Topic Introduction

Immunoassays

Thomas O. Kohl and Carl A. Ascoli

The enzyme immunoassay (EIA) is one of the most powerful of all immunochemical techniques. First described in the early 1970s, these assays are now used routinely in laboratory analyses and diagnostics. In biology and biotechnology, the EIA is a valuable and versatile tool used to detect and quantitate antigens and antibodies. Application of the appropriate EIA permits rapid quantification of different antigens and antibodies (referred to here as analytes) present at very low concentrations within a mixture. These assays are extremely sensitive and provide valuable information that would be difficult to determine by other techniques. Here we detail the development and optimization of the enzyme-linked immunosorbent assay (ELISA), a term generally used for any plate-based immunoassay that incorporates enzyme-, chemiluminescence-, or fluorescence-based reporters. It is amenable to standardization, automation, and large-scale sampling.

INTRODUCTION

Enzyme-linked immunosorbent assay (ELISA) assays are performed under conditions in which either the antibody is in excess, the antigen is in excess, or there is antibody competition or antigen competition. Assays performed with excess antibody (i.e., antigen competition) allow for the rapid detection and quantification of antigens. Conditions allowing for antigen excess (i.e., antibody competition) are used for the detection and quantification of antibodies present in samples.

There are several key factors to consider when developing an immunoassay. Select the microtiter plate that will allow for successful analyte (i.e., antigen or antibody) immobilization by considering properties of the analyte such as size and hydrophobicity. For the analyte adsorption process—referred to as plate coating—to be successful, it is necessary to determine parameters such as the analyte concentration, the coating time, and the temperature at which the process is to be performed, together with the pH, ionic strength, and constituents (e.g., detergents) of the coating buffer. The amount of antibody or antigen bound to the well surface is critical to the assay's sensitivity and has to be carefully controlled. Depending on the type of microtiter plate used, select a blocking buffer that will provide complete coverage of the remaining binding surface to minimize nonspecific background reactivity. Several commonly used blocking buffers are discussed below.

The optimal immunoassay format also depends on the type of antigen-specific antibodies used (e.g., polyclonal antibodies, affinity-purified polyclonal antibodies, single or multiple monoclonal antibodies), as well as whether the antigen preparations are homogeneous or heterogeneous. Table 1 can serve as a guide when evaluating different types of assay protocols. It presents possible assay choices in the order of general usefulness. When considering whether to use polyclonal or monoclonal antibodies, be aware that there are significant differences in lot-to-lot variability and

From the Antibodies collection, edited by Edward A. Greenfield.

Cite this introduction as *Cold Spring Harb Protoc*; doi:10.1101/pdb.top093690

TABLE 1. Choosing an assay protocol

Determination	Antibody availability	Antigen	Assay format[a]
Antigen presence or quantification	Polyclonal antibody	Homogeneous	Antigen capture (Ag competition); antibody capture (Ag competition)
		Heterogeneous	Antibody capture (Ab excess)
	Affinity-purified polyclonal antibody	Homogeneous	Two-antibody sandwich ELISA; antigen capture (Ag competition); antibody capture (Ag competition)
		Heterogeneous	Two-antibody sandwich ELISA; antibody capture (Ab excess)
	One monoclonal antibody	Homogeneous	Antigen capture (Ag competition); antibody capture (Ag competition)
		Heterogeneous	Antibody capture (Ab excess)
	Multiple monoclonal antibodies	Homogeneous	Two-antibody sandwich ELISA; antigen capture (Ag competition); antibody capture (Ag competition)
		Heterogeneous	Two-antibody sandwich ELISA; antibody capture (Ab excess)
Antibody presence or quantification[b]	Affinity-purified polyclonal antibody	Homogeneous	Antibody capture (Ag excess)
		Heterogeneous	—
	One monoclonal antibody	Homogeneous	Antibody capture (Ag excess)
		Heterogeneous	—
	Multiple monoclonal antibodies	Homogeneous	Antibody capture (Ag excess)
		Heterogeneous	—

[a]The choice of assay within each group is listed in order of preference.

[b]For most assay formats used to determine antibody levels, the antibody is detected using an anti-immunoglobulin antibody, and thus the antibody becomes the antigen.

consistency, increased sensitivity versus high specificity, and the time and costs required to prepare each type of antibody.

DETECTING AND QUANTITATING ANTIGENS

The most useful method to detect and quantify antigens is the sandwich ELISA (see Protocol: **Immunometric Antibody Sandwich ELISA** [Kohl and Ascoli 2017a]). This assay is quick and reliable and can be used to determine the relative level of most antigens. However, it requires either two monoclonal antibodies that bind to independent sites on the antigen or affinity-purified polyclonal antibodies.

If two monoclonal antibodies or affinity-purified polyclonal antibodies are not available, the next most useful assay for quantifying antigens is the competition assay. For a competition assay, a sample of pure or nearly pure antigen is required. There are two choices for assays using antigen competition. In an antigen capture assay (see Protocol: **Direct Competitive ELISA** [Kohl and Ascoli 2017b]), the antigen is labeled, and a constant amount of labeled antigen is mixed with the test solution containing an unknown amount of the antigen. Solutions are then allowed to bind to a subsaturating amount of antibody bound to the microtiter plate well. High levels of antigen in the test solution will reduce the amount of labeled antigen that can bind. In an antibody capture assay (see Protocol: **Indirect Competitive ELISA** [Kohl and Ascoli 2017c]), a sample of pure or partially pure antigen is bound to the microtiter plate well. Antigen in the test solution is mixed with a preparation of antibody, and both are added to the microtiter well. High levels of antigen in the test solution will block the binding of the antibody to the immobilized antigen.

If pure or partially pure antigen preparations are not available, the next most useful assay for antigen detection will be an antibody capture assay. Here the test solution is bound to the microtiter plate well, and a saturating amount of labeled antibody is used to detect the antigen. The use of highly specific monoclonal antibodies or affinity-purified polyclonal antibodies will be more accurate than polyclonal antibodies when the antigen is rare. As the antigen becomes more abundant, the specificity of the antibodies becomes less important.

