Chapter

Optimization, Validation and Standardization of ELISA

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Abstract

The enzyme-linked immunosorbent assay (ELISA) is a commonly used analytical immunochemistry assay based on the specific bond between the antigen and the antibody. The application of this test has significantly changed the practice of medical laboratories in which it is used for detection and quantification of molecules such as hormones, peptides, antibodies, and proteins. Various technical variants of this test can detect antigen (native or foreign) or antibody, determine the intensity of the immune response whether pathological or not; the type of induced immune response as well as the innate immunity potential; and much more. These capabilities, as well as the high sensitivity and robustness of the test and a small price, make it possible to quickly and reliably diagnose diseases in most laboratories. Besides, ELISA is a test that is also used in veterinary medicine, toxicology, allergology, food industry, etc. Despite the fact that it has existed for almost 50 years, different ELISA tests with different technical solutions are still being developed, which improves and expands the application of the this exceptional test. The aim of this chapter is to empower the rider to optimize, standardize and validate an enzyme linked immunosorbent assay.

Keywords: ELISA, optimization, standardization, validation, accuracy, precision

1. Introduction

Enzyme linked immunosorbent assay (ELISA) has existed for 50 years and ELISAs with different technical solutions are still being developed, which improves and expands the range of application.

The test was first described by Engvall and Perlmann in 1971 [1–3] and was based on the work of Avrameas, who used enzyme linked antibodies in histochemistry [4, 5]. The method was quickly developed for sero-diagnosis of trichinosis [6] and antibodies to *Plasmodium vivax* and *P. falciparum* [7], to be used in epidemiological studies of malaria.

Since the discovery there have been numerous applications of ELISA, used to detect both antigens and antibodies. Besides the detection of protein antigens ELISAs that permit the determination of antibodies to native and denatured DNA [8, 9], polysaccharide antigens [10–12] and phospholipids [13] have been optimized. In fact, sometimes the name ELISA is applied to tests in which there are no antibodies, but instead specific protein–protein interactions are used. From the perspective of optimization, validation and standardization such tests can be treated in the same way. Regarding protein antigens the sensitivity of ELISA is usually in the pg/ml range [14].

When developing a diagnostic test, precise and optimal performance conditions must be found for all the steps within the test protocol. This ensures that the entire

procedure is optimal. Before routine usage in diagnostics, for example, the newly developed, or a newly modified procedure must be proven to be accurate, precise and reproducible. Also, in order to measure the values obtained with the test, it is necessary to standardize the test. Therefore, optimization, validation and standardization (OVS) of ELISA are extremely important and necessary, especially if it is to be used in clinical or veterinary medicine. This chapter will present the procedures by which ELISA is characterized in an understandable and precise way.

Reviewing the literature, we noticed that the described boundaries between optimization, standardization and validation are not clear enough. The reason for this is that in certain situations performing a single ELISA can lead to a completion of both validation and optimization characteristics, which is completely valid. Before going into more details and in order to avoid confusion it is suitable to clearly define these three terms.

According to Merriam-Webster dictionary,

Optimization is: "an (act, process, or methodology of making something such as a design, system, or decision) as fully perfect, functional, or effective as possible."

Validation is: "an act, process, or instance of validating *especially*: the determination of the degree of validity of a measuring device."

Standardization is**:** "to bring into conformity with a standard especially in order to assure consistency and regularity ... to compare with a standard: to determine the strength, value, or quality of (something) by comparison with a standard."

ELISA most often serves to measure the presence or quantity of antibodies or antigens, or biomolecules in general which can be recognized by antibodies. In biological matrices (such as serum, plasma, blood, urine and saliva) ELISA is an important diagnostic tool used to detect various antigens and antibodies. Indirect or direct ELISAs are used in medical product development, particularly for testing vaccines and new drugs. ELISA with specific antibodies can be designed to measure impurities within the medical products resulting from the production process. Antibody assays against these impurities should also be developed and validated for testing the levels of the impurities, which should be kept at a minimum in order to avoid adverse immune responses. For immunogenic substances with expected low concentrations, such as cytokines, hormones, toxins etc., sandwich ELISA is used.

Irrespective of the ELISA design (indirect, direct or sandwich), OVS principles are the same. Of paramount importance for any bioanalytical method is that it is well characterized, fully validated and documented to a satisfactory standard in order to yield reliable results.

The first step in ELISA development is optimisation, which is followed by standardization and finaly validation.

2. ELISA optimization

Optimization of an ELISA is essential to its success. Since ELISA is a multistep procedure, each component can be individually tested prior to the start of an experiment.

ELISA procedure consists of antigen or antibody coating, saturation, analyte application, detection with appropriate antibodies, primary or secondary and signal detection. Between each step the plate is washed. A variety of samples can be tested with ELISA, and the choice of assay conditions will depend upon the complexity of the sample and the expected amount of analyte present. Optimization is the establishment of ideal concentrations of each assay reagent and ideal conditions for each step and that must be done empirically. The cornersotne of any ELISA is the

selection of the protocol type: direct, indirect or sandwich; which is dependent on the type of sample, avaliable reagents and the concentration of the analyte, keeping in mind that the procedure should be as straight forward as possible.

