Global Journal of Biotechnology & Biochemistry 16 (1): 01-08, 2021
ISSN 2078-466X

DOI: 10.5829/idosi.gjbb.2021.16.01.15435

# Review: Invention, Development, Types and Application of Enzyme Linked Immuno-Sorbent Assay

*Girma Tsegaye*

Jimma University College of Agriculture and Veterinary Medicine, Jimma, Ethiopia

**Abstract:** ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying peptides, proteins, antibodies and hormones. ELISA is an antigen-antibody reaction that was developed by Peter Perlmann and Eva Engvall in1971 as an alternative to radioimmunoassay methods. ELISAs are typically performed in 96-well polystyrene plates, which will passively bind antibodies and proteins. It is this binding and immobilization of reagents that makes ELISAs so easy to design and perform. For development antigen-antibody reaction in Elisa some enzymes like alkaline phosphatase, horseradish peroxidase and beta-galactosidase and specific substrate such as ortho-phenyl diamine dihydrochloride (for peroxidase), para-nitrophenyl phosphate (for alkaline phosphatase) to give colored end product. ELISA is a powerful diagnostic tool that allows the detection of all types of biological molecules at very low concentrations and quantities. There are four types of ELISA to detect specific antibodies and soluble antigens and a variety of ELISA and types have been developed to increase the specificity of measurement. Nowadays ELISA was widely used in medical laboratories because it provides relatively rapid, sensitive, specific, accurate, robust, simple and cost-effective analytical methods to the detection of proteins and antigens produced by microorganisms and helps in guiding the clinicians in diagnosing and monitoring diseases that inflict biological systems exhibits.

**Key words:** ELISA · Antibody · Antigen · Enzymes · Proteins

## INTRODUCTION

Quantitative analytical methods that show antigen-antibody reactions by the color change gained through an enzyme-linked conjugate and enzyme-substrate that serve to detect the presence and concentration of molecules in biological fluids are generally called enzyme immune tests [enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA)] [1].

Enzyme immunoassay and enzyme-linked immunosorbent assay are both generally used as diagnostic techniques and as quality control methods in several industries; they are also used as analytical tools in biomedical research for the identification and quantification of specific antigens or antibodies in a given sample. ELISA is a biochemical assay that uses antibodies and an enzyme-mediated color change to identify the presence of either antigen (proteins, peptides, hormones, etc.) or antibody in a given sample [2].

In other expressions when we have the antigen that we know to be specific to a certain substance, we can detect the kind and quantity of its antibody and when we have the antibody, we can find out its specific antigen and the amount of antigen, using this method. All techniques and methods of analysis using enzymes to show antigen-antibody reactions are generally referred to as enzyme immuno-tests [1].

Immunoenzyme techniques having been effectively applied to the localization of intracellular antigens both at the light and electron microscope level, the identical overall standard was performed to identify soluble antigens and antibodies in body fluids. Immunoenzyme assays were thus developed as options to radioimmune assays. Specific antibodies can be estimated quantitatively by ELISA. After incubating the test serum in an antigen-coated polystyrene tube or plate, an enzyme labeled anti-immunoglobulin is added and the enzyme then remaining in the tube or plate after washing offers a

**Corresponding Author:** Girma Tsegaye, Jimma University College of Agriculture and Veterinary Medicine, Jimma, Ethiopia. Tel: +251917343958.

measure of the amount of specific antibody in the serum. The test relies on the insolubilization of antigens by passive adsorption to a solid phase [3].

Immunoassays are antibody-based analytical methods for quantitative/qualitative analysis. Since the principle of immunoassays is based on specific antigen-antibody reactions, the assays have been utilized globally for diagnosis, pharmacokinetic studies by drug monitoring and the quality control of commercially available products [4].