Last, if rare antigens must be detected without the use of a pure antigen sample, the assay must be combined with a secondary technique to differentiate the antigen from assay-associated background. Commonly used secondary techniques include immunoprecipitation, immunoblotting, and cell staining.

DETECTING AND QUANTIFYING ANTIBODIES

If pure or nearly pure antigen preparations are available, the antibody presence and level can be determined using a variation of the antibody capture assay (see Protocol: **Indirect Immunometric ELISA** [Kohl and Ascoli 2017d]). Using this assay, purified or partially purified antigen is bound to the microtiter plate well, and the test solution containing an unknown amount of antibody is allowed to bind the immobilized antigen. The amount of bound antibody is determined by incubation with a labeled secondary reagent such as an anti-immunoglobulin antibody.

In many cases, it is easiest to determine the presence or level of antibodies by considering them to be a special class of antigen and to use anti-immunoglobulin antibodies to detect them. In these cases, many of the assays discussed above can be used to detect these "antigens."

ELISA detection methodology can be classified as either direct or indirect. The direct detection method uses an antigen-specific, reporter-labeled primary antibody for direct detection of the antigen. It is often used for analyte detection of complex antigen mixtures, such as host cell protein lysates, using polyclonal antibodies capable of recognizing high- and low-molecular-weight components. The host cell protein ELISA (Kohl and Ascoli 2017a) is commonly used in the pharmaceutical industry to show clearance of host cell proteins from finished preparations of biological drugs or to determine the lot-to-lot equivalence of therapeutic antibodies.

The indirect detection method uses a reporter-labeled secondary antibody specific for the detection of the primary antibody. Secondary antibodies are commercially available from a variety of sources including Jackson ImmunoResearch, KPL, Rockland Immunochemicals, and Southern Biotechnology. These secondary antibodies are generally cross-adsorbed; thus, they display minimal or negligible cross-reactivity to the capture antibody in the sandwich ELISA application. However, specificity to the primary detection antibody is generally achieved by use of antigen-specific capture and detection antibodies derived from different host species. Representative schematics of direct and indirect detection methods are shown in Figure 1.

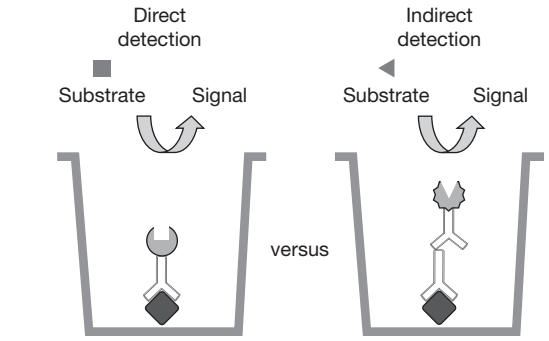
The use of reporter-labeled secondary antibodies greatly amplifies the signal because multiple secondary antibodies bind specifically to the primary antibody, thus elevating the sensitivity of assay. Even greater signal amplification can be achieved through use of biotinylated secondary antibodies in combination with reporter-labeled streptavidin, a tetrameric protein containing four biotin-binding sites. Streptavidin is superior to hen egg white avidin because the former shows less background reactivity. Addition of reporter-labeled streptavidin to the assay leads to the formation of streptavidin–biotin complexes, further increasing the presence of reporter and resulting in maximum signal amplification.

SELECTING THE APPROPRIATE MICROTITER PLATE

Immobilizing the analyte is a complex process affected by many different parameters, of which selection of a suitable plate is only one part of the process. In-depth knowledge of the analyte of interest's structure and available functional groups aids in selecting the appropriate microtiter plate surface for successful immobilization.

Microtiter plates come in a variety of formats ranging from the standard six-well configuration commonly used for tissue culture to the familiar 96- to 384-well layouts to strip plates and so-called single break strip plates used in the development and performance of ELISAs. Plates are generally molded from polystyrene, a long-carbon-chain molecule displaying pendant benzene rings on every other carbon. The surface chemistry of the plate wells is an integral part of the ELISA that is often overlooked. The surface area of the well and its chemical properties facilitate the immobilization of the analyte. Some analyte denaturation at the solid–liquid and solid–liquid–gas interfaces during the adsorption and washing processes is inevitable (Gibbs 2001); thus, selection of the appropriate well surface to ensure optimal adsorption of the analyte is crucial for the development of sensitive, precise, and accurate immunoassays.

Cite this introduction as *Cold Spring Harb Protoc;* doi:10.1101/pdb.top093690



Advantages

- Rapid protocol—use of only one antibody requiring fewer steps in the ELISA
- Elimination of potential secondary antibody cross-reactivity

Disadvantages

- Potentially decreased antibody immunoreactivity resulting from conjugation to a reporter
- Costly and time-consuming labeling procedure
- Limited choice of labeled primary antibody between experiments
- Minimal signal amplification

Advantages

- Multitude of commercially available secondary antibodies
- Versatility—use of one labeled secondary antibody for the detection of multiple different antibodies generated in a single host species
- Substitution of different reporter labeled secondary antibody
- Noncompromised immunoreactivity
- Increased sensitivity through signal amplification

Disadvantages

- Potential cross-reactivity resulting in nonspecific signal
- Additional steps in the ELISA procedure

FIGURE 1. Direct versus indirect ELISA detection methods.