Numerous factors should be tested, such as the concentration of antigen, or antibody used for coating, temperature, the duration of individual steps the type of coating buffer, such as phosphate-buffered saline (PBS) or carbonate buffer, sample preparation methods (with or without EDTA, decomplementation, serum or plasma or whole samples). Plate saturation is also a step which requires optimization such as different concentration of bovine serum albumine (BSA), nonfat-dried milk, or whole serum from different animals. Here we will discuss the most important steps of the optimization procedure.

2.1 Antigen coating

The first step in ELISA is coating wells with antigen or capturing antibodies. Most often this consists of applying a protein solution in PBS or carbonate buffer to microttiter plate wells. The microtiter plates for coating with proteins are special plates with modified surface, i.e. highly charged polystyrene surface with high affinity to molecules with polar or hydrophilic groups. This kind of surface has a high binding capacity for proteins, including globular antibodies and ensures proper antibody orientation. On the other hand ELISA for lipid antigens is performed on a hydrophobic surface, suited for non-protein antigens, which are not soluble in PBS or carbonate buffer, but are dissolved in an apropriate alcohol. Irrespective of the type of antigen the whole surface of the well bottom must be covered. If the whole surface is not covered the absorbance read will be lower, and if excess antibody/antigen is present, layers of antibody/antigen may form and wash away in subsequent steps, which again leads to lower signal. **Figure 1** shows the dependance of absorbance on the ammount of antibody/antigen used for coating. For the optimized protocol it is important to select that antigen/antibody concentraion that gives the highest

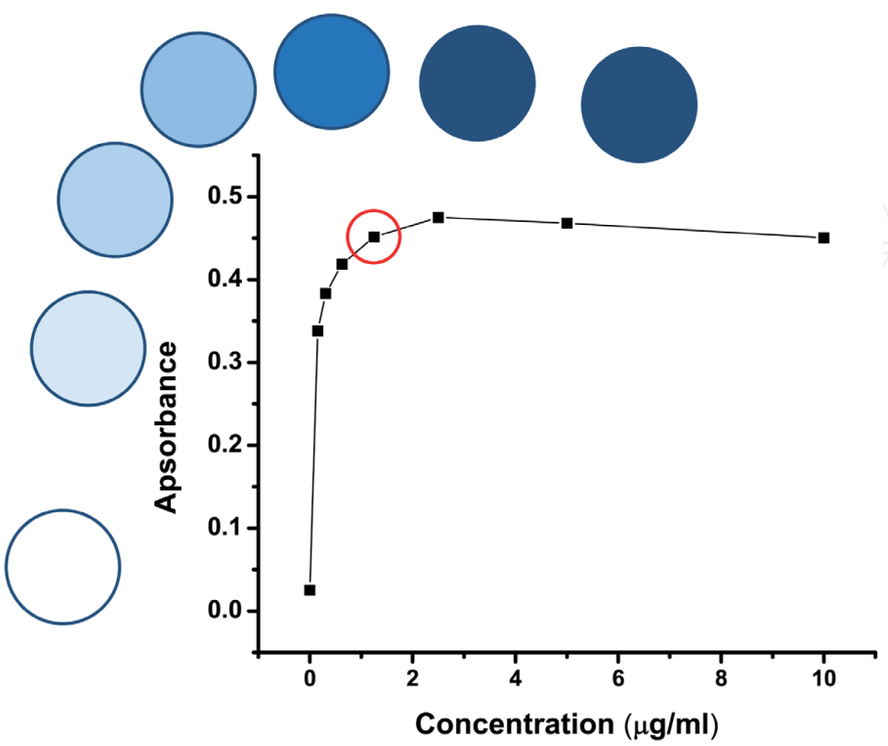

Figure 1.
Dependance of absorbance on the ammount of antibody/antigen used for well coating in ELISA.

absorbance, marked with a red circle in **Figure 1**, which ensures that the complete well surface availiable for binding is covered in a monolayer. This principle should be followed regardless of the type of antigen/antibody or the ELISA type. For axample, in sandwith ELISA the wells are covered with capture antibodies, either whole IgG or Fab fragments and in direct and indirect ELISA with the antigens.

2.2 Saturation-blocking

The process of coating an ELISA plate with antigen relies on the binding activity of the solid phase of the well, which immobilizes biomolecules on the well surface. Step after that must be blocking. During blocking free binding sites at the bottom of the wells become saturated with a blocking buffer in order to prevent the possibility of nonspecific binding and the residual binding capacity of the wells, thus greatly improving the signal-to-noise ratio and specificity. Without appropriate blocking the detection antibody could bind nonspecifically alongside the antigen, resulting in high background signal and low sensitivity.

There is a variety of blocking buffers, to choose from, not one of which is ideal for every situation. Although these buffers are called blocking buffers they usually contain a blocking component such as BSA, nonfat-dried milk, casein or whole serum. Every blocking buffer represents a compromise between reducing the background and maintaining specificity. Whole sera and serum protein albumin can cause non-specific ELISA signals in certain circumstances [15].

Even different BSA preparations show variations in the blocking activity of non-specific binding in ELISA. To prevent false positive results from cross reactive antibodies or non-specific binding of ELISA reagents to BSA, alternative blocking agents can be used and even no protein can be included in the blocking buffer [1]. These different blocking agents, (as well as their different concentration, incubation time, etc) should be tested in parallel, to discover the best way of saturation for each individual ELISA system.