ELISA could be a plate principally established test used for detective work and measuring substances like peptides, proteins, antibodies and hormones. ELISA uses a variety of enzymes like alkaline enzyme, horseradish oxidase and beta-galactosidase. Specific substrates like ortho phenyl diamine dihydrochloride (for peroxidase), paranitrophenyl phosphate (for alkaline phosphate) are used that are hydrolyzed on top of enzymes to grant colored outcome [5].

ELISA was widely used in medical laboratories because it exhibits many advantages like simple procedure, high specificity and sensitivity because of an antigen-antibody reaction. High proficiency, as the concurrent investigation, can be implemented without complicated sample pre-treatment. Cost-effective assay, as low-cost reagents are used. Generally ELISA technique is harmless and eco-friendly, since radioactive elements and great quantities of organic solvents are not required.

Both “indirect” and “sandwich” ELISAs allow the detection of antigen or antibody at very low concentrations. The competitive method identifies compositional differences in complex antigen combinations with a great sensitivity, even when the specific detecting antibody is exist in relatively small amounts.

The goal of this review is to describe ELISA, its invention, development, types and its applications in the current real world.

## Literature Review

**Invention of ELISA:** Berson and Yalow were the first to develop an immunoassay, known as radioimmunoassay (RIA), for detecting endogenous plasma insulin [6]. However, radioactive labels have been slowly substituted by enzyme labels for the reason of safety concerns related to radioactivity since the study by Avrameas in 1969, who coupled antigens or antibodies and enzymes using glutaraldehyde [7]. Described first by Engvall and Perlmann [3]. ELISA is a modification of the radioimmunosorbent technique (RIST), in which an enzyme is substituted for the radiolabel of the antiserum. Engvall and Perlmann published their first paper on ELISA in 1971 [3] and established quantitative measurement of IgG in rabbit serum with alkaline phosphatase as the reporter label.

The invention of enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) provided more medical aspect advantage and were established independently and simultaneously by the research group of Peter Perlmann and Eva Engvall at Stockholm University in Sweden and by the research group of Anton Schuurs and Bauke van Weemen in The Netherlands. Nowadays, wholly automated tools in medical research laboratories everywhere in the world use immunoassay techniques with an enzyme as the reporter label for routine determination of innumerable analytes in patient samples [8].

The other third group, a team at the Pasteur Institute, also developed an enzyme-immunologic method, as one technique for the measurement of serum IgG, with the aid of iimmunoadsorbent sand enzyme-labeled antigens and published in 1971 [9]. The groups in Stockholm and Oss vigorously expanded on their first findings of ELISA and EIA and applied them to a wide variety of analytes in clinical chemistry/laboratory medicine in the years after their publications in 1971 [10].

## ELISA Development

**Principle of ELISA:** ELISA allows the highly sensitive and selective quantitative/qualitative analysis of antigens, including proteins, peptides, nucleic acids, hormones, herbicides and plant secondary metabolites [4]. The antibodies in ELISA method can either be monoclonal or polyclonal antibodies, which bind to a very specific binding site and multiple binding sites of antigens, respectively. The binding of the antigen and antibody is detected using an enzyme-linked to a secondary antibody, which turns the antigen-antibody complex into a colored product when enzyme substrate is added [11].

ELISA can detect protein residues even at a very low level including micrograms per gram (μg/g) or parts per million (ppm). To detect these molecules, an antigen or antibody is labeled using enzymes, the so-called enzyme immunoassay, in which alkaline phosphatase (ALP) [12] horseradish peroxidase (HRP) [13] and β-galactosidase are commonly used. ELISA holds four steps, consisting of coating, blocking, reacting of antigen and antibody and developing color [11].

A general ELISA procedure takes the following five-stage system [14] Coating of micro-titer plate wells with substance, Blocking of each single unbound web site to anticipate false-positive results, adding of essential antibodies (e.g. Rabbit being antibodies) to the wells, adding auxiliary antibodies conjugated to a macromolecule (e.g. Hostile to mouse IgG) and observing of reaction of a substrate with the catalyst to form a hued item.