Analyte adhesion is achieved through a variety of different mechanisms. Polystyrene is a hydrophobic compound displaying surface properties that naturally facilitate the transient binding of analytes through hydrophobic interactions and hydrogen bonding—termed passive adsorption. This surface property, in conjunction with the pH of the coating buffer and the incubation time and temperature of the reaction, permits larger analytes such as immunoglobulins to be immobilized and used in a wide variety of applications. Table 2 presents the properties of microtiter plates whose surface chemistries range from completely hydrophobic to very hydrophilic, thus facilitating the binding of different analytes possessing a range of hydrophobic and hydrophilic domains. High-binding polystyrene, generated through modification of the surface by radiation or amination, facilitates adsorption of analytes by ionic and covalent bonding. This surface is less denaturing to the analyte and promotes low nonspecific binding through specific bond formation by appropriate functional groups and/or cross-linkers. Microtiter plates that facilitate specific covalent binding of tagged analytes by affinity capture technique display defined and consistent binding characteristics. Functional groups or cross-linkers are capable of selectively adsorbing tagged analytes and, therefore, provide site-directed covalent linkage of the analyte to its specific immobilizer, resulting in a high signal-to-noise ratio while minimizing background leaching. Detailed functionalities of microtiter plate well surfaces are provided by plate manufacturers. This information along with Table 2 should aid in the selection of the best surface chemistry to use for the successful adsorption of a desired analyte.

TABLE 2. Microtiter plate surface properties

	Modification	Surface properties	Analyte–surface interaction	Application properties	Binding capacity	Analyte denaturation
Hydrophobic	Unmodified polystyrene	Medium binding	Passive adsorption	Immobilization of larger analytes (>20 kDa) displaying abundant hydrophobic domains	Low ~100–200 ng IgG/cm^2	Yes, biomolecules unfold to expose hydrophobic regions
	Radiation	High binding	Passive adsorption; Ionic, mediated by incorporation of carboxylic acid on benzene ring-exposed carbons	Immobilization of positively charged medium to large analytes (>10 kDa) with or without hydrophobic domains	Increased ~400–500 ng IgG/cm^2	Yes, possible steric interference
Hydrophilic	Maleic anhydride/ maleimide activated	High binding	Hydrophilic Ionic or covalent, mediated by bifunctional cross-linkers such as bis-sulfosuccinimidyl suberate, carbodiimide, or monomeric gluteraldehyde	Immobilization of negatively charged small analytes or molecules displaying a functional amine, carboxyl, sulfhydryl, or thiol group	Increased ~2 × 10^{13} reactive sites/cm^2	Reduced
	Precoated	High binding	Protein A, G, or A/G; Protein L; streptavidin; biotin; nickel; copper; glutathione; anti-GFP; anti-GST; secondary antibodies	Immobilization of tagged proteins or antibodies	Increased	Reduced

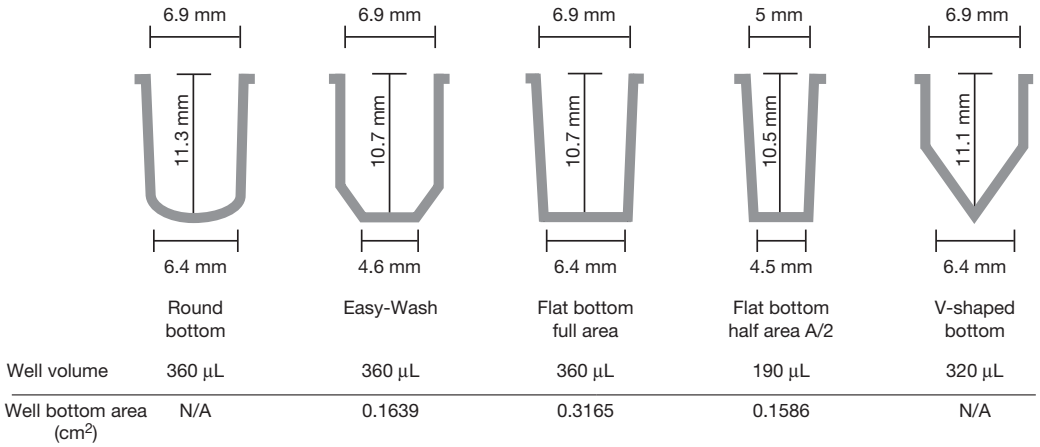


FIGURE 2. Microtiter plate well shape and dimensions.

Aside from differences in surface chemistry, microtiter wells are molded in different shapes (Fig. 2). The standard 96-well microtiter plate is most commonly used in general laboratory ELISA applications. Individual wells of a 96-well plate hold 320–360 µL. The recommended working volume for an ELISA is 300 µL per well. Analyte adsorption in a total volume of 100 µL covers a well surface area ranging from 0.84 to 0.94 cm^2 depending on its shape. The half-area (*A*/2) well has been designed to facilitate performance of an ELISA under limiting analyte availability or cost-prohibitive conditions and accommodates a working volume of 50 µL (Gibbs 2001). Flat-bottom wells, which are used most often, allow for exceptional optical transmission with reduced background absorbance. Their use in immunoassays results in the retention of up to 10 µL of residual liquid along the sharp edges of the well bottom, rendering them suitable for immunoassays in which the analyte might be overly susceptible to extensive drying. V-shaped bottom wells are optimally suited for magnetic bead or agglutination assays because their cone shape facilitates trouble-free removal of well contents after concentration by centrifugation at up to 6000*g*. The round or U-bottom well improves analyte mixing and ensures complete liquid removal during washing steps in the ELISA. It enhances the overall assay sensitivity in fluorescent applications. The Easy-Wash (Corning) well is a combination of the flat and round bottom wells. While maintaining exceptional optical transmission properties, the round-to-narrowed flat well bottom significantly improves washing and liquid removal steps in the ELISA. All well shapes are compatible with microplate washers and readers, although adjustments have to be made to accommodate the rounded shape and are dependent on the available plate washer in use.

Clear or transparent microtiter plates are manufactured from pure polystyrene. Independent of their defined surface binding properties, these chemically resistant plates facilitate greater sample visibility and are best for colorimetric ELISAs. Solid black or white microtiter plates are recommended for use in fluorescence and biochemiluminescence or chemiluminescence assays, respectively. Black plates significantly reduce background caused by autofluorescence, whereas white plates offer low background luminescence. Black and white plates are also manufactured with clear well bottoms, making them extremely useful for ELISAs in which simultaneous colorimetric and either fluorescence or chemiluminescence detection might be required.

