. Understand the performance goals for the assay system.
3. Develop the assay to the point that there is a detectable concentration response curve.
4. Perform method development/robustness testing.
5. Prepare the reference/calibration standard and control and assess stability.
6. Establish assay procedures, appropriate controls and limits, assay and sample acceptance criteria, and instrumentation.
7. Determine method performance, and qualify method for accuracy, specificity, precision, and robustness, including qualification of all applicable sample types to be analyzed.
8. Validate the assay.
9. Implement the method (technology transfer) in the testing laboratory, including training and qualification of analysts.
10. Monitor assay performance.

During assay development, the critical parameters and reagents that are required for the assay should be assessed and set at levels that yield desired assay performance. In many instances several parameters may be evaluated, and well-designed experiments can accelerate assay development, particularly for assessing the potential interaction of several inputs.

Many ELISA procedures are product specific, and external reference/calibration standards may not be available. The preparation and stability of reference/calibration standards should be considered early in assay development.

Assay Validation

Assay validation is executed according to guidances from appropriate regulatory bodies [e.g., International Council for Harmonisation (ICH) Q2] to demonstrate that the particular test used for an analyte is appropriate for its intended use. More information about assay validation can be found in *Validation of Compendial Procedures* ⟨1225⟩ or ⟨1033⟩ if the ELISA is used as a surrogate potency assay. See *Appendix 2: Additional Sources of Information about Specific Topics in Validation and Data Analysis*.

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DATA ANALYSIS

The analysis of ELISA data can be simple (e.g., a linear calibration with inverse regression) or complex (e.g., a nonlinear calibration curve with inverse regression). The type and rigor of data analysis depend largely on the assay system and the intended uses of the assay. For example, data reduction may estimate a concentration (e.g., ng/mL) of an unknown sample using a calibration curve. Other approaches include estimation of the half-maximal inhibitory concentration (IC_{50}) or effective concentration (EC_{50}), estimation of the amount of a sample that yields the same response as the EC_{50} (or IC_{50}) on a standard curve, and an estimate of the relative activity of a test sample compared to a reference/calibration standard. More extensive guidance about statistical methods for potency analysis is given in ⟨1032⟩ and ⟨1034⟩.

In general, ELISA curves are characterized by a nonlinear relationship between the concentration of the analyte of interest and the calculated mean response. Typically, this response curve is defined by a sigmoidal relationship of response to concentration. A wide range of mathematical models can fit standard/calibration curves, and analysts should take care in the selection of an appropriate curve-fitting algorithm. In other cases, ELISA assays are used for qualitative purposes to determine whether a sample is positive or negative based on a sensitivity threshold.

Basic Statistical Analysis

Basic statistical methods are not detailed here. *Analytical Data—Interpretation and Treatment* ⟨1010⟩ addresses important fundamentals, including data handling; computation of means, standard deviations, and standard errors; detection of and methods to address nonconstant or nonnormal variation; detection of and management of outliers; and procedures for and interpretation of statistical tests and confidence intervals. The concepts behind validation, goals, designs, analysis, and practical methods for validation are described in ⟨1010⟩, ⟨1225⟩, and ⟨1033⟩. *Design and Analysis of Biological Assays* ⟨111⟩ contains guidance on combining results from independent assays, ▲outliers, and confidence intervals.▲ (USP 1-Dec-2019)

Nonlinear Statistical Analysis

Nonlinear calibration for immunoassays draws on many sources for statistical design and analysis. These include methods for assessing and addressing nonconstant variance, designs and analysis methods for experiments with complex structures, and validation. The concepts behind linear calibration design, analysis, and inverse regression apply in nonlinear calibration, and professional statisticians can help apply these appropriately.

Reporting Results

Reported estimates of concentration should be understood as having an associated confidence interval based on the results of the validation. The reported value or estimate used to describe a sample can be based on a combined result from multiple assays.

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APPENDICES

Appendix 1: Abbreviations

Ab: Antibody
ABTS: 2,2′-Azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]diammonium salt
Ag: Antigen
Anti-Ig: Anti-immunoglobulin
AP: Alkaline phosphatase
BSA: Bovine serum albumin
CSPD: Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate

EIA: Enzyme immunoassay
ELISA: Enzyme-linked immunosorbent assay
HRP: Horseradish peroxidase
Ig: Immunoglobulin
ITM: Immunological test method
MG: 4-Methylumbelliferyl galactoside
NG: Nitrophenyl galactoside
OPD: *o*-Phenylenediamine dihydrochloride
PBS: Phosphate-buffered saline
pNPP: *para*-Nitrophenyl phosphate
PVDF: Polyvinylidene fluoride
TMB: 3,3′,5,5′-Tetramethylbenzidine

Appendix 2: Additional Sources of Information about Specific Topics in Validation and Data Analysis

	Analytical Data—Interpretation and Treatment ⟨1010⟩	**Design and Analysis of Biological Assays** ⟨111⟩	**Validation of Compendial Procedures** ⟨1225⟩	**Biological Assay Chapters** ⟨1032⟩, ⟨1033⟩, and ⟨1034⟩
Means	X	—	—	—
Standard deviations	X	—	—	—
Standard errors	X	—	—	—
Non-normality	X	—	—	X
Nonconstant variance	X	—	—	X
Outliers	X	▲X▲ (USP 1-Dec-2019)	—	X
Tests	X	—	—	—
Confidence intervals	X	▲X▲ (USP 1-Dec-2019)	—	X
Validation	—	—	X	X
Combining results from multiple assays	—	X	—	X