PLOS ONE

PLoS One. 2016; 11(5): e0153317.
Published online 2016 May 5. doi: 10.1371/journal.pone.0153317

PMCID: PMC4858304
PMID: 27149415

Measuring Digital PCR Quality: Performance Parameters and Their Optimization

A. Lievens,* S. Jacchia, D. Kagkli, C. Savini, and M. Querci

Kelvin Yuen Kwong Chan, Editor

Molecular Biology and Genomics Unit, European Commission - Joint Research Centre, Institute for Health and Consumer Protection, 21027 Ispra (VA), Italy, Hospital Authority, CHINA,

Competing Interests: The authors have declared that no competing interests exist.

Conceived and designed the experiments: AL SJ. Performed the experiments: AL SJ DK. Analyzed the data: AL. Contributed reagents/materials/analysis tools: AL SJ DK. Wrote the paper: AL. Contributed in proofreading the main body of the text: MQ. Contributed in proofreading the tables and checking the statistics: CS.

* E-mail: antoon.lievens@ec.europa.eu

Received 2015 Dec 1; Accepted 2016 Mar 28.

Abstract

Digital PCR is rapidly being adopted in the field of DNA-based food analysis. The direct, absolute quantification it offers makes it an attractive technology for routine analysis of food and feed samples for their composition, possible GMO content, and compliance with labelling requirements. However, assessing the performance of dPCR assays is not yet well established. This article introduces three straightforward parameters based on statistical principles that allow users to evaluate if their assays are robust. In addition, we present post-run evaluation criteria to check if quantification was accurate. Finally, we evaluate the usefulness of Poisson confidence intervals and present an alternative strategy to better capture the variability in the analytical chain.

Introduction

About two decades ago, digital PCR was developed as a potential alternative quantification strategy [1, 2] and in the wake of technological advances in the field of nanofluidics, several digital PCR platforms have recently entered the market. Digital PCR is based on the concept of limiting dilutions. Practically a reaction is split into a large number of (nanoliter) sub-reactions so that individual target copies are separated by the process of partitioning. After thermal cycling and read-out this leads to the classification of each partition as either positive (containing target) or negative (no target present).

The distribution of the target molecules across the partitions can be seen as a Poisson process (the targets end up in partitions independently and with a fixed rate). Poisson statistics thus allow the calculation of the initial number of targets from the number of positive and negative partitions. As a consequence, digital PCR is an absolute quantification strategy by default. This is very different from qPCR, which is based on the proportionality between fluorescence & DNA mass and where quantification is always relative. Therefore, calibration curves are needed to invoke absolute quantification in a qPCR setting.

A recent survey among European food analysis laboratories indicated that a growing proportion of them are investing in digital PCR platforms and that this technique is increasingly applied in a routine setting (*e.g.* GMO analysis, food fraud, species identification). This ongoing permeation of digital PCR into a legislative setting signals the need for application guidelines and for a harmonization effort. Here, we have established several criteria to measure the performance of digital PCR assays and have set the limits for the corresponding parameters. Further, we have explored a range of theoretical and practical aspects of dPCR in order to gauge which practices may help establish a robust and reliable framework for digital quantification of the DNA targets in food and feed samples.

Material and Methods

DNA Samples and PCR reactions

All DNA extracts were quantified fluorometrically using Picogreen (Molecular Probes) and a fluorometer (Biorad Versafluor). The amount of template copies was calculated from the DNA quantities using haploid genome weights [3].

Samples were prepared from dry materials (CRM and seed material). DNA was extracted using a CTAB based method adopted from [4]. The exact protocol for DNA extraction is available in S1 File.

real time PCR reactions were performed in 25μl using primers from [5–13] (see S1 Table). Probe-based reactions were run using Taqman Universal Mastermix (Life Technologies), primers and probes were ordered from Eurogentec. All reactions were amplified in ABI microamp 96-well plates using either an Applied Biosystems ABI7900 or ABI7500 (Life Technologies). A single thermal cycling protocol was used for all real time PCR reactions: 10min 95°C, 60× (15sec 95°C, 1min 60°C). Results were analysed & exported using the SDS 2.4.1 software.

digital PCR reactions were performed using the Biorad QX200 digital droplet platform using Twin.Tec 96 well PCR plates (Eppendorf). Initial volume of the reaction mixture was 20μl which, together with the droplet generating oil, resulted in a final PCR volume of approx. 45μl. Reactions were set up using Probe Supermix (Biorad), primers and probes were ordered from Eurogentec. Thermal cycling was performed on a Biorad C1000 using the following thermal cycling protocol: 10min 95°C, 45× (15sec 95°C, 1min 60°C), 10min 98°C. Results were analysed & exported using the Quantasoft 1.6.6.320 software.

digital touchdown PCR was performed as described in [14]. The final thermal cycling protocol used was: 10min 95°C, 30× (15sec 95°C, 1min 63°C), 15× (15sec 95°C, 1min 60°C), 10min 98°C.

regulation of normal cellular differentiation, as well as some unique aspects of control of differentiation of vascular SMCs. We will start with a simple definition of cellular differentiation. Although this may be obvious to the majority of readers, we nonetheless review it here since we continually encounter statements in the literature that convey some confusion in this area.