ELISA BLOCKING BUFFERS

During the initial plate-coating step, the analyte is adsorbed onto the microtiter plate. The next step is to block the wells properly. Blocking is critical in the performance of a reliable ELISA because it prevents nonspecific binding of other molecules to the well surface and the adsorbed analyte, thus

reducing background interference. Critically, the blocking step also stabilizes the immobilized analyte and enhances interactions between the antigen and the antibody (Huber et al. 2009).

No blocking buffer is ideal for every immunoassay. The optimal blocking buffer to use depends on the goals of the ELISA and the blocking buffer's compatibility with other assay components. All other things being equal, the most suitable blocking buffer in a particular assay is the one that aids in generating the highest signal-to-noise ratio. Insufficient blocking will result in high backgrounds, whereas an excess of blocking can prevent antigen–antibody interactions or inhibit functionality of the assay-specific reporter. Regardless of which blocking buffer you choose, it should be optimized for every new ELISA. This is best done during development of the assay. The following blocking buffers are commonly used.

BLOTTO Blocking Buffer

BLOTTO blocking buffer is used in many different types of ELISA. It consists of a 5% (w/v) nonfat powdered milk solution prepared in Tris-buffered saline (TBS) or phosphate-buffered saline (PBS). Although available as a ready-to-use 1× TBS solution, BLOTTO is easy to prepare in-house, thus saving money and permitting dilution of assay-specific antibodies in a recommended 2% (w/v) BLOTTO solution (Alegria-Schaffer et al. 2009). BLOTTO blocking buffer is not recommended in streptavidin/avidin-based techniques because of the presence of endogenous biotin in the buffer.

Bovine Serum Albumin (BSA)-Based Blocking Buffer

Stock solutions of bovine serum albumin (BSA)-based blocking buffers are prepared by dissolving 10% (w/v) BSA, Fraction V (which has very low Ig content), in either TBS or PBS. The working solution is 5% (v/v) BSA. BSA blocking buffer has the advantage over BLOTTO blocking buffer in that it contains only one purified protein. This minimizes potential cross-reactions in the ELISA and improves assay sensitivity by reducing the background signal. Devoid of endogenous biotin, BSA blocking buffer can be used in ELISAs that incorporate biotin and avidin.

Casein-Based Blocking Buffers

Casein-based blocking buffers contain casein protein purified from milk by the Hammarsten method (Van Slyke and Baker 1918). Commercially available 1% (w/v) ready-to-use solutions in TBS or PBS are the optimal concentration for most ELISA blocking steps. As with BSA-based blocking buffers, this buffer also consists of only one purified protein in solution, thus limiting cross-reactions in the ELISA. Some casein blocking buffer preparations contain 0.1% sodium azide and should not be used as diluents for the preparation of horseradish peroxidase (HRP)-conjugated antibodies.

Serum

Although not used routinely in ELISAs, nonimmune sera from goat, human, mouse, goose, and rabbit serve as blocking agents for tissue immunohistochemical staining and various other cellular imaging methods. Delipidated sera contain the endogenous complement of serum proteins capable of adequately blocking nonspecific binding sites and provide a natural chemical environment for antibodies when applied as diluents.

Gelatin

A commonly applied agent for efficient blocking of ELISA microtiter plates is derived from fish skin extracts. Fish gelatin is commercially available and fish gel-based blocking buffer can be used in the ELISA as a 3% (v/v) preparation stabilized in PBS. Gelatin displays several advantages over other common blocking buffers because its protein content is unlikely to facilitate any binding interactions with analytes of mammalian origin and it effectively stabilizes solutions, thereby facilitating antibody-binding interactions. Application of gelatin in the ELISA results in a reduction or elimination of background reactivity leading to increased signal-to-noise ratios.

Cite this introduction as *Cold Spring Harb Protoc;* doi:10.1101/pdb.top093690

Protein-Free Blocking Buffers

Protein-free blocking buffers are commercially available as ready-to-use preparations in either TBS or PBS, with or without 0.05% (v/v) Tween 20. These proprietary nonprotein blocking buffers can be used in an ELISA to ensure complete blocking of available binding surfaces. Because of the absence of proteins in these buffers, they have been shown to eliminate the analyte cross-reactivity that is commonly associated with protein-based blocking buffers. These buffers have been used in many other immunochemical applications, such as the Western blot, where, under optimized conditions, they generate extremely low background and a high signal-to-noise ratio.

ELISA WASH BUFFERS

The washing buffer often used in many ELISAs is PBS or TBS, with or without detergent. Depending on the assay format and assay-specific reagents, a nonionic detergent such as Tween 20, Tween 80, Triton X-100, Triton X-114, Nonidet P-40, Brij-35, or Brij-58 is included in the ELISA wash buffer. Detergents are typically added to concentrations between 0.2% and 1.0%.

REPORTER-LABELED SECONDARY ANTIBODIES

The preferred method of analyte detection in an ELISA is indirect detection using reporter-labeled secondary antibodies. Depending on the ELISA, a variety of different reporter labels are commercially available, eliminating the time required to produce them in-house and often reducing the overall cost of running the ELISA. Secondary antibodies have been raised against numerous species-specific primary antibodies and are available for detection of whole antibody molecules or fragments. Although it is not feasible to generate secondary antibodies capable of self-recognition (e.g., chicken/chicken) or of closely related species (e.g., goat/bovine), an appropriate selection among the many chromophore- or fluorophore-labeled secondary antibodies should aid in generating the best results from your ELISA. For example, the selection highlighted in bold in Table 3 would represent a whole molecule goat antibody targeting chicken IgM conjugated to peroxidase. Table 3 lists combinatorial arrangements resulting in some of the most common secondary antibodies used in the detection step of an ELISA.