2.3 Sample preparation

It is almost always necessary to dilute samples for ELISA test, so the choice of the diluent is important. Generally, standard diluent should be as similar as possible to the matrix of the sample. For example, PBS with BSA is a good serum replacement in ELISA and is most often used for biologycal samples. The next important diluent component is non-ionic detergent (Tween 20, Triton X-100, CHAPS) that, in low concentrations, prevents non-specific (hydrophobic) protein–protein interactions. The specific binding is usually more resistant to the detergent. Detergents in one step do not provide a permanent barrier to biomolecule non-specific attachment in the following steps because it washes away with water or aqueous buffer, so in certain situations, detergents should be present in all the diluents/buffers.

It may be necessary to choose a different diluent than PBS/Tween/BSA, if the analyte is not serum. In that case, it is necessary to check the standard curve and linearity of dilution for the experimantal sample. The reason for this is the influence of the components of a standard diluent or matix on antigen/antibody interactions. In such cases spike-and-recovery or linearity-of-dilution experiments should be performed.

The goal in assay development is to achieve high signal-to-noise ratio while maintaining optimal responses. The sample matrix may contain interfeering components that affect assay response to the analyte by introducing a difference in comparison to the standard diluent. In order to asses this phenomenon, spike-and-recovery experiment is designed.

The idea of spike-and-recovery is that you add (spike) a certain amount of standard into the sample buffer or the samples, and measure them in parallel with samples with no standard added. Sometimes one can compare the same amount of analyte added into the natural test sample matrix and identical spike added to the standard diluent. So it can be seen whether you can measure (recover) the exact amount again, and how much you can recover from it in percentages. If, for any reason, you can not recover the same amount in comparison to a control, this means that something in the test solution is not in favor of the assay, so one should proceed with finding the right standard diluent.

Linearity-of-dilution experiments provide information about the precision of the assay results for different diluted samples in the chosen sample diluent. These experiments are performed to demonstrate that highly concentrated samples can be accurately measured by diluting into the assay's quantitative range and the concentration can be calculated by multiplying the measured concentration by the dilution factor. Linearity-of-dilution experiment in practise means the measurement of at least three dilutions in the appropriate range in the selected diluent. There are two different ways to perform a linearity-of-dilution experiment, both with the same outcome. The usual method implies using a highly concentrated sample and then testing several different dilutions of that sample in the chosen sample diluent. Alternatively one can first prepare several different dilutions of a low concentration sample and then spike it with the same amount of the analyte before testing. If a sample does not exhibit linear dilution (i.e. linear dependence of absorbance on dilution), the situation can be that one has missed the range of linearity, as generally speaking linearity rarely or never exists over the entire range of concentrations; or that the matrix component is interfering with the measurement at the given dilution. Sometimes, matrix interference occurs if an interfering factor is present at concentrations above a certain threshold, and when the sample is diluted, interference is no longer observed. This kind of testing of a novel bioanalytical method is required by the EMA [16, 17].

When testing an experimental sample it is important to test several dilutions, all in duplicate or triplicate in conjunction with a known standard to ensure that the final results fall within the linear portion of the standard curve. This ensures the accuracy of the result. In highly concentrated samples underestimation of the concentration can occur, while in highly diluted samples overestimation can occur. Prepare different concentrations of the sample, keeping in mind the detection limit of the substrate. At this point, it is very suitable to detect maximal quantity of sample that can be detected, that is the last concentration after which there is no further absorbance increases (the same principle as for antigen coating optimization), **Figure 1**. This way the upper limit of the method is determined which enables the optimization of the next step.

At this point of optimisation, if sample is sera, high unspecific absorbance can occur, which is not related to the concentration of the sample/analyte. This can occur if the sera is not decomplemented, because active complement binds to antibody Fc. Heat-inactivation of serum for 30 minutes at 56°C eliminates complement activity, but one must keep in mind that different immunoglobulin isotypes and immunologbulins from different species show different sensitivity to heat treatment [18]. So, it is important to carefully consider or test the inactivation step.

2.4 The choise of the detecting antibody

ELISA is largely dependent on the choice of antibodies used, so antibodies should be carefully chosen. Based on the type of sample and the expected analyte

concentration, the choice of monoclonal or polyclonal antibodies, or even the combination of both, should provide optimal signal-to-noise ratio [19]. Each antibody type offers distinct advantages.

The interaction between antibodies and their antigens is described by specificity, affinity, and avidity.

Specificity is an indication of whether an antibody binds solely to a unique epitope from a single antigen in a single species, or whether it binds to similar epitopes present on several molecules from the same or a few different species, i.e. whether it is cross-reactive. Specificity is the most important quality of an antibody, and this is the principle that ELISA is based upon, so a carefull selection should be made.

Affinity describes the strength of binding of an antibody with an antigen. This binding is a reversible interaction and affinity determines how much antigen is bound by an antibody at any particular moment, which is dependent upon how quickly this binding occurs, and for how long the interaction lasts. High affinity antibodies should be used in all types of immunoassay because they rapidly produce a large number of stable interactions and provide the most sensitive detection.

Avidity is a less intuitive term than affinity as it is based on affinity, but is highly influenced by the the total number of antigen binding sites or valency, which determines the overall stability of the antibody–antigen interaction. Therefore, avidity varies with antibody isotype and whether it is intact or fragmented. Additional factors which determines avidity are the structure of the antibody, the length and motility in the hinge region and the space between the Fab fragments.