Cellular differentiation is simply the process by which multipotential cells in the developing organism acquire those cell-specific characteristics that distinguish them from other cell types. Although the process of cellular differentiation is quite complex, in the final analysis it can be subdivided into the following three major regulatory components: *1*) selective activation of the subset of genes required for the cell's differentiated function or functions; *2*) coordinate control of expression of cell-selective/specific genes at precise times and stochiometries; and *3*) continuous regulation of gene expression through effects of local environmental cues on the genetic program that determines cell lineage, including control of chromatin structure or epigenetic programming that can influence the ability of transcription factors to access regulatory regions of genes. In addition, it is important to recognize that an understanding of the differentiation of any cell type not only involves elucidating cell autonomous mechanisms that control gene expression patterns and functional properties (i.e., specialization of inividual cells), but also must encompass how the cell interacts with its environment (i.e., other cells, matrix, etc.) and the complex processes that control overall tissue and organ morphogenesis.

A major challenge in understanding differentiation of the SMC is that it can exhibit a wide range of different phenotypes at different stages of development, and even in adult organisms the cell is not terminally differentiated and is capable of major changes in its phenotype in response to changes in its local environment (see reviews in Refs. 191, 225) (Fig. 1.) For example, during early stages of vasculogenesis SMCs are highly migratory and undergo very rapid cell proliferation. Indeed, recent live videos of vascular development, the SMC investment process, and vascular remodeling in zebrafish (118) and avian systems (45) indicate that there is a remarkable amount of movement of SMCs and SMC progenitor cells as part of the complex morphogenic events that result in formation of the cardiovascular system. During vascular development, SMCs also exhibit very high rates of synthesis of extracellular matrix components including collagen, elastin, proteoglycans, cadherins, and integrins that comprise a major portion of the blood vessel mass. At this stage of development, SMCs form abundant gap junctions with endothelial cells, and the process of investment of endothelial tubes with SMCs or pericytes is critical for vascular maturation and vessel remodeling (113). In contrast, in adult blood vessels the SMC shows an exceedingly low rate of proliferation/turnover, is largely nonmigratory, shows a very low rate of synthesis of extracellular matrix components, and is a cell virtually completely committed to carrying out its contractile function. Indeed, the mature fully differentiated SMC expresses a repertoire of appropriate receptors, ion channels, signal transduction molecules, calcium regulatory proteins, and contractile proteins necessary for the unique contractile properties of the SMC (191). However, upon vascular injury, "contractile" SMCs are capable of undergoing transient modification of their phenotype to a highly "synthetic" phenotype (see sect. III), and they play a critical role in repair of the vascular injury. Upon resolution of the injury, the local environmental cues within the vessel return to normal, and SMCs reacquire their contractile phenotype/properties. Taken together, the model that has emerged is that SMCs within adult mammals are highly plastic cells that are capable of rather profound alterations in their phenotype in response to changes in local environmental cues important for their differentiation (Fig. 1). Key questions are thus, *1*) What genes and gene products serve as appropriate markers with which to study SMC differentiation/maturation? *2*) What are the key environmental cues or signals that control the expression of these SMC-specific/selective marker genes?

Before considering these questions, we wish to briefly consider several lines of evidence challenging the dogma that repair of vascular injury is carried out principally (or exclusively) by reversible phenotypic modulation of preexisting SMCs. Two alternative mechanisms have been proposed, although in reality none is mutually exclusive. The first line of evidence is that circulating bone marrow-derived SMC progenitor cells play a major role in normal vascular injury repair (84, 221, 230) (see also sect. III). Note that we are excluding consideration of the possible role of recipient-derived stem cells in normal or transplant atherosclerosis (24, 102) in the present discussion, although we will consider this very interesting topic in section III. The second line of evidence is that SMC populations within blood vessels are extremely heterogeneous with resident stable populations of preexisting SMCs that are phenotypically distinct from the classical definition of a contractile SMCs (64, 86) and that these cells carry out injury repair. We will briefly consider each of these issues in the next two paragraphs.

A number of relatively recent studies have provided evidence showing that circulating cells, presumably derived from bone marrow, can contribute to neointima formation and repair following vascular injury (84, 221, 230). However, for the most part, studies in animal models have either involved very extensive damage to medial SMCs (indeed, nearly complete destruction of the media and SMC death), and/or transplantation-associated immunological injury due to genetic mismatch of host and donor tissues combined with lack of adequate immuno-

HHS Public Access
Author manuscript
Methods Mol Biol. Author manuscript; available in PMC 2020 January 01.

Published in final edited form as:
Methods Mol Biol. 2019 ; 1897: 289–298. doi:10.1007/978-1-4939-8935-5_25.