TABLE 3. Reporter-labeled secondary antibodies

Raised in	Antibody form	Raised against	Immunoglobulin target	Conjugated reporter
		Bovine	IgG1	
		Cat	IgG2a	Unconjugated
		Chicken	IgG2b	Enzyme-labeled
		Dog	IgG3	• ***Peroxidase***
Chicken		Donkey	IgG4	• *Alkaline phosphatase*
Dog		Ferret	IgG	• β-*Lactamase*
Donkey		Goat	IgA	Biotinylated
Goat		Guinea Pig	**IgM**	Dyes
Guinea pig	**Whole molecule**	Hamster, Armenian	IgD	• *Texas Red*
Hamster, Armenian		Hamster, Golden Syrian	IgE	• *Rhodamine*
Hamster, Golden Syrian	$F(ab')_2$	Horse	IgG, IgA, IgM	• *Fluorescein*
Horse		Human	IgG1 Fc region	• *IRDye*
Mouse	Fab	Monkey	IgG2 Fc region	• *ATTO-TEC*
Rabbit		Mouse	IgG2 Fd region	• *DyLight*
Rat		Rabbit	IgG3 hinge region	• *CyDye*
Sheep		Rat	IgG4 Fc region	• *Biotium CF*
		Sheep	IgY	• *Brilliant Violet*
		Swine	IgM Fc5 μ region	• *Chromeo*
		Turkey	κ light chain	
			λ light chain	

IMMUNOASSAY SUBSTRATES

The use of enzyme-linked secondary antibodies in an ELISA requires a suitable substrate for detection. Substrates varying in their degree of sensitivity, ease of use, and compatibility with imaging equipment are commercially available for chromogenic, chemiluminescence, chemifluorescence, and fluorescence detection methods. Chromogenic substrates are available for alkaline phosphatase (AP)- and HRP-labeled antibodies. These substrates are described below, in order of decreasing sensitivity.

Chromogenic (Colorimetric) Substrates

The most sensitive chromogenic substrate for detection of HRP-labeled antibodies in an ELISA is 3,3′,5,5′-tetramethylbenzidine (TMB), which is available from a variety of commercial sources (e.g., Sigma-Aldrich, SurModics, and Rockland Immunochemicals). This ready-to-use soluble substrate rapidly yields a blue color on enzymatic processing and delivers a measurable maximum optical density (OD) at 652 nm (OD_{652}). The reaction is halted by adding hydrochloric, sulfuric, or phosphoric acid, resulting in an immediate color change with a maximum absorbance at 450 nm (OD_{450}). TMB substrate sensitivity ranges from ~20 to 80 ng/mL depending on the dilution factors of the primary and secondary antibodies. Use of TMB in the presence of excess antibody can contribute to an increased background signal. Note that the insoluble form of TMB typically used for immunoblotting will not work as a substrate in an ELISA.

O-Phenylenediamine dihydrochloride (OPD) requires preparation in substrate buffer before use in an ELISA. HRP-mediated processing of OPD results in the development of a yellow/orange color with a maximum absorbance at 450 nm. Addition of 2.5 M sulfuric acid terminates the reaction and causes a color change from yellow/orange to green, with maximal absorbance at 490 nm. OPD can be detected down to ~70 ng/mL.

2,2′-Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) is a slow-reacting substrate displaying significantly decreased background reactivity. HRP-mediated substrate hydrolysis results in green color development measurable at 410 and 650 nm. Its detection sensitivity is ~2.5 ng/mL.

para-Nitrophenyl phosphate (pNPP) disodium salt is the most commonly used substrate for the detection of alkaline phosphatase-conjugated antibodies in an ELISA. In the presence of alkaline phosphatase, pNPP generates a yellow color measurable at 405 nm following addition of 2 N NaOH. The detection limit of pNPP is ~100 ng/mL. Working solutions should be prepared just before use, although commercially available ready-to-use formulations are stable for up to twelve months at 4°C. Alkaline phosphatase activity is optimal at pH 8–10. The enzyme reaction is inhibited by inorganic phosphates, cyanides, arsenate, and divalent cation chelators such as EDTA.

Less commonly used substrates include soluble *O*-nitrophenyl-β-D-galactopyranoside (ONPG) in an ELISA incorporating β-galactosidase-conjugated reagents. In conjunction with naphthol-AS-Bl-β-D-galactopyranosidase (Nap-Gal) and 4-methyl-umbelliferyl-β-D-galactopyranosidase (MUm-Gal), these substrates result in a yellow color development measurable at a maximal absorbance of 405 or 410 nm after the addition of 1 M sodium carbonate. A 3 mM ONPG solution in phosphate-buffered saline containing 10 mM $MgCl_2$ and 0.1 mM 2-mercaptoethanol must be prepared immediately before use in an ELISA.

Chemiluminescent Substrates

Chemiluminescent ELISA substrates for use with HRP-labeled antibodies offer sensitivity in the femtogram per milliliter (fg/mL) range, which is significantly greater than any of the chromogenic substrates. High-performance luminol-based chemiluminescent substrates are available from several sources (e.g., Thermo/Pierce, Invitrogen, and Rockland Immunochemicals) and display sensitivities to subpicogram levels at optimal antibody concentrations, yielding high signal-to-noise ratios with minimal background. These substrates offer reduced substrate development time with maximum signal detection measurable at an emission (E_{max}) wavelength of 425 nm within 1 min of incubation.

 Cite this introduction as *Cold Spring Harb Protoc;* doi:10.1101/pdb.top093690

Pierce's SuperSignal ELISA Pico Chemiluminescent Substrate is suitable for use in black or white opaque plates, providing greater flexibility in choosing which type of assay to use.