When available, one should always choose monoclonal antibodies over polyclonal antibodies, in fact, commercial ELISA kits almost always utilize monoclonal antibodies. Monoclonal antibodies have specificity for a single epitope, usually a small part of the antigens' surface. Monoclonal antibodies are therefore less likely to interact with closely-related proteins and are not generally expected to trigger non-specific signals in an immunoassay. Polyclonal antibodies are a mixture of antibodies with increased specificity to the antigen, therefore they bind different epitopes. Commercial polyclonal antibodies are often affinity purified or cross-adsorbed, but still the posibility of crossreactivity is higher. In addition, polyclonal antibody preparations can show batch to batch variations which should not be the case with monoclonal antibodies.

The advantage of using polyclonal antibodies is that they rarely fail to bind to the antigen due to a single blocked antibody binding site, antigen configuration change, or misfolding, although the latter are more important in tests other than ELISA. When combining monoclonal antibodies as in sandwich ELISA it is important to check literature or to test experimentaly the compatibility of the antibodies in terms that they do not share an epitope or for steric hinderance. Matched pairs are the basis of many sandwich ELISAs, either in kits or for in house assay set up. Matched antibody pairs means they are capable of detecting different epitopes on the same protein antigen, so they can be used together in a sandwich ELISA.

Sometimes the ELISA sensitivity can be increased by using indirect detection with polyclonal antibodies instead of direct detection with a monoclonal antibody, due to higher levels of polyclonal antibody binding to the target antigen. For cost reduction it can also be the combination of monoclonal capture with polyclonal detection.

After careful antibody selection, serial dilutions of capture antibodies should be carefully prepared for proper titration of antibody concentration. This is performed according to the previously mentioned principle of detecting maximum ammount of the component (in this case detection antibody) after which there is no further absorbance increase, **Figure 1**. Again, the ideal concentration should provide the highest signal and lowest noise.

As ELISA is a method which basicaly consists of overlaying different components which specificaly interact in each step (except washing) an optimization is required which follows the principle of titration until the complete coverage of the previous layer. Often the enzyme conjugate, i.e. enzyme responsible for color development, is already chemically bound to the detecting antibody, thereby enabling its direct use as a detection antibody in immunoassays. If this is not the case then enzyme concentration should be optimized too.

2.5 The enzyme conjugate selection

In this step, the first point is choosing the apropriate enzyme conjugate, depending on the needs of the researcher. The enzymes should be stable at typical assay temperatures: 4°C, 25°C, and 37°C; have a shelf life greater than six months when stored at 4°C; be inexpensive and commercially available. The enzymes should also survive the necessary conjugation conditions and yield productive conjugation. The enzymes should have an easily measurable activity; with high substrate turnover number. Horse radish peroxidase (HRP) and calf intestine alkalne phosphatase (ALP) are two most widely used enzymes for detection in ELISA assays [20]. HRP is usually conjugated to an antibody in a 4:1 ratio. For ALP the ratio is a little more unfavorable, 2:1, but the conjugate is more stable [21]. These enzymes are typically used because they each meet most, if not all, of the criteria necessary to produce a sensitive, inexpensive, and easily performed assay.

All enzyme-linked immunoassays, imply the usage of the enzyme substrate. Colorimetric ELISAs usually require soluble colored reaction products. The decision which substrate to choose depends on the desired sensitivity, reaction time, and the detection device. For colorimetric detection the most desirable substrates quickly produce intensely colored reaction products. When the analyte amounts span a wide range of concentrations (large dynamic range), then it is more suitable to use substrates that produce color over a longer time period (15 to 30 minutes) because then, one is able to detect the wider range of analyte-dependent color intensities. For assays with a timed endpoint, the reaction is stopped with an inhibitor suitable for the specific enzyme substrate combination after a defined time period that stops further color development. This allows detection to be performed within a reasonable time; for this, a substrate that has a "slow" reaction rate (15 to 30 minutes to completion) is optimal.

Both HRP and ALP have substrates that yield soluble colored reaction products.

The most common substrates that produce soluble reaction products with HRP are: TMB (3,3′,5,5′-Tetramethylbenzidine), ABTS (2,2′-azino-di[3-ethylbenzthiazoline] sulfonate), and OPD (o-phenylenediamine). TMB is a highly sensitive substrate, safe for laboratory workers. Due to its rapid reaction rate, it is ideally suited for on-line kinetic analysis. TMB can also be used in endpoint assays by stopping the reaction with 1 M phosphoric acid. ABTS is considered an all-purpose substrate. Although it is less sensitive than either TMB or OPD, it has the widest working range of any substrate currently available for peroxidase or alkaline phosphatase. Its reaction rate is suitable for endpoint assays and is easily stopped with 1% SDS (sodium dodecyl sulfate), which does not change the color or the absorbance of the reaction product. OPD was once the most popular substrate for peroxidase. It is slightly less sensitive than TMB, but it is cancerogenic.

The most commonly used substrate that produces a soluble reaction product with ALP is p-NPP (p-nitrophenylphosphate). pNPP is a substrate with a low reaction rate, so it usually takes 30 to 60 minutes for the dye to develop optimally. This property makes it possible to increase the sensitivity by increasing the reaction time period. At the same time, this property makes the pNPP substrate unsuitable for kinetic analysis [22].