An Introduction to the Performance of Immunohistochemistry

Shino Magaki[1], **Seyed A. Hojat**[1], **Bowen Wei**[1], **Alexandra So**[1], **William H. Yong**[1,2,3,*]

[1]Department of Pathology and Laboratory Medicine (Neuropathology), David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

[2]Brain Research Institute, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

[3]Jonsson Comprehensive Cancer Center, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

Summary

i.

Immunohistochemistry (IHC) is a powerful technique that exploits the specific binding between an antibody and antigen to detect and localize specific antigens in cells and tissue, most commonly detected and examined with the light microscope. A standard tool in many fields in the research setting, IHC has become an essential ancillary technique in clinical diagnostics in anatomic pathology (1) with the advent of antigen retrieval methods allowing it to be performed conveniently on formalin fixed paraffin embedded (FFPE) tissue (2, 3) and automated methods for high volume processing with reproducibility (4). IHC is frequently utilized to assist in the classification of neoplasms, determination of a metastatic tumor's site of origin and detection of tiny foci of tumor cells inconspicuous on routine hematoxylin and eosin (H&E) staining. Furthermore, it is increasingly being used to provide predictive and prognostic information, such as in testing for *HER2* amplification in breast cancer (5) in addition to serving as markers for molecular alterations in neoplasms, including *IDH1* and *ATRX* mutations in brain tumors (6). In this section we describe the basic methods of immunohistochemical staining which has become an essential tool in the daily practice of anatomic pathology worldwide.

Keywords

Immunohistochemistry; Antibodies; Antigens; Antigen retrieval; Light microscopy

1. Introduction

Immunohistochemistry (IHC) is a widely used ancillary testing method in anatomic surgical pathology for cell classification and diagnosis and utilizes antibodies targeted against certain antigens in specific tissues and cells to facilitate determination of cell type and organ of origin. The method is most commonly performed on formalin fixed paraffin embedded (FFPE) tissue which has the advantage of being amenable to easy storage, although it was first developed on frozen sections and can also be done on plastic embedded tissue (7, 8).

*Corresponding author: William Yong MD, Brain Tumor Translational Resource, David Geffen School of Medicine at UCLA, CHS 13-145B, 10833 Le Conte Avenue, Los Angeles, CA, 90095; wyong@mednet.ucla.edu.

The use of IHC has recently further expanded to assess predictive and prognostic biomarkers in many malignancies including those of the breast, gastrointestinal tract, lung, hematolymphoid and central nervous systems (9, 10). Guidelines for the standardization and analytic validation of immunohistochemical tests have been established by the College of American Pathologists (1, 11).

The sequential steps in IHC can be summarized as follows: antigen retrieval (AR), addition of primary antibody, application of a secondary antibody that binds the primary antibody, and addition of a detection reagent to localize the primary antibody (Fig 1). The first step in IHC is usually antigen retrieval (AR), which involves the pretreatment of tissue to retrieve antigens masked by fixation and make them more accessible to antibody binding (12). This technique, first described by Shi et al, has significantly increased the sensitivity of IHC and consequently greatly expanded its application (3, 13). There are various methods of antigen retrieval depending on the specific target antigen and antibody (Table 1), but most generally involve the breaking of protein cross-links caused by fixation, such as formalin, through chemical or physical means. Physical treatments include heat and ultrasound while chemical methods include enzyme digestion and denaturant treatment, although many utilize both, such as chemical treatment with heat. Currently, the most popular method is heat induced antigen retrieval (HIAR) using microwave ovens most commonly, as well as pressure cookers, autoclaves and water baths (14).

The primary antibody, which can be monoclonal or polyclonal, is titrated to optimize contrast between positively staining tissue and nonspecific background staining, with the highest primary antibody dilution to prevent waste (1, 7). In general, monoclonal antibodies, which target a single epitope, tend to be more specific while polyclonal antibodies, which can bind many different epitopes, tend be more sensitive (2). Usually starting with the dilution recommended by the manufacturer or published in the literature for the tissue of interest, a more concentrated and less concentrated dilution flanking the recommended dilution is tested on a series of tissues with the appropriate positive control. This may be combined with various combinations of dilutions of the secondary antibody in the setting of the particular AR method and chromogen to produce optimum staining (7). For the initial titration, an antibody concentration of 1 to 5 μg/mL is usually recommended (1).

To visualize the antigen antibody interaction under light microscopy, either the primary antibody or secondary antibody, which is targeted against the immunoglobulin of the species in which the primary antibody was produced, must be labeled (2). In the direct method, the primary antibody is labeled and applied to the tissue in a quick one step process; however, this method is not commonly used due to lack of signal amplification and thus the requirement for a higher concentration of antibody as well as the need to label each primary antibody. In the indirect method, the secondary antibody is labeled, allowing for signal amplification and use with many different primary antibodies. There are various labels that can be used, such as fluorescent molecules and enzymes such as horseradish peroxidase or alkaline phosphatase which produce a colored product after incubation with a chromogenic substrate such as diaminobenzidine (DAB) (2, 7). Immunofluorescence techniques using fluorescent compounds are also available but require a fluorescence microscope (2). The avidin-biotin-peroxidase method suffers from high background staining due to binding of

The use of IHC has recently further expanded to assess predictive and prognostic biomarkers in many malignancies including those of the breast, gastrointestinal tract, lung, hematolymphoid and central nervous systems (9, 10). Guidelines for the standardization and analytic validation of immunohistochemical tests have been established by the College of American Pathologists (1, 11).