**Various Quality Elements in ELISA Development**

**Antibody Production:** Antibody production requires a process called immunization, whereby a selected antigen is injected into a laboratory animal through subcutaneous and/or intramuscular routes with an appropriate adjuvant. The purpose of using adjuvant is to improve the immune response of the animal by increasing the immunogenicity and the efficiency of antigen presentation to increase the number of antibody-secreting B-cells. A good quality antiserum, characterized by its high affinity and avidity, is the key to success for an immunoassay. Starting from a low dose of the immunogen for primary injection is necessary to produce antibodies with high affinity and avidity [15]. Besides, to maintain the production of an antibody with a high titer, booster injections are applied at approximately three to four-week intervals after the first injection [16]. A good quality antigen-specific antibody, used as capture and detection antibody, is crucial for a sensitive and specific ELISA.

**Optimizing Assay Operating Conditions of ELISA:** Assay optimization is an important stage to reach the best test performance of ELISA for proteins (substances) identification. Once the assay format has been selected along with good quality antibody production, a number of required reagents and circumstances are required to achieve the ELISA examination.

**Solid-Phase Support and Coating Reagents:** Solid-phase support as coating carrier is one of the essential elements to begin the ELISA analysis. A 96-well polystyrene micro-titer plate (in a 12 x 8 format) is commonly used in ELISA. The micro-titer plate is made from highly hydrophobic material composed of a long carbon chain with benzene rings attached to every alternate carbon. This material gives the micro-titer plate a greater capacity to bind proteins (antigen and antibody) through hydrophobic interactions between the non-polar structure of the protein and the solid matrix [17].

**Buffers, Time and Temperature:** The choice of buffers for coating, extracting, blocking and washing; in terms of composition, concentration and pH plays a key role in providing optimum conditions in each processing step. The most common buffers used in ELISA are a pH 9.6 carbonate or bicarbonate buffer for coating, pH 7.4 phosphate-buffered saline solutions for regular extraction and washing buffers and pH 7.4 phosphate buffer for blocking [11]. The blocking step is performed after the coating and washing steps to fill up any space or gap between coated proteins on the plate; hence, preventing non-specific binding for any protein to the plate.

Furthermore, time and temperature can be other factors affecting detection in the assay. In each step, adequate time is necessary for the interactions of proteins with the plate (for coating) and antibody to the antigen, as well as the enzymatic reactions for color development. The optimum binding of an antibody to antigen can be achieved within 1 or 2 hours. Temperature can also affect the binding and dissociation rate of the proteins. A plate is typically coated at 4°C overnight or 37°C for one hour depending on the desired working schedule. However, the subsequent process of the assay including blocking, washing, sample or antigen adding and primary and secondary antibodies incubations are usually conducted at 37° C for an hour [18].

**Assay Validation:** After a successful assay optimization, the quality and the performance of the assay are assessed by a series of analytical test procedures for validation. Validation is crucial in ELISA development to ensure that the assay complies with established specifications for ELISA‘s use in achieving proper standards of accuracy and reliability. The validation of an assay requires large-scale intra- and inter-laboratory trials with the replicated procedure to obtain sufficient data to support and document the validity. The tests or procedures conducted for a validation study are necessary to characterize the performance of the assay. The characteristics that require consideration during validation include accuracy, precision, specificity, detection limit, quantitation limit and robustness of the assay [19].

**Types of ELISA**

**Direct ELISA:** The technique was simultaneously developed in 1971 by Engvall and Perlmann [3] and by Van Weemen and Schuurs [20] the technique lead the way to other ELISA types. Direct ELISA technique is appropriate for determining the amount of high molecule-

weight antigens. The surface of the plate is coated directly with the antibody or antigen. An enzyme-tagged antibody or antigen enables the measurement. Incubation is followed by washing which eliminates the unbound antigens or antibodies from the medium. Then proper substrate is added to the medium to create a signal through coloration. The signal is measured to determine the amount of the antigen or antibody.