Chemifluorescent Substrates

10-Acetyl-3,7-dihydroxyphenoxazine (ADHP)–based red chemifluorescent substrates for use with HRP-labeled antibodies in an ELISA include, among others, QuantaRed Enhanced Chemifluorescent HRP (Thermo Scientific-Pierce), Amplite (ATT Bioquest), and Amplex Red (Invitrogen) (Held 2003), all of which yield soluble resorufin. The excitation/emission maxima for each of the commercial substrates are ~572/588 nm (QuantaRed), 571/585 nm (Amplite), and 530/590 nm (Amplex Red). These ready-to-use substrates provide fluorescence detection levels in the picogram-to-femtogram (0.16–4 to 2500 pg/mL) range, sensitivities comparable to enhanced chemiluminescence detection methods. Results can also be evaluated by colorimetric measurement at wavelengths of 572 and 560 nm, respectively.

p-Hydroxyphenyl compounds, including 3-(*p*-hydroxyphenyl) propionic acid [HPPA], *p*-hydroxyphenethyl alcohol, hordenine, *p*-ethylphenol, 3-(*p*-hydroxyphenyl)-1-propanol, *p*-*n*-propylphenol, and *p*-hydroxyphenyllactic acid are excellent substrates for HRP-mediated reactions in a fluorogenic ELISA (Zaitsu and Ohkura 1980; Matsumoto et al. 1984). One commercially available version of these substrates is QuantaBlu Fluorogenic Peroxidase Substrate (Thermo Scientific-Pierce), which is available in two separate formulations to allow for the performance of kinetic and nonkinetic ELISAs with sensitivities exceeding that of chromogenic substrates. QuantaBlu excitation at 325 nm yields a maximum emission measurable at 420 nm; however, flexible excitation wavelengths from 315 to 340 nm result in measurable emission between 370 and 470 nm.

As with other components of an ELISA, each immunoassay substrate should be optimized for use within the specific ELISA during development of the assay. Highly sensitive substrates are more difficult to optimize because nonspecific binding or inefficient blocking of the microtiter well can skew the signal-to-noise ratio. Thus it is best to choose ELISA substrates that can function at or below an assay-specific detection limit followed by subsequent optimization of both assay-specific antibody dilutions and reaction/reading times to achieve usable assay results (Held 2003).

ELISA PARAMETERS

ELISA development guidelines set forth by the NIH Chemical Genomics Center determine the strengths and limitations of designed assays throughout the development process. Not all ELISAs are the same, and some assays show high specificity and sensitivity, whereas others do not. The intrinsic properties of the analyte ultimately determine many assay limitations; however, the variation of assay-specific parameters aids in the optimization of assay performance. Among ELISA parameters, the limit of detection (LoD) for an analytical procedure is defined by the point at which the analysis is just feasible (Fig. 3). An assay is simply not capable of accurately measuring analyte concentrations down to 0. A sufficient analyte concentration must be present to produce an analytical signal that can be reliably distinguished from the analytical noise, or limit of blank (LoB)—the signal produced in the absence of analyte. The LoD of any assay can be determined by either a statistical approach in which negative (i.e., blank) samples are measured in replicate to determine the LoB—a reasonable starting point for the estimation of the LoD—or by the alternative empirical approach entailing the measurement of progressively more dilute concentrations of the analyte. Furthermore, the limit of quantification (LoQ) is defined as the analyte concentration at which quantitative results can be reported with a high degree of confidence. Not only is it defined by the lowest analyte concentration that can be reliably detected, but it also fulfills predefined goals for bias and imprecision (Armbruster and Pry 2008).

The limit of linearity (LoL) of an ELISA is defined as the analyte concentration at which the calibration curve departs from linearity by a specified amount. A deviation of ~5% is usually considered the upper limit and is frequently observed at higher analyte concentrations. The range between

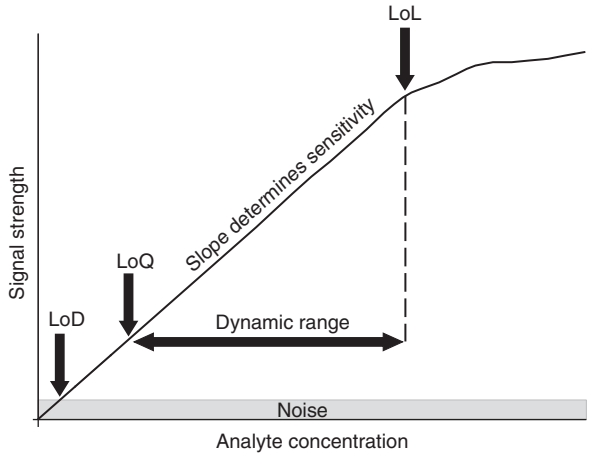


FIGURE 3. Calibration curve indicating the LoD (limit of detection), the LoQ (limit of quantification), the LoL (limit of linearity), the assay sensitivity, and the dynamic range.

the LoQ and LoL is referred to as the "dynamic range" in which the sensitivity of the ELISA is determined by the interplay between analyte concentration and signal strength. Once developed, an ELISA is validated to determine precision and suitability as well as other predetermined characteristics of the assay.

ELISA VALIDATION PARAMETERS

The validation of an analytical ELISA method is the process by which it is established, ensuring that the performance characteristics of the method meet the requirements for the intended analytical application. ELISA performance characteristics are expressed in terms of elements including, but not limited to, the individual parameters discussed below.

The validity of an analytical ELISA method can only be verified by laboratory studies. Therefore, the documentation of the successful completion of such studies forms the basic requirement for determining whether the method is suitable for the intended application. The guidelines, described in this section, on the fundamental validation parameters for immunoassays are presented in the U.S. Pharmacopeia Chapter 1225, *Validation on Compendial Methods* (Pharmacopeia 2009), and the ICH *Q2 (R1) on the Validation of Analytical Procedures: Text and Methodology* (ICH 2005) and aid in the laboratory-based development and validation of robust immunoassays for detection of analytes. Method requirements with acceptance criteria defined within a protocol must be met to ensure method validation including the individual elements as described below.

Precision

Defined as the degree of agreement between a series of measurements obtained from multiple sampling of the identical homogenous sample, the precision encompasses the anticipated range of the assay and should be considered under the following criteria:

- **Reproducibility/repeatability**: Results obtained when the analysis is performed in a laboratory by an operator using a piece of equipment over a relatively short time span. At least six determinations of three different matrices at two or three different concentrations should be performed and the relative standard deviation (RSD) calculated.
- **Intermediate precision**: Results generated by a different analyst using different equipment followed by evaluation and documentation.

Cite this introduction as *Cold Spring Harb Protoc*; doi:10.1101/pdb.top093690

Accuracy

Accuracy is defined as the extent to which test results generated by the method and the true value agree. Accuracy is the closeness of test results obtained by the analytical method to the true value. Accuracy is reported as the percent recovery of known added amounts of analyte in the sample matrix. If certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration of analyte. Each spike and blank sample will be analyzed, and the blank result will be subtracted from the spiked result. The percent recovery is expressed relative to the targeted spike concentration of analyte added and is assessed using a minimum of six determinations over a minimum of three concentration levels in the representative pool of data obtained from multiple analyses. Accuracy is reported as the percent recovery by the assay of a known added amount of analyte in the sample or as the difference between the mean and the accepted true value, together with the confidence intervals.

Linearity

Linearity is defined as the ability to elicit test results that are directly proportional to the concentration of analyte in samples within a given range. Linearity can be shown directly on the analyte by dilution of a standard stock solution by a series of three to six analyses of five or more analyte standards whose concentrations span 80%–120% of the expected concentration range. The response should be directly proportional to the concentrations of analyte. A linear regression equation applied to the results should have an intercept not significantly different from 0. If a significant nonzero intercept is obtained, it should be shown that this has no effect on the accuracy of the method. Linearity should yield a correlation coefficient >0.9; should this not be the case, the ELISA should be repeated to properly determine the limits of quantification and detection.

Quantification Limit

The LoQ is defined as the minimum amount of analyte that produces quantitative measurements within the target matrix with acceptable precision. Several samples with decreasing amounts of analyte are analyzed six times. The calculated RSD percent of the precision is plotted against the analyte amount. The amount that corresponds to the previously defined required precision is equal to the LoQ. It is important to use not only pure analyte standards for this test but also spiked matrices that closely represent unknown samples. The percentage recovery is calculated for each preparation and the mean recovery documented.

Limit of Detection

The limit of detection is defined as the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. The LoD is the amount that results in a peak with a height at least two or three times as high as the baseline noise level. Analyte standards must be prepared and evaluated to verify the LoD. Analyte must be detected in all analyses for the concentration to meet the LoD requirement. The analyte LoD might need to be determined by trial and error and the concentration documented.

Sensitivity

Sensitivity is defined as the lowest concentration of analyte that the analytical procedure can reliably differentiate from background noise. The sensitivity is expressed as the slope of the linearity regression.

Specificity

Specificity is defined as the ability to assess analyte unequivocally in the presence of components that may be expected to be present. Typically, these might include impurities, degradation products, and the matrix.

Range

The range is defined as the interval between the upper and lower analyte concentration in the sample for which it has been shown that the analytical procedure has a suitable level of precision, accuracy, and linearity.

TYPES OF IMMUNOASSAYS

Variations in ELISA methodology determine the criteria under which an immunoassay can be performed. Qualitative and quantitative assays share common principles and individual steps; however, the variable being analyzed might change. For example, by varying certain key conditions within the procedure, an assay can be altered to determine different levels of antigen or antibody within biological samples.

It is not always apparent which assay format should be chosen, particularly to the novice. This section is designed to guide the researcher on the appropriate selection of an assay for different applications. Immunometric assays are divided into noncompetitive and competitive formats. The noncompetitive two-antibody sandwich ELISA captures an antigen using an immobilized antigen-specific antibody before detection with a reporter-labeled second antigen-specific antibody (see Protocol: **Immunometric Double-Antibody Sandwich ELISA** (Kohl and Ascoli 2017e). The signal strength that is measured is directly proportional to the concentration of antigen present in the sample, which permits rapid quantification of the analyte. In the noncompetitive antibody capture assay (Kohl and Ascoli 2017d), the plate-immobilized antigen facilitates binding of the antigen-specific antibody and serves to determine the antibody concentration present in the sample.

The one-step competitive immunoassay uses surface immobilization of the capture antibody followed by the addition of a proportional mixture of unlabeled antigen and reporter-labeled antigen to individual wells (Pekka 1991; Kohl and Ascoli 2017b). Both antigen species compete for a limited number of antibody-binding sites. Because all immunoassay formats include washing steps during which unbound analytes are removed, a lower level of detectable reporter-labeled antigen in the competitive assay is indicative of the presence of a greater amount of unlabeled antigen. Thus, the resultant signal strength is inversely proportional to the antigen concentration present in the sample.

In the two-step competitive immunoassay (Pekka 1991; Kohl and Ascoli 2017c), the antibody concentration exceeds the unlabeled antigen concentration, thus allowing for the formation of antigen:antibody complexes before adding the mixture to antigen that has been immobilized to the wells of the microtiter plate. Available antibody binds to the immobilized antigen as a function of free antigen concentration. A reduced signal indicates that there is a greater concentration of antigen present within the sample. This assay provides several-fold increased specificity over the one-step format described above.