The sequential steps in IHC can be summarized as follows: antigen retrieval (AR), addition of primary antibody, application of a secondary antibody that binds the primary antibody, and addition of a detection reagent to localize the primary antibody (Fig 1). The first step in IHC is usually antigen retrieval (AR), which involves the pretreatment of tissue to retrieve antigens masked by fixation and make them more accessible to antibody binding (12). This technique, first described by Shi et al, has significantly increased the sensitivity of IHC and consequently greatly expanded its application (3, 13). There are various methods of antigen retrieval depending on the specific target antigen and antibody (Table 1), but most generally involve the breaking of protein cross-links caused by fixation, such as formalin, through chemical or physical means. Physical treatments include heat and ultrasound while chemical methods include enzyme digestion and denaturant treatment, although many utilize both, such as chemical treatment with heat. Currently, the most popular method is heat induced antigen retrieval (HIAR) using microwave ovens most commonly, as well as pressure cookers, autoclaves and water baths (14).

The primary antibody, which can be monoclonal or polyclonal, is titrated to optimize contrast between positively staining tissue and nonspecific background staining, with the highest primary antibody dilution to prevent waste (1, 7). In general, monoclonal antibodies, which target a single epitope, tend to be more specific while polyclonal antibodies, which can bind many different epitopes, tend be more sensitive (2). Usually starting with the dilution recommended by the manufacturer or published in the literature for the tissue of interest, a more concentrated and less concentrated dilution flanking the recommended dilution is tested on a series of tissues with the appropriate positive control. This may be combined with various combinations of dilutions of the secondary antibody in the setting of the particular AR method and chromogen to produce optimum staining (7). For the initial titration, an antibody concentration of 1 to 5 μg/mL is usually recommended (1).

To visualize the antigen antibody interaction under light microscopy, either the primary antibody or secondary antibody, which is targeted against the immunoglobulin of the species in which the primary antibody was produced, must be labeled (2). In the direct method, the primary antibody is labeled and applied to the tissue in a quick one step process; however, this method is not commonly used due to lack of signal amplification and thus the requirement for a higher concentration of antibody as well as the need to label each primary antibody. In the indirect method, the secondary antibody is labeled, allowing for signal amplification and use with many different primary antibodies. There are various labels that can be used, such as fluorescent molecules and enzymes such as horseradish peroxidase or alkaline phosphatase which produce a colored product after incubation with a chromogenic substrate such as diaminobenzidine (DAB) (2, 7). Immunofluorescence techniques using fluorescent compounds are also available but require a fluorescence microscope (2). The avidin-biotin-peroxidase method suffers from high background staining due to binding of

The use of IHC has recently further expanded to assess predictive and prognostic biomarkers in many malignancies including those of the breast, gastrointestinal tract, lung, hematolymphoid and central nervous systems (9, 10). Guidelines for the standardization and analytic validation of immunohistochemical tests have been established by the College of American Pathologists (1, 11).

The sequential steps in IHC can be summarized as follows: antigen retrieval (AR), addition of primary antibody, application of a secondary antibody that binds the primary antibody, and addition of a detection reagent to localize the primary antibody (Fig 1). The first step in IHC is usually antigen retrieval (AR), which involves the pretreatment of tissue to retrieve antigens masked by fixation and make them more accessible to antibody binding (12). This technique, first described by Shi et al, has significantly increased the sensitivity of IHC and consequently greatly expanded its application (3, 13). There are various methods of antigen retrieval depending on the specific target antigen and antibody (Table 1), but most generally involve the breaking of protein cross-links caused by fixation, such as formalin, through chemical or physical means. Physical treatments include heat and ultrasound while chemical methods include enzyme digestion and denaturant treatment, although many utilize both, such as chemical treatment with heat. Currently, the most popular method is heat induced antigen retrieval (HIAR) using microwave ovens most commonly, as well as pressure cookers, autoclaves and water baths (14).

The primary antibody, which can be monoclonal or polyclonal, is titrated to optimize contrast between positively staining tissue and nonspecific background staining, with the highest primary antibody dilution to prevent waste (1, 7). In general, monoclonal antibodies, which target a single epitope, tend to be more specific while polyclonal antibodies, which can bind many different epitopes, tend be more sensitive (2). Usually starting with the dilution recommended by the manufacturer or published in the literature for the tissue of interest, a more concentrated and less concentrated dilution flanking the recommended dilution is tested on a series of tissues with the appropriate positive control. This may be combined with various combinations of dilutions of the secondary antibody in the setting of the particular AR method and chromogen to produce optimum staining (7). For the initial titration, an antibody concentration of 1 to 5 μg/mL is usually recommended (1).