**Indirect ELISA:** Indirect ELISA systems have been developed based on direct ELISA to evaluate the presence of antibody in antisera [21]. In this technique what determines and separates the antigen to be measured is not the primary antibody, but another antibody that is placed in the medium. Therefore, due to this the method is called as indirect method. In this technique, the diseased serum is added to the antigen-coated wells and the plates are incubated. During this incubation, the antibodies produced against the antigens in the diseased serum plaque create an antigen-antibody complex. To render the antigen-antibody complex visible, a secondary antibody that distinguishes the antibody in the serum and which is labeled with the enzyme is added. Then the substrate of the enzyme is added to the medium to create color and the concentration is determined. This technique exploited to detect antigen is used more commonly in endocrinology [22].

**Sandwich ELISA:** In this method, the antigen is detected via anchoring between two antibodies, which identify different epitopes, or the so-called sandwich method [23]. In this ELISA technique, the wells are coated with a capture antibody and blocked. The sample is added to the micro-plate wells coated with the antibody; then, the plate is incubated for some time and washed. Washing removes the unbound antigens. When the antigen-specific to the bound antibody is found, these antigens cannot be removed. Following the washing step, antibodies that are tagged with the enzyme specific to the antigen are added and incubated.

After incubation and washing, if there are antigens in the medium, these cannot be removed as the enzyme-tagged antibodies are bound to them. To reveal the enzyme activity, an enzyme substrate is added to the medium and coloration is ensured. Coloration shows a positive result, while lack of coloration indicates a lack of enzymes or a negative result. As the relevant protein is stuck between two antibody molecules, this method is called Sandwich ELISA. Sandwich ELISAs have been reported to be 2-5 times more sensitive than all other ELISAs [22].

**Competitive ELISA:** In 1973, Belanger developed competitive ELISA to detect rat á-fetoprotein, which involved the development of indirect ELISA and sandwich ELISA [23]. The key event of competitive ELISA is the competitive reaction between targets (antigen or antibody) in the sample and enzyme-labeled targets (antigen or antibody) against corresponding immobilized antibody or antigen. To detect the antigen in competitive ELISA, an enzyme-labeled antigen is used to compete with the target antigens against the immobilized antibody. The main advantage of competition ELISA is its high sensitivity to compositional differences in complex antigen mixtures, even when the specific detecting antibody is present in relatively small amounts [24].

**Application of ELISA**

**In Diagnosis:** In the area of diagnosis, ELISA has proven to be a capable platform applied worldwide for detecting a variety of disease types in humans and animals. Even to date, ELISA-based infectious serology marks one of the most reliable means for accurate diagnosis and prognosis. Even in plant pathology, ELISA technique is attracting increasing attention. ELISA has successfully overcome the drawbacks of the previous serological analyses performed in phyto-diagnosis [25].

**For Detection of Diseases:** There is a broad range of developed and marketed state-of-the-art assays for the detection of infectious agents. Several different commercial ELISA kits are available in the market for detection of HIV [26], Influenza [27], Dengue fever [28], Ebola [29], Chagas disease [30], including for the detection of parasitic diseases in animals like Trichinosis, Schistosomiasis, Trypanosomiasis, Filariasis and Toxoplasmosis [31]. The technique was widely applicable in the detection of major animal infectious diseases like Brucellosis, FMD and CBPP [32].

**For Pregnancy Test:** Many different bio-molecular entities including human chorionic gonadotropin, luteinizing hormone, follicle-stimulating hormone, estriol and thyrotrophin-stimulating hormone [33] can be expressed due to the pregnancy. ELISA can detect some of these proteins from the maternal blood, saliva, or urine at the early stages of the pregnancy [34]. HCG is one of the common hormones that can be detected by ELISA during the first month after fertilization. Another biomolecule associated with pregnancy is estriol, which can be detected with ELISA in the saliva at the sixth week of pregnancy. Specific ELISA pregnancy tests were developed for animals as well [35].

**In Immunology:** The defender of the body, the immune system, can operate in cellular or humoral (innate or adaptive) modes [36]. Measuring and monitoring the changes of the immune response underlay the foundation for understanding immune disease. Various studies have demonstrated ELISA as the gold standard method that is rapid and cost-effective for such measurements and monitoring [37]. A great number of examples for ELISA applications in immunology are reported, while some efforts were directed to optimize ELISA protocols further and to validate/establish their accuracy, sensitivity and specificity to support the clinical practice [38].

**In Food Industry:** ELISA plays a major role in the food industry. It is the main platform for identifying food allergens such as those present in milk, peanuts, walnuts, almonds and eggs [39] developed a monoclonal antibody-based sandwich ELISA for the detection of ovalbumin in food, which is the most frequent cause of food allergy, especially in children. ELISA can also be employed to corroborate the authenticity of the food products [39]. This technique is of great help to avoid possible economic losses caused by fraudulent substitution. In the case of meat and meat-based products, ELISA has proven to be a reliable technique that provides careful monitoring of the product, especially when religious considerations in the choice of food are concerned. ELISA is also an essential technique for quality control of fish, milk (as well as their sub-products), genetically modified foods, irradiated foods, or other harmful food components that can be transferred to a human, such as bovine spongiform encephalopathy [40].

**In Drug Monitoring and Pharmaceutical Industry:** ELISA techniques have also found a variety of applications in screening certain classes of drugs in plasma. The conventional therapeutic drug monitoring (TDM) strategies monitor drug levels in the plasma samples [41]. TDM also provides information regarding the treatment procedure allowing physicians to examine if the medication is present in the patient's body [42].

**In Vaccine Development:** ELISA serves as a great candidate for vaccine development. The sera sample from an immunized animal or human model can be tested to detect the presence of antibodies against certain types of antigens, which were intentionally injected into the host [43]. Normally different antigens are used to produce immune reactions in the host, among which those that elect higher protection response with less adverse effects can be selected [44].

**In Toxicology:** Toxicology involves studying the adverse effects of chemical compounds on living organisms. This area covers diagnosis and curing the effects of toxins (antigenic agents from plant or animal origins) as well as toxicants (toxic substances released into the environment) Competitive ELISA has a long history of being applied for detection of aflatoxin B1, one of the known toxins from rice. Developed immunoassay for aflatoxin monitoring is rapid and straightforward while offering desirable specificity and sensitivity. The competitive assay developed for this purpose was also reported to have a considerably longer shelf life for at least 12 months at room temperature [45].

## CONCLUSIONS AND RECOMANDATIONS

ELISA method was developed in 1971 as an alternative technique to radioimmunoassay methods to reduce radiation hazards originating from radioimmunoassay techniques. Quantitative analytical methods that show antigen-antibody reactions through the color change obtained by using an enzyme-linked conjugate and enzyme-substrate and that serve to identify the presence and concentration of molecules in biological fluids are generally called enzyme immune tests. Basically, ELISA holds four principle steps, consisting of coating, blocking, reacting of antigen and antibody and developing color. The binding of the antigen and antibody is detected using an enzyme-linked to a secondary antibody, which turns the antigen-antibody complex into a colored product when enzyme substrate is added.

There are about four types of Elisa that have been developed to detect specific antibodies, soluble antigens and to increase the specificity of measurement. ELISAs have been widely used for the measurement of polyclonal and monoclonal antibodies in biological fluids or in culture media. It was most commonly used in medical laboratories because it provides relatively rapid, sensitive, specific, accurate, robust, simple and cost-effective analytical methods for the detection of proteins and antigens produced by microorganisms and helps in guiding the clinicians in diagnosing and monitoring diseases.

In the area of diagnosis, ELISA has proven to be a capable platform applied worldwide for detecting a variety of disease types in humans and animals. Even to date, ELISA-based infectious serology marks one of the most reliable means for accurate diagnosis and prognosis. Apart from aiding in diagnosis, it was widely applied in the food industry, vaccine development, toxicology drug Monitoring and Pharmaceutical Industry.

The enzymes to be used in the method must not be present in the biological sample to be analyzed, nor must any other substance interfere with the analyzed biological sample; as the ELISA results may be wrong due to the possibility of interference. ELISA would be more familiar to us if the antibody or antibody-mimicking probes that are alternatively used in ELISA could be obtained more easily.

## REFERENCES


1. Hornbeck, P.V., 2015. Enzyme-linked immunosorbent assays. Current Protocols in Immunology, 110(1): 2-1.
2. Singh, H., M. Shimojima, T. Shiratori, L. Van An, M. Sugamata and M. Yang, 2015. Application of 3D printing technology in increasing the diagnostic performance of enzyme-linked immunosorbent assay (ELISA) for infectious diseases. Sensors, 15(7): 16503-16515.
3. Engvall, E. and P. Perlmann, 1971. Enzyme-linked immunosorbent assay (ELISA). Protides of the Biological Fluids, pp: 553-556.
4. Sakamoto, S., W. Putalun, S. Vimolmangkang, W. Phoolcharoen, Y. Shoyama, H. Tanaka and S. Morimoto, 2018. Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. Journal of Natural Medicines, 72(1): 32-42.
5. Baid, A., 2016. ELISA-A mini review. Res. Rev. J. Pharm. Anal, 5: 1-8.
6. Yalow, R.S. and S.A. Berson, 1960. Immunoassay of endogenous plasma insulin in man. The Journal of Clinical Investigation, 39(7): 1157-1175.
7. Avrameas, S., 1969. Coupling of enzymes to proteins with glutaraldehyde: use of the conjugates for the detection of antigens and antibodies. Immunochemistry, 6(1): 43-52.
8. Lequin, R.M., 2005. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clinical Chemistry, 51(12): 2415-2418.
9. Avrameas, S. and B. Guilbert, 1971. Enzymo-immunological determination of proteins with the aid of immunoadsorbants and enzyme-labelled antigens. Comptes rendus hebdomadaires des seances de l'Academie des sciences. Serie D: Sciences Naturelles, 273(25): 2705-2707.
10. Iorio, R., A. Giannattasio, E. Lamberti, C. Della Corte, E. Nicastro and M.I. Spagnuolo, 2006. Hyper-γ-Glutamyltransferase Is Commonly Present in Non–Breast-Fed Infants with Biliary Atresia Successfully Treated with Portoenterostomy. Clinical Chemistry, 52(7): 1430-1430.
11. Lim, P.W., 2010. Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of pistachio residues in processed foods. M.S.Thesis, University of Nebraska, Lincoln, NE, USA.
12. Avrameas, S., T. Ternynck and J.L. Guesdon, 1978. Coupling of enzymes to antibodies and antigens. Scandinavian Journal of Immunology, 8: 7-23.
13. Nakane, P.K. and A. Kawaoi, 1974. Peroxidase-labeled antibody a new method of conjugation. Journal of Histochemistry & Cytochemistry, 22(12): 1084-1091.
14. Bellia, F., R. Ragusa, R. Barkin and F. Elisabeth, 2016. The Mock Code an Educational Tool in International Medical Missions. J. Nurs. Care, 5(361): 2167-1168.
15. Zhao, J., B. Dynlacht, T. Imai, T.A. Hori and E. Harlow, 1998. Expression of NPAT, a novel substrate of cyclin E–CDK2, promotes S-phase entry. Genes & Development, 12(4): 456-461.
16. Koppelman, S.J. and S.L. Hefle, eds., 2006. Detecting allergens in food. Woodhead Pub.
17. Dai, J.R., B. Liu, D.R. Feng, H.Y. Liu, Y.M. He, K.B. Qi, H.B. Wang and J.F. Wang, 2011. MpAsr encodes an intrinsically unstructured protein and enhances osmotic tolerance in transgenic Arabidopsis. Plant Cell Reports, 30(7): 1219-1230.
18. Hadi, A.R.D.A., 2012. Development of a Rapid Diagnostic Technique for Equine Influenza (Doctoral dissertation, Universiti Putra Malaysia).
19. Lipp, M., R. Shillito, R. Giroux, F. Spiegelhalter, S. Charlton, D.Pinero and P. Song, 2005. Polymerase chain reaction technology as analytical tool in agricultural biotechnology. Journal of AOAC International, 88(1): 136-155.
20. Van Weemen, B.K. and A.H.W.M. Schuurs, 1971. Immunoassay using antigen-enzyme conjugates. FEBS Letters, 15(3): 232-236.