Factors that affect the measurement of enzymatic activity are temperature, buffer composition (pH, ionic strength), build-up of product inhibitors, the increase in back-reaction as the product concentration increases, stability of the enzyme and sometimes exposure to light. As most of these facors such as pH and substrate depletion, are known, commercially available reagents are optimized for composition and concentration in order to control these parameters. For novel ELISA optimization of the most concern are reaction time and temperature.

If the antigen can clearly be detected then the substrate is appropriate. If the antigen is below the threshold for detection then one should select a more sensitive substrate.

2.6 Signal detection methods

It should be noted that the detection methodologies for ELISA are few, but the most prevalent in the laboratories is colorimetric. In addition, fluorescent and luminescent are also used.

In colorimetric detection the amount of color in each well is read by a spectrophotometer and samples are compared relative to one another or with the use of a standard curve derived from known analyte concentrations.

Fluorescent substrates [23] for ALP and HRP can potentially yield a higher signal, leading to increased sensitivity and broader dynamic range. This kind of detection requires black plates, which are also availiable with various degrees of hydrophobicity and a fluorescent plate reader is required. Fluorescence yielding substrates have a shorter half-life than colorimetric substrates, so the signal is declining over time. This kind of ELISA is useful for measuring immune responses because of broader dynamic range [19].

The same detection antibodies conjugated with ALP or HRP, can also be used for chemiluminescent assays [24]. In this type of experiment, ALP, for example, will modify a substrate, forming a chemiluminiscent product which creates light emission. ALP chemiluminiscent substrates can have pg/ml sensitivity. The signal can be read in black or white opaque ELISA plates and a luminometer is required. The advantages of this detection type are typically a higher dynamic range and lower background signal. The signal is not as stable as the colorimetric or fluorescent detection and must be read within a short time of generating the signal.

The type of substrate used depends on several factors, most notably the desired assay sensitivity and signal to bakground ratio.

3. ELISA standardization

Many laboratories have independently developed ELISA techniques for their own purposes. For results to be valid they must be comparable with results of the same ELISA test performed in different laboratories. Consistency in the assessment of ELISA results in different areas of application (diagnostics, production control, scientific research, immunogenicity assessment etc.) requires standardized and acknowledged methodological protocols. Protocol harmonization progress with respect to the international standardization and validation of this technique has been made.

Today, leading regulatory agencies for specific guidance on immunogenicity assessment of biotherapeutic products are part of EMA and WHO, [25] and there are other agencies. The National Institute for Biological Standards and Control (NIBSC), for example, part of UK Medicines and Healthcare products Regulatory Agency (MHRA), is of great importance to the field of biological standardization. It produces over 90% of the biological international standards in use around the

world. The WHOs' Biological Reference Materials are established through a standard procedure, [26] in which representative materials are tested by participating laboratories using their own methodologies and coordinated by a responsible WHO Collaborating Center [27]. Upon establishment of the reference preparation by the Expert Committee on Biological Standardization (ECBS), the material is assigned a unitage and serves as the comparator against which results from laboratories can be standardized and compared, irrespective of the location or the methods employed. This enables the results of bioanalytical methods, including ELISA, to be comparable. Based on international standards„ working standard" (i.e. in-house or secondary standards) are evaluated and compared, and subsequently adequately used.

At first glance, it is very simple to explain the process, i.e. the term of standardization in ELISA: comparing the absorbance of a sample with the absorbance of the known concentration of the standard (in-house or commercial) and based on that, determining the unknown concentration.

If the ELISA is intended for the measurement of the final detectable dilution, as in titration experiments, and not for the measurement of biomolecule quantity a reference standard may not exist.

Then the need exists for establishing a reference standard. For any ELISA, consideration must be given to the selection of standards which represent, on average, what would be expected of an immune response of the organism in question. Immunogenicity assessment relies on the measurement of antigen induced antibodies in serum or plasma. Such antibodies are heterogeneous in terms of classes, subclasses and alotypes, concentration as well as antigenic specificity. Some will neutralize the biological activity of the antigen, others will not, despite the high affinity/avidity. Irrespective of the type of ELISA system used, endpoint titration is a function of both antibody concentration and avidity. And finally, as every sample is unique with vast individual differences among humans, for example, it is not possible to make a straightforward comparison with standard antibodies. Nevertheless, although the ideal is unreachable, if wanting to produce valid and reproducible data a reference standard must be established.

The physical quantity to be measured in ELISA is absorbance. Absorbance is influenced by test parameters and photometric instrumentation, so raw, corrected or normalized OD values [28] cannot be used for inter-laboratory standardization. This is why end-point titration or determination of highest serial dilution which demonstrates a minimum of antibody activity is often used for measuring the immune response in diagnostics and vaccinology. Under some circumstances, quantitative data are not required for diagnostic purposes and sometimes end-point titration is sufficient, with an adequate semi-quantitative standard. End-point titrations are labor-intensive, costly and impractical for most routine diagnostic purposes.

In order to overcome the relativity of the measured absorbance a notion of "percent positivity" (PP) is accepted, this way the absorbance of each sample tested is expressed as a percentage of a highly positive reference standard. Although semi-quantitative, PP is expressed on a continuous scale of 0–100 and has two major advantages, first, it requires only a single dilution and second, it does not assume parallelism or uniform background activity. Therefore, it may be used for inter-laboratory standardization.

Even with measurements with qualitative standard curve, it is not correct to determine the result from a single sample dilution measurement. This can only be acceptable if there is a parallelism in dilution curves between the sample and the standard. If more quantitative data are needed, PP values can be converted to units which are directly proportional to antibody activity.

Sometimes an elegant and appropriate way to quantify samples is competitive or inhibitory ELISA. When performing competitive ELISA, one applies the

sample preincubated with the same antigen used for plate coating and measures the amount of non inhibited antibodies. There is a negative relationship between color intensity and the amount of test sample antibody inhibited by antigens. Percent inhibition (PI) of the color produced by the standard competing antibody is more widely used. The development of consistent standard curves for this kind of assay is extremely difficult, but still possible.

The specific guidance on immunogenicity assessment of biotherapeutic products has been elaborated by leading regulatory agencies such as the EMA and U.S. Food and Drug Administration (US FDA) [29–32].

4. ELISA validation

Validated analytical methods such as ELISA for quantification of biomarkers, drugs, biological products, and their metabolites in a given biological matrix (e.g. blood, plasma, serum, or urine) are critical for the successful conduct of nonclinical and clinical studies. Validating the analytical method ensures that the data are reliable [33]. Validated methods provide critical data to support the safety and effectiveness of drugs and biological products.

Although there is abundant literature relating to immunochemical methods, [34] EMEA [35, 36] and US FDA [8] have clearly defined the characteristics of the validation procedure for bioanalytical methods, which also applies to the validation of ELISAs, which are intended for use in diagnostics, toxicology, basic or applied research [37] or production control [38]. Metodology for the validation of bioanalytical methods must follow clear recomendations from reference institutions such as the EMEA [35, 39] or the WHO because that provides important measurements to be of satisfactory quality all over the world.

ELISA validation according to these recommendations means determining the following method caracteristics:

1. Specificity

2. Linearity – *Range - Limit of detection (LOD)*

3. Sensitivity

4. Accuracy

5. Precision (repetability = intra assay, inter assay, reproducibility = inter laboratory assay)

6. Robustnes

Acceptance criteria should be prospectively defined based on the intended use of the method.

4.1 Specificity

Specificity means that the method must differentiate the targeted analyte from all other matrix components. Which is why it is important to test wether *"related molecules"*, e.g. endogenous compounds, isoforms, variant forms of the analyte, or physico-chemically similar compounds interfere with the results by giving false positivity. Specificity can be confirmed by adding increasing concentrations of

available "related molecules" or drugs, into drug-naive sample matrix and measuring the amount of the macromolecule of interest within the working range. Specificity can also be tested by testing samples (serum) of unimmunized subjects (negative immunization control), or sometimes it is convinient to prove specificity with competitive (inhibitory) ELISA.

Evaluation of specificity may be conducted during optimization and validation, when more data on the behavior of the analyte become available. Specificity should be tested with quality control (QC) samples [40]. QC samples are the samples with known amounts of the analyte, in identical matrix like the sample. These are usually in-house produced samples, with a lower amount of the analyte. When the method is performed with these QC samples and satisfactory results are obtained, then the method is also good, i.e. valid. If the method does not give good enough results with the QC samples, it means that the method is not of sufficient quality, so it must be investigated why the method worked poorly. The shortcomings must be corrected, and then again checked with QC samples. Still it needs to be defined what is satisfactory. The criterion for accepting the results obtained with QC samples is that the measured value does not deviate by more than 25% from the nominal value [40].

4.2 Linearity

Linearity is the ability of the analytical method to produce results by calculating a direct proportion, within the working range. Linearity is described by range and detection limits.

Linearity is a function of values that can be graphically represented by a straight line. The linearity of an analytical method can be explained as its capability to show "results that are directly proportional to the concentration of the analyte in the sample" [39].

Unfortunately, the analytical response of a method is not always linear. Sometimes when the data are not linear they can be mathematically transformed, e.g. by applying logarithms but in some cases or some range of immunoassays transformation is not appropriate.

Linearity is important as it confirms the sensitivity of the method for the analysis of concentration within a defined range. According to the EMEA International Council for Harmonization ICH Q2(R1) guideline, linearity of a given response must be evaluated using a minimum of 5 concentrations of the analyte (multi-point calibration). Then, the collected data must be statistically analyzed, by performing regression analysis using the method of the least squares, in order to mathematically determine the line that best fits a set of data. For linearity, the results are required to be represented as linear equation (Eq. (1)).

$$y = kx + n \tag{1}$$

In a linear regression line, the regression coefficient is the constant "k" that represents the rate of change of one variable "y" as a function of change in the other "x" (thus the slope), while "n" is the Y-intercept. The correlation coefficient r, a value without units, expresses the precision of the linearity fit of the experimental data. In case of a value being less than 0.95, it may either be a result of a broad spreading during measurement or due to a non-linear correlation. Often, the coefficient of determination (R^2) is used, which is the square r. For most methods applied at $R^2 \geq 0.98$ can be achieved. If there is a perfect linear relationship, it has a value of 1 (100%). Linearity studies are important because they define the range of the method within which the results are obtained accurately and precisely.

To summarize, linearity is one major aspect in the quantitative method validation procedures. It describes the range of concentrations for which the method can function reliably. If the data are non-linear, transformation into a linear form may be performed, or the data can be accepted as is while demonstrating a clear relation between the analyte concentration and the measured absorbance [41].

Range. As mentioned previously range is determined from linearity and the data obtained which fall within the determined range should be of satisfactory accuracy and precision. The range is limited by upper and lower detection level.

Upper limit of quantification (ULOQ): is defined as a mean value of 10 duplicates of maximally achieved absorbances in the linear part of the standard curve, from which three standard deviations have been subtracted. Subtraction of the multiplied standard deviations achieves accuracy in this range from 80–120%.

Lower limit of quantification (LLOQ): is the lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision. In practise this is a mean value of the smallest result measured in the linear part of the curve to which three standard deviations have been added.

4.3 Sensitivity

Senzitivity or limit of detection, (LD) for ELISA is defined in the same way as for other bionalaytical methods. At this point, it is appropriate to underline the difference between the limit of detection (LD) and lower limit of quantification nominal (LLOQ). LD is the lowest analyte concentration that can be distinguished from the assay background, while the LLOQ is the lowest concentration at which the analyte can be quantitated at defined levels for precision and accuracy. LD is determined from standard deviation of the sample blank and the slope of the linear curve (Eq. (2)).

$$L_D = 3.3(SD(b)/k) \tag{2}$$

L_D—LD (detection limit) nominal
k—slope of the linear curve Eq. (1)
SD(b)—standard deviation of the blank [39]

There are bioanalytical methods which have the same values for LD and LLOQ, but with ELISA, especially when biologycal samples are measure this is not the case, and LD is lower than LLOQ. For liretature reference of these terms one should read Armbruster and Pry [42].

4.4 Accuracy

The accuracy of an analytical method describes the closeness of the value determined by the method to the nominal concentration. In practice, as the reference material is precious and universally needed, the first step is to make a sufficient amount of the quality control (QC) samples, previously standardized against the reference material. Then the QC sample can be used for determining validation characteristics. Accuracy should be assessed on samples spiked with known amounts of the analyte, the QC samples. The accuracy can be expressed as the difference between the obtained experimnental value and the nominal value (which is acurate), using the absolute or even better the relative error.

Absolute error is the difference between the experimantal result and the nominal value, (Eq. (3)):

$$\Delta x_i = \left| \mu - x_i \right| \quad (3)$$

Δx_i—absolute error of individual measurement
μ— nominal value
x_i—measured value

It is important to perform multiple measurements for a single sample, in order to present the absolute error as the mean value of absolute errors of individual measurements (Eq. (4)).

$$\Delta x = [\left| \mu - x_1 \right| + \left| \mu - x_2 \right| + \ldots\ldots + \left| \mu - x_{n-1} \right| + \left| \mu - x_n \right|]/n \quad (4)$$

n—number of measurements
Δx—mean value of absolute or standard error

Because of the numerical nature, the absolute value of the difference does not give insight into its significance for the accuracy of measurement, so it is always important to calculate the relative error as well.

Relative error (δ) is a quotient of the absolute error and the actual (nominal) value (Eq. (5)), it is without units and can be expressed in percentages by multiplying with 100.

$$\delta = \Delta x/\mu \quad (5)$$

The level of accuracy must be determined for the whole range of the analytical procedure. Minimal requirements for this are three concentrations one close to ULOQ, one close to LLOQ and one in the middle of the range, each in triplicate.

Today it is common practise to develop an ELISA as an internal laboratory assay without the standards or the QC samples or for titration experiments for the determination of the last measurable dilution. In this situation there is no measurable quantitifier for accuracy testing. For accuracy to be calculated as % that shows how much the obtained results corresponds with the actual value, it is necessary to use concrete, absolute and measurable quantity such as analyte concentration. In practise this can be achieved [43] with inhibitory ELISA, which is based on the dependance of the absorbance on inhibitor concentration. The difference between the described calculations is in the reverse proportion, as described in the ELISA standardization section [37].

4.5 Precision

Precision is a validation characteristic which describes the reproducibility of the measurement, in other words the closeness of two measurements of the same sample. Precision is higher if the results are closer to one another. At first glance it is easy to confuse accuracy with precision, because in both cases it is about the absolute and the relative error of the obtained results. **Figure 2** shows the difference between accuracy and precicion, where accuracy describes the deviation from the actual (nominal) value, while precision describes the deviation from the mean value. Precision is determined by simply repeating the measurement.

Standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision (intra, intermediate or inter) investigated [35]. The three parameters are dependent on the closeness of individual results to the mean value, and give the complete picture of the precision of the test.

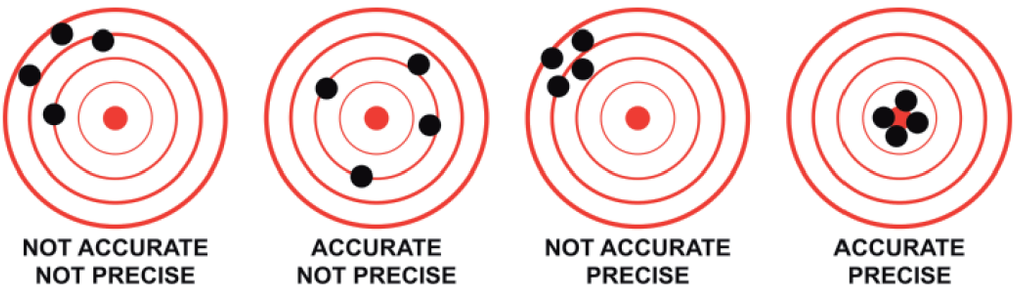

Figure 2.
Accuracy and precision defined.

A. DEVIATION is the difference between the measured value from the mean value, and has the same units as the measured value (Eq. (6)).

$$d_i = \left| \bar{x} - x_i \right| \tag{6}$$

$\bar{x}$—mean value of repeated measurements of the same sample
x_i—one measured value
d_i—deviation, the difference between the mean value and one measurement
Standard deviation is the mean value of all measurement deviations Eq. (7).

$$SD = \sqrt{\frac{1}{N}\sum_{i=0}^{n}(di)} \tag{7}$$

B. COEFFICIENT OF VARIATION CV (relative standard deviation) is standard deviation expressed in percentages and is calculated based on the measured mean value $\bar{x}$ (Eq. (8)).

$$CV(\%) = (SD: \bar{x}) * 100 \tag{8}$$

C. CONFIDENCE INTERVAL (CI) is the range of values within which the "actual" result is found. A CI of 95% means that if the measurement was to be repeated an infinite number of times, 95% of the results would fall within this range of values. For validation purpose, higher CI, 95% or 99% is needed, with optimal performance within the middle part of the range. A wide CI can be caused by small number of samples or by a large variance between sample measurements. Range of values for the given CI shows precision. This parameter is easily calculated by statistical programmes, or by a profesional statistician.

4.5.1 Intra-assay precision (repetability)

Intra-assay validation shows the reproducibility between wells within an assay plate. Data resulting from intra-assay validation helps ensure that repeated measurement of the same sample on a single plate gives comparable results. Repeatability should be assessed using a minimum of 6 determinations covering the specified range for the procedure (e.g. 3 concentrations, 2 replicates each), or a minimum of 6 determinations at 100% of the test concentration [39].

The % CV for each sample is calculated by finding the standard deviation of multiplicate results dividing that by the multiplicate mean, and multiplying the

result by 100 (Eq. (8)). The average of the individual CVs is reported as the intra-assay CV ($CV_{intra\text{-}assay}$).

Usually, $CV_{intra\text{-}assay}$ of 10% or less is considered satisfactory [44].

4.5.2 Intermediate precision

Intermediate precision (sometimes called within-lab reproducibility) shows the reproducibility between assays done on different days, or different plates. Satisfactory inter-assay precision is typically <10% [44].

For example, to monitor plate-to-plate variation the same samples are analyzed in quadruplicate on ten different plates. The plate means are calculated and then used to calculate the overall mean, standard deviation, and % CV. Overall % CV is calculated by dividing the SD of the plate means with mean of the plate means and multiplying by 100 (Eq. (8)). The average of the all plates % CV represents the inter-assay CV ($CV_{intermediate}$). In order to monitor daily variation quadruplicate samples are analyzed in ten different days and analyzed in the same way.

4.5.3 Reproducibility (inter-laboratory assay precision)

Reproducibility is assessed by means of an inter-laboratory trial. The outcome of the cross validation is critical in determining whether the obtained data are reliable and whether they can be compared and used. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopeias.

Satisfactory value for $CV_{inter\text{-}assay}$ is 10–15% [43].

Analyzing the literature it can be seen that the term inter assay is sometimes used for precision assessment on different days or on different plates, and sometimes for testing in different laboratories. Acording to EMEA, the term inter assay precision describes precision of the measurement assessment in different laboratories. If it is to be used in a different context it shold be described.

4.6 Robustness

Robustness testing involves monitoring the effects of small unintentional errors on the quantitative and qualitative characteristics of the method, where the errors relate to the internal parameters described in the method prescription. For example, buffer temperature, incubation temperature, sample incubation time, secondary antibody incubation time, number of washes before color development, color development time, and the like. This feature shows the reliability of the method despite minor deviations in performance.

There is also the notion of rigidity - as a sub-notion of robustness - which monitors the effects of changes in external parameters such as other lots of chemicals, other people working, other instruments used and the like.

Practically, this property is not measured or calculated in a certain way, but is established during the development of the method (optimization). Data on this can also be collected during operation.

This guideline describes full validation methodology. In case when method is already validated, when a smaller change to the protocol is instated, a full validation may not be necessary. It is possible to perform partial validation, and the nature of the modification will determine the extent of validation required. All modifications should be reported and the scope of revalidation or partial validation justified [34].

5. Conclusion

In our experience ELISA is an excellent analytical method which can be used for the detection and quantification of numerous biomolecules. No matter what this specific biomolecule is, the basis of ELISA is the antigen–antibody interaction. The existence of this specific interaction usually enables the construction of different ELISA protocols, dependent on your prior knowledge and imagination. After careful protocol optimization, determination of validation characteristics and the acquirement of an appropriate standard you can get a reliable and inexpensive analytical method useful in diagnostics, research or biomedicine in general.

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Conflict of interest

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

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