Cell-Based ELISA

Cell-based ELISA (see Protocol: **Direct and Indirect Cell-Based ELISA** [Kohl and Ascoli 2017f]) has developed into a popular alternative immunoassay for the rapid detection of expressed cell-surface antigens or receptors. It is used to determine cell surface antigen expression profiles using existing reporter-labeled antibodies. The target specificity of newly developed antibodies can be determined by the indirect approach, which screens hybridoma supernatants for antibody reactivity against the target

Cite this introduction as *Cold Spring Harb Protoc;* doi:10.1101/pdb.top093690

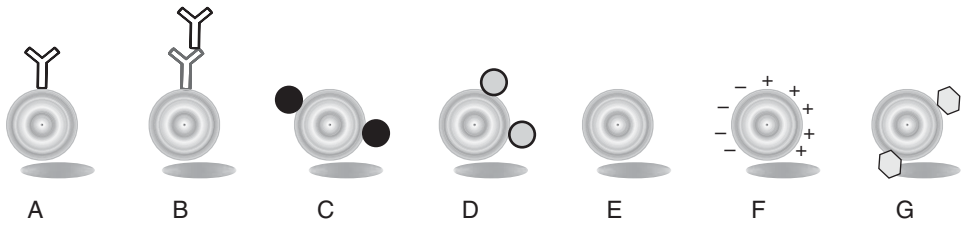


FIGURE 4. Beads for use in multiplex ELISA applications with different surface functionalities. (*A*) Beads coupled with target-specific primary antibody. (*B*) Beads coupled with species-specific secondary antibody. (*C*) Beads coupled with Protein A. (*D*) Beads coupled with Protein G. (*E*) Beads with a specific surface functionality for direct coupling of the analyte of interest. (*F*) Beads with a surface charge. (*G*) Beads coupled with streptavidin.

antigen of interest. The cell-based ELISA acts as a surrogate for the identification of immunohistochemistry-reactive antibodies and aids in hybridoma-derived antibody identification, which is used for further ELISA development. Cell-based ELISAs have detection sensitivities comparable to quantitative analyses by flow cytometry and can be developed in multiplex format using different reporters for the analysis of cell populations.

Bead-Based ELISA

The microtiter plate-based ELISA has long been the standard for quantitative analysis of antigens and antibodies but is a poor choice for high-throughput multiplex analyses. An alternative—the multiplex bead array—has the ability to provide simultaneous quantitative measurement of large numbers of soluble antigens within a small sample volume using an automated 96-well plate format. In the bead-based ELISA, microscopic beads coated with antibodies that capture antigens (or antigen-coated beads to capture antibodies) replace the antibody-coated microtiter plate wells. The beads are available in various sizes and with different surface functionalities, enabling them to be used in a wide variety of multiplex ELISA applications (Fig. 4).

First, color-coded beads are coated with either antibodies or antigens, and the beads are added to microtiter plate wells. Standards and test samples are added to the coated beads and incubated. Unbound analytes are washed away, and then an analyte-specific biotinylated antibody cocktail is added. The beads are washed and reporter-labeled streptavidin is added. Unbound streptavidin is washed away, and the beads are resuspended in buffer and measured using a Luminex analyzer. The reporter-derived signal is directly proportional to the concentration of analyte present within the sample (Kala et al. 1997; Elshal and McCoy 2006).

REFERENCES

Alegria-Schaffer A, Lodge A, Vattem K. 2009. Performing and optimizing western blots with an emphasis on chemiluminescent detection. *Methods Enzymol* **463:** 573–599.

Armbruster D, Pry T. 2008. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev* **29:** S49–S52.

Elshal MF, McCoy JP. 2006. Multiplex bead array assays: Performance evaluation and comparison of sensitivity to ELISA. *Methods* **38:** 317–323.

Gibbs J. 2001. *Immobilization principles: Selecting the surface*. ELISA technical bulletin no. 1. Corning Incorporated Life Sciences, Tewksbury, MA.

Held P. 2003. *Determination of horseradish peroxidase (HRP) using Amplex® Red and the Synergy™ HT multi-mode microplate reader*. Application note, April 21. BioTek Instruments, Winooski, VT.

Huber D, Rudolf J, Ansari P, Galler B, Führer M, Hasenhindl C, Baumgartner S. 2009. Effectiveness of natural and synthetic blocking reagents and their application for detecting food allergens in enzyme-linked immunosorbent assays. *Anal Bioanal Chem* **394:** 539–548.

ICH. 2005. ICH harmonised tripartite guideline: Validation of analytical procedures: Text and methodology Q2(R1). International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva.

Kala M, Bajaj K, Sinha S. 1997. Magnetic bead enzyme-linked immunosorbent assay (ELISA) detects antigen-specific binding by phage-displayed scFv antibodies that are not detected with conventional ELISA. *Anal Biochem* **254:** 263–266.

Kohl TO, Ascoli CA. 2017a. Immunometric antibody sandwich ELISA. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot093716.

Kohl TO, Ascoli CA. 2017b. Direct competitive ELISA. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot093740.

Kohl TO, Ascoli CA. 2017c. Indirect competitive ELISA. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot093757.

Kohl TO, Ascoli CA. 2017d. Indirect immunometric ELISA. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot093708.

Kohl TO, Ascoli CA. 2017e. Immunometric double-antibody sandwich ELISA. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot093724.

Kohl TO, Ascoli CA. 2017f. Direct and indirect cell-based ELISA. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot093732.

Matsumoto T, Furuta T, Nimura Y, Suzuki O. 1984. 3-(*p*-Hydroxyphenyl) propionic acid as a new fluorogenic reagent for amine oxidase assays. *Anal Biochem* **138:** 133–136.

Pekka V. 1991. One- and two-step non-competitive avidin–biotin immunoassays for monomeric and heterodimeric antigen. *J Immunol Methods* **136:** 77–84.

Pharmacopeia (USP). 2009. *Validation of compendial methods*, USP 32–NF 27, General Chapter 1225. United States Pharmacopeia, Rockville, MD.

Van Slyke LL, Baker JC. 1918. The preparation of pure casein. *J Biol Chem* **35:** 127–136.

Zaitsu K, Ohkura Y. 1980. New fluorogenic substrates for horseradish peroxidase: Rapid and sensitive assays for hydrogen peroxide and the peroxidase. *Anal Biochem* **109:** 109–113.

Cite this introduction as *Cold Spring Harb Protoc;* doi:10.1101/pdb.top093690

Immunoassays

Thomas O. Kohl and Carl A. Ascoli

Cold Spring Harb Protoc; doi: 10.1101/pdb.top093690