21. Lindström, P. and O. Wager, 1978. IgG autoantibody to human serum albumin studied by the ELISA-technique. Scandinavian Journal of Immunology, 7(5): 419-425.
22. Aydin, S., 2015. A short history, principles and types of ELISA and our laboratory experience with peptide/protein analyses using ELISA. Peptides, 72: 4-15.
23. Belanger, L., C. Sylvestre and D. Dufour, 1973. Enzyme-linked immunoassay for alpha-fetoprotein by competitive and sandwich procedures. Clinica Chimica Acta, 48(1): 15-18.
24. Dobrovolskaia, E., A. Gam and J.E. Slater, 2006. Competition enzyme-linked immunosorbant assay (ELISA) can be a sensitive method for the specific detection of small quantities of allergen in a complex mixture. Clinical & Experimental Allergy, 36(4): 525-530.
25. Boonham, N., J. Kreuze, S. Winter, R.van der Vlugt, J. Bergervoet, J. Tomlinson and R. Mumford, 2014. Methods in virus diagnostics: from ELISA to next generation sequencing. Virus Research, 186: 20-31.
26. Nandi, S., S. Maity, S.C. Bhunia and M.K. Saha, 2014. Comparative assessment of commercial ELISA kits for detection of HIV in India. BMC Research Notes, 7(1): 436.
27. Tarigan, S., R. Indriani, P.A. Durr and J. Ignjatovic, 2015. Characterization of the M2e antibody response following highly pathogenic H5N1 avian influenza virus infection and reliability of M2e ELISA for identifying infected among vaccinated chickens. Avian Pathology, 44(4): 259-268.
28. Hunsperger, E.A., S. Yoksan, P. Buchy, V.C. Nguyen, S.D. Sekaran, D.A. Enria, S. Vazquez, E. Cartozian, J.L. Pelegrino, H. Artsob and M.G. Guzman, 2014. Evaluation of commercially available diagnostic tests for the detection of dengue virus NS1 antigen and anti-dengue virus IgM antibody. PLoS Neglected Tropical Diseases, 8(10): 1-11.
29. Schieffelin, J., L.M. Moses, J. Shaffer, A. Goba and D.S. Grant, 2016. Clinical validation trial of a diagnostic for Ebola Zaire antigen detection: Design rationale and challenges to implementation. Clinical Trials, 13(1): 66-72.
30. Aria, L., M.E. Acosta, Y. Guillen, A. Rojas, T. Meza and B. Infanzón, 2016. ELISA Chagas test IICS V. 1 evaluation for the diagnosis of Chagas disease. Memorias del Instituto de Investigaciones en Ciencias de la Salud, 14(3): 7-13.
31. PMC, J.E. and G.R. API, 1976. The enzyme-linked immunosorbent assay (ELISA).
32. Birhanu, T. and E. Nekemte, 2014. Prevalence of the major infectious animal diseases affecting livestock trade industry in Ethiopia. Prevalence, 4(17): 76-82.
33. Wingeier, M., P. La Marca-Ghaemmaghami, R. Zimmermann and U. Ehlert, 2017. Is salivary estriol detectable in very early pregnancy?. The Journal of Maternal-Fetal & Neonatal Medicine, 30(2): 228-232.
34. Wilcox, A.J., D.D. Baird, D. Dunson, R. McChesney and C.R. Weinberg, 2001. Natural limits of pregnancy testing in relation to the expected menstrual period. Jama, 286(14): 1759-1761.
35. Karen, A., N.M. De Sousa, J.F. Beckers, Á.C. Bajcsy, J. Tibold, I. Mádl and O. Szenci, 2015. Comparison of a commercial bovine pregnancy-associated glycoprotein ELISA test and a pregnancy-associated glycoprotein radiomimmunoassay test for early pregnancy diagnosis in dairy cattle. Animal Reproduction Science, 159: 31-37.
36. Hosseini, S., P. Vázquez-Villegas, M. Rito-Palomares and S.O. Martinez-Chapa, 2018. General Overviews on Applications of ELISA. In Enzyme-Linked Immunosorbent Assay (ELISA) (pp: 19-29). Springer, Singapore.
37. Orsolini, G., M.R. Snyder, C.S. Crowson, J.L. Frinack and M.G. Kevin, 2016. THU0327 Comparison of Immunoenzymatic Assay and Crithidia Immunofluorescence Test for The Detection of anti-Double Strand DNA Antibodies in Patients with Systemic Lupus Erythematosus.
38. Uchida, K., K. Nakata, B. Carey, C. Chalk, T. Suzuki, T. Sakagami, D.E. Koch, C. Stevens, Y. Inoue, Y. Yamada and B.C. Trapnell, 2014. Standardized serum GM-CSF autoantibody testing for the routine clinical diagnosis of autoimmune pulmonary alveolar proteinosis. Journal of Immunological Methods, 402(1-2): 57-70.
39. Peng, J., X. Meng, X. Deng, J.Zhu, H. Kuang and C. Xu, 2014. Development of a monoclonal antibody-based sandwich ELISA for the detection of ovalbumin in foods. Food and Agricultural Immunology, 25(1): 1-8.
40. Asensio, L., I. González, T.García and R. Martín, 2008. Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). Food Control, 19(1): 1-8.

41. Verweel, G., D.M. Burger, N.L. Sheehan, A.S. Bergshoeff, A. Warris, L.C. van der Knaap, G. Driessen, R.de Groot and N.G. Hartwig, 2007. Plasma concentrations of the HIV-protease inhibitor lopinavir are suboptimal in children aged 2 years and below. Antiviral Therapy, 12(4): 453.
42. Nso, A.P., B. Larru, J.M. Bellón, M.J. Mellado, J.T. Ramos, M.I. González, M.L Navarro, M.Á. Muñoz-Fernández and M.I. De José, 2010. Comparison of levels of antiretroviral drugs with efficacy in children with HIV infection. The Indian Journal of Pediatrics, 77(4): 397-402.
43. Pizza, M., V. Scarlato, V. Masignani, M.M. Giuliani, B. Arico, M. Comanducci, G.T. Jennings, L. Baldi, E. Bartolini, B. Capecchi and C.L. Galeotti, 2000. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science, 287(5459): 1816-1820.
44. Miura, K., A.C. Orcutt, O.V. Muratova, L.H. Miller, A. Saul and C.A. Long, 2008. Development and characterization of a standardized ELISA including a reference serum on each plate to detect antibodies induced by experimental malaria vaccines. Vaccine, 26(2): 193-200.
45. Kolosova, A.Y., W.B. Shim, Z.Y. Yang, S.A. Eremin and D.H. Chung, 2006. Direct competitive ELISA based on a monoclonal antibody for detection of aflatoxin B 1. Stabilization of ELISA kit components and application to grain samples. Analytical and Bioanalytical Chemistry, 384(1): 286-294.