To visualize the antigen antibody interaction under light microscopy, either the primary antibody or secondary antibody, which is targeted against the immunoglobulin of the species in which the primary antibody was produced, must be labeled (2). In the direct method, the primary antibody is labeled and applied to the tissue in a quick one step process; however, this method is not commonly used due to lack of signal amplification and thus the requirement for a higher concentration of antibody as well as the need to label each primary antibody. In the indirect method, the secondary antibody is labeled, allowing for signal amplification and use with many different primary antibodies. There are various labels that can be used, such as fluorescent molecules and enzymes such as horseradish peroxidase or alkaline phosphatase which produce a colored product after incubation with a chromogenic substrate such as diaminobenzidine (DAB) (2, 7). Immunofluorescence techniques using fluorescent compounds are also available but require a fluorescence microscope (2). The avidin-biotin-peroxidase method suffers from high background staining due to binding of

endogenous biotin, and the method is now largely obsolete. Polymer based methods utilize many peroxidase molecules and secondary antibodies which are attached to a dextran polymer backbone and allows for increased sensitivity (7).

Background staining may be due to nonspecific antibody binding, more common in polyclonal antibodies, and endogenous peroxidase activity, more problematic in tissues with abundant hematopoietic elements such as bone marrow. Nonspecific antibody binding can be decreased by preincubation with normal serum from the same species as the secondary antibody or with a commercially available universal blocking agent. Endogenous enzyme activity can be inhibited by pretreating the tissue with solutions containing hydrogen peroxide prior to application of the antibody (2).

Quality control is critical, and a positive and negative control should be performed with each run. Positive controls are tissues that contain an antigen known to stain with a certain antibody, and ideally should be run on the same slide as the tissue of interest so that the control tissue undergoes the same reaction conditions as the sample tissue. To eliminate the possibility of nonspecific antibody binding with the secondary antibody, negative controls consist of the sample tissue that undergoes the identical staining conditions minus the primary antibody or with a non-immune immunoglobulin from the same species (7). False positives and negatives can be due to the immunohistochemical method itself but also to a myriad of other factors including preparation and fixation (see Notes section).

2. Materials

2.1. Solutions

1. Deionized and distilled water
2. Xylene
3. Ethanol, anhydrous denatured, histological grade (100, 95, 80, and 70%)
4. Hematoxylin solution
5. Tacha's Bluing solution: for bluing hematoxylin stained nuclei
6. Wash buffers: follow vendor recommendations.
7. Antigen retrieval buffer, depends on specific antigen retrieval method: for HIAR e.g. 10X Antigen Decloaker (Biocare, Pacheco, CA, USA) diluted 1:10 with deionized water
8. 0.1% TBS-Tween
9. 3% hydrogen peroxide (for blocking endogenous peroxidase); can also use a ready-to-use peroxidase blocking solution available from various manufacturers
10. Blocking reagent to decrease nonspecific background staining e.g. Background Sniper (Biocare, Pacheco, CA, USA)
11. Charged or adhesion slides to promote tissue retention onto the slide

endogenous biotin, and the method is now largely obsolete. Polymer based methods utilize many peroxidase molecules and secondary antibodies which are attached to a dextran polymer backbone and allows for increased sensitivity (7).

Background staining may be due to nonspecific antibody binding, more common in polyclonal antibodies, and endogenous peroxidase activity, more problematic in tissues with abundant hematopoietic elements such as bone marrow. Nonspecific antibody binding can be decreased by preincubation with normal serum from the same species as the secondary antibody or with a commercially available universal blocking agent. Endogenous enzyme activity can be inhibited by pretreating the tissue with solutions containing hydrogen peroxide prior to application of the antibody (2).

Quality control is critical, and a positive and negative control should be performed with each run. Positive controls are tissues that contain an antigen known to stain with a certain antibody, and ideally should be run on the same slide as the tissue of interest so that the control tissue undergoes the same reaction conditions as the sample tissue. To eliminate the possibility of nonspecific antibody binding with the secondary antibody, negative controls consist of the sample tissue that undergoes the identical staining conditions minus the primary antibody or with a non-immune immunoglobulin from the same species (7). False positives and negatives can be due to the immunohistochemical method itself but also to a myriad of other factors including preparation and fixation (see Notes section).

2. Materials

2.1. Solutions

1. Deionized and distilled water
2. Xylene
3. Ethanol, anhydrous denatured, histological grade (100, 95, 80, and 70%)
4. Hematoxylin solution
5. Tacha's Bluing solution: for bluing hematoxylin stained nuclei
6. Wash buffers: follow vendor recommendations.
7. Antigen retrieval buffer, depends on specific antigen retrieval method: for HIAR e.g. 10X Antigen Decloaker (Biocare, Pacheco, CA, USA) diluted 1:10 with deionized water
8. 0.1% TBS-Tween
9. 3% hydrogen peroxide (for blocking endogenous peroxidase); can also use a ready-to-use peroxidase blocking solution available from various manufacturers
10. Blocking reagent to decrease nonspecific background staining e.g. Background Sniper (Biocare, Pacheco, CA, USA)
11. Charged or adhesion slides to promote tissue retention onto the slide

Peptides 72 (2015) 4–15

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA

Suleyman Aydin *

Firat University, School of Medicine, Department of Medical Biochemistry (Firat Hormones Research Group), 23119 Elazig, Turkey

ARTICLE INFO

Article history:
Received 13 March 2015
Received in revised form 7 April 2015
Accepted 8 April 2015
Available online 20 April 2015

Keywords:
Analytical error
ELISA
General protocol
Peptide analysis

ABSTRACT

Playing a critical role in the metabolic homeostasis of living systems, the circulating concentrations of peptides/proteins are influenced by a variety of patho-physiological events. These peptide/protein concentrations in biological fluids are measured using various methods, the most common of which is enzymatic immunoassay EIA/ELISA and which guide the clinicians in diagnosing and monitoring diseases that inflict biological systems. All the techniques where enzymes are employed to show antigen–antibody reactions are generally referred to as enzymatic immunoassay EIA/ELISA method. Since the basic principles of EIA and ELISA are the same. The main objective of this review is to present an overview of the historical journey that had led to the invention of EIA/ELISA, an indispensible method for medical and research laboratories, types of ELISA developed after its invention [direct (the first ELISA method invented), indirect, sandwich and competitive methods], problems encountered during peptide/protein analyses (pre-analytical, analytical and post-analytical), rules to be followed to prevent these problems, and our laboratory experience of more than 15 years.

Introduction

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 immunotests [enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA)] [16]. Very low-concentration molecules such as peptides/proteins, hormones, vitamins and drugs display a high level of specificity against antibodies or antigens developed for them [12,16,23]. This is because it is almost impossible for an antibody to be bound to a molecule other than its own antigen. Thus, this method can be used to measure even substances in very low concentrations with hardly any risk of interference. In other words, when we have the antigen which we know to be specific to a certain substance, we can identify the type and amount 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 immunotests [12,16].

History of ELISA

Although the basic principle of ELISA and radioimmunoassay (RIA) techniques dates back to 1941 [11], RIA method was first used by Yalow and Berson in 1960s to measure the endogenous plasma insulin level [41]. In fact, ELISA method was invented simultaneously by two research teams at the same time [13,39]. However, ELISA method was pioneered largely by the Swiss scientists Engvall, and Perlmann who died in 2005 [13]. These two researchers developed the ELISA method in 1971 by modifying the RIA method [13]. In other words, they devised the immunological ELISA method by conjugating the tagged antigen and antibody radioisotopes in RIA with enzymes rather than radioactive iodine 125. They employed this new method to determine the levels of IgG in rabbit serum [13]. In the same year, a different research team succeeded in quantifying human chorionic gonadotropin amounts in the urine by using horseradish peroxidase (EC 1.11.17) enzyme with the EIA method [39]. The researchers applied for a patent both in the USA and Europe.

Abbreviations: AST, aspartate aminotransferase; CK, creatinine kinase; CZ, Czech Republic; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; HCl, hydrochloric acid; H_2SO_4, sulfuric acid; LDH, lactate dehydrogenase; NaOH, sodium hydroxide; PRC, The People's Republic of China; QC1, quality control; RIA, radioimmunoassay; SA, South Africa; UK, The United Kingdom; USA, The United States of America.

* Tel.: +90 5334934643; fax: +90 424 2379138.
E-mail address: saydin1@hotmail.com

http://dx.doi.org/10.1016/j.peptides.2015.04.012
0196-9781/

Following the invention of ELISA, a number of researchers used it: Carlson and colleagues in 1972 [10], Holmgren and Svennerholm in diagnostic microbiology in 1973 [15], Ljungstrom and colleagues to identify the presence of trichinosis in parasitology in 1974 [26], and Voller et al. to diagnose malaria in 1975 [40]. Bishai and Galli, Leinikki et al. and Ukkonen et al. made use of the ELISA method to identify infections caused by influenza, parainfluenza and mumps viruses in 1978, 1979, and 1981, respectively [6,22,38]. In 1980, Siegle et al. modified the ELISA test and incorporated microtitration plates to identify the concentrations of various hormones, peptides, and proteins [35]. The method which has found different fields of application and grown beyond infancy over time has become a routinely used method in research and diagnosis laboratories around the world.

How does the ELISA method work?

The antigen utilized in the ELISA method is bound to a solid phase. Tubes and microplates made of rigid polystyrene, polyvinyl and polypropylene are used as the solid phase. The microplates used must be able to appropriately adsorb the antigen and the antibody, but not adsorb the components in the other phases [13,41]. The enzymes that can be employed in ELISA include beta galactosidase, glucose oxidase, peroxidase, and alkaline phosphatase. Alkaline phosphatase can be stored at 4 °C with its conjugate sodium azide. Alkaline phosphatase and P-nitro-phenyl phosphate are used as substrates, are available in safe tablet forms, and produce a yellow color in positive reactions. For the peroxidase conjugate, 5 amino salicylic acid and orthophenylenediamine are used as the substrates and the production of a brown color is considered a positive reaction. If beta galactosidase is used, the sample must be read in a fluorometer. The catabolic effects of enzymes determine both the acceleration and the specificity of the immunological reaction during the enzyme-substrate reaction [12]. The enzyme-substrate reaction is usually completed within 30–60 min. The reaction can be stopped using sodium hydroxide (NaOH), hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) [16]. The results are read on a spectrophotometer and at 400–600 nm depending on the characteristics of the conjugate used.

Types of ELISA

Enzymatic immunoassay methods are considered under two general headings as homogeneous enzymatic immunoassay methods and heterogeneous enzymatic immunoassay methods [27] (Fig. 1). In the homogeneous enzymatic immunoassay methods, enzymes become inactivated when they are bound to the antibody, and thus, there is no stage (washing) where the antigen is separated from the medium. Homogeneous enzymatic immunoassay method is usually employed to measure substances in small quantities, like therapeutic drugs [27]. Homogeneous method is expensive and has low sensitivity. The only advantage it possesses is its ease of use.

As heterogeneous enzymatic immunoassay methods are more commonly used [27], the methods and types of this method are detailed in the following paragraphs. In this method, in order to the prevent interference of any molecule in the medium with it after the binding of the antigen and the antibody, the antigen–antibody complex is bound to the walls of the experiment tubes and anything other than the complex is removed from the medium through washing procedures. In other words, in heterogeneous enzymatic immunoassay methods, it is essential to have a washing stage to separate the bound antigen from the free antigen after the antigen–antibody interaction. Since the heterogeneous method is more sensitive than the homogeneous one, it is more commonly used. ELISA is a heterogeneous immunoassay technique used to detect specific antibodies and soluble antigens, and since the structure and the characteristics of the substances to be measured are not always the same, a variety of ELISA types have been developed to increase the specificity of measurement [27]. Schematic description of the homogeneous enzymatic immunoassay and heterogeneous enzymatic immunoassay methods is presented in Fig. 2.

Direct ELISA (antigen screening)

The technique was simultaneously developed in 1971 by Engvall and Perlmann [13] and by Van Weemen and Schuurs [39], the technique pioneered other ELISA types. Direct ELISA method is suitable 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 removes the unbound antigens or antibodies from the medium. Then the appropriate substrate is added to the medium to produce a signal through coloration. The signal is measured to determine the amount of the antigen or antibody [12,16].

Indirect ELISA

The technique was developed in 1978 Lindström and Wager [25], who were inspired by the direct ELISA method. The researchers reported measuring porcine IgG using this method. The reason why this method is called the indirect method is that what determines and separates the antigen to be measured is not the primary antibody, but another antibody that is placed in the medium. In this method, the diseased serum is added to the antigen-coated wells and the plates are incubated. During this incubation, the antibodies formed against the antigens in the diseased serum plaque produce an antigen–antibody complex. In order to render the antigen–antibody complex visible, a secondary antibody that recognizes the antibody in the serum and that is tagged with the enzyme is added. Then the substrate of the enzyme is added to the medium to produce color and the concentration is determined. This method utilized to identify antigens is used more commonly in endocrinology.

Sandwich ELISA (antibody screening)

The technique was developed in 1977 Kato and his co-workers [20]. In this ELISA method, the wells are coated with a capture antibody and blocked. The sample is added to the microplate 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. In order to reveal the enzyme activity, enzyme substrate is added to the medium and coloration is ensured. Coloration shows a positive result, while lack of coloration indicates 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.

Competitive ELISA (antigen/antibody screening)

The technique was developed in 1976 Yorde and his coworkers [42]. In this method, the surface of the wells is coated with the antigen-specific antibody or antibody-specific antigen. The sample to be measured and the enzyme-tagged antigen or antibody

Following the invention of ELISA, a number of researchers used it: Carlson and colleagues in 1972 [10], Holmgren and Svennerholm in diagnostic microbiology in 1973 [15], Ljungstrom and colleagues to identify the presence of trichinosis in parasitology in 1974 [26], and Voller et al. to diagnose malaria in 1975 [40]. Bishai and Galli, Leinikki et al. and Ukkonen et al. made use of the ELISA method to identify infections caused by influenza, parainfluenza and mumps viruses in 1978, 1979, and 1981, respectively [6,22,38]. In 1980, Siegle et al. modified the ELISA test and incorporated microtitration plates to identify the concentrations of various hormones, peptides, and proteins [35]. The method which has found different fields of application and grown beyond infancy over time has become a routinely used method in research and diagnosis laboratories around the world.

How does the ELISA method work?

The antigen utilized in the ELISA method is bound to a solid phase. Tubes and microplates made of rigid polystyrene, polyvinyl and polypropylene are used as the solid phase. The microplates used must be able to appropriately adsorb the antigen and the antibody, but not adsorb the components in the other phases [13,41]. The enzymes that can be employed in ELISA include beta galactosidase, glucose oxidase, peroxidase, and alkaline phosphatase. Alkaline phosphatase can be stored at 4 °C with its conjugate sodium azide. Alkaline phosphatase and P-nitro-phenyl phosphate are used as substrates, are available in safe tablet forms, and produce a yellow color in positive reactions. For the peroxidase conjugate, 5 amino salicylic acid and orthophenylenediamine are used as the substrates and the production of a brown color is considered a positive reaction. If beta galactosidase is used, the sample must be read in a fluorometer. The catabolic effects of enzymes determine both the acceleration and the specificity of the immunological reaction during the enzyme-substrate reaction [12]. The enzyme-substrate reaction is usually completed within 30–60 min. The reaction can be stopped using sodium hydroxide (NaOH), hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) [16]. The results are read on a spectrophotometer and at 400–600 nm depending on the characteristics of the conjugate used.

Types of ELISA

Enzymatic immunoassay methods are considered under two general headings as homogeneous enzymatic immunoassay methods and heterogeneous enzymatic immunoassay methods [27] (Fig. 1). In the homogeneous enzymatic immunoassay methods, enzymes become inactivated when they are bound to the antibody, and thus, there is no stage (washing) where the antigen is separated from the medium. Homogeneous enzymatic immunoassay method is usually employed to measure substances in small quantities, like therapeutic drugs [27]. Homogeneous method is expensive and has low sensitivity. The only advantage it possesses is its ease of use.

As heterogeneous enzymatic immunoassay methods are more commonly used [27], the methods and types of this method are detailed in the following paragraphs. In this method, in order to the prevent interference of any molecule in the medium with it after the binding of the antigen and the antibody, the antigen–antibody complex is bound to the walls of the experiment tubes and anything other than the complex is removed from the medium through washing procedures. In other words, in heterogeneous enzymatic immunoassay methods, it is essential to have a washing stage to separate the bound antigen from the free antigen after the antigen–antibody interaction. Since the heterogeneous method is more sensitive than the homogeneous one, it is more commonly used. ELISA is a heterogeneous immunoassay technique used to detect specific antibodies and soluble antigens, and since the structure and the characteristics of the substances to be measured are not always the same, a variety of ELISA types have been developed to increase the specificity of measurement [27]. Schematic description of the homogeneous enzymatic immunoassay and heterogeneous enzymatic immunoassay methods is presented in Fig. 2.

Direct ELISA (antigen screening)

The technique was simultaneously developed in 1971 by Engvall and Perlmann [13] and by Van Weemen and Schuurs [39], the technique pioneered other ELISA types. Direct ELISA method is suitable 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 removes the unbound antigens or antibodies from the medium. Then the appropriate substrate is added to the medium to produce a signal through coloration. The signal is measured to determine the amount of the antigen or antibody [12,16].

Indirect ELISA

The technique was developed in 1978 Lindström and Wager [25], who were inspired by the direct ELISA method. The researchers reported measuring porcine IgG using this method. The reason why this method is called the indirect method is that what determines and separates the antigen to be measured is not the primary antibody, but another antibody that is placed in the medium. In this method, the diseased serum is added to the antigen-coated wells and the plates are incubated. During this incubation, the antibodies formed against the antigens in the diseased serum plaque produce an antigen–antibody complex. In order to render the antigen–antibody complex visible, a secondary antibody that recognizes the antibody in the serum and that is tagged with the enzyme is added. Then the substrate of the enzyme is added to the medium to produce color and the concentration is determined. This method utilized to identify antigens is used more commonly in endocrinology.

Sandwich ELISA (antibody screening)

The technique was developed in 1977 Kato and his co-workers [20]. In this ELISA method, the wells are coated with a capture antibody and blocked. The sample is added to the microplate 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. In order to reveal the enzyme activity, enzyme substrate is added to the medium and coloration is ensured. Coloration shows a positive result, while lack of coloration indicates 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.

Competitive ELISA (antigen/antibody screening)

The technique was developed in 1976 Yorde and his coworkers [42]. In this method, the surface of the wells is coated with the antigen-specific antibody or antibody-specific antigen. The sample to be measured and the enzyme-tagged antigen or antibody

Following the invention of ELISA, a number of researchers used it: Carlson and colleagues in 1972 [10], Holmgren and Svennerholm in diagnostic microbiology in 1973 [15], Ljungstrom and colleagues to identify the presence of trichinosis in parasitology in 1974 [26], and Voller et al. to diagnose malaria in 1975 [40]. Bishai and Galli, Leinikki et al. and Ukkonen et al. made use of the ELISA method to identify infections caused by influenza, parainfluenza and mumps viruses in 1978, 1979, and 1981, respectively [6,22,38]. In 1980, Siegle et al. modified the ELISA test and incorporated microtitration plates to identify the concentrations of various hormones, peptides, and proteins [35]. The method which has found different fields of application and grown beyond infancy over time has become a routinely used method in research and diagnosis laboratories around the world.

How does the ELISA method work?

The antigen utilized in the ELISA method is bound to a solid phase. Tubes and microplates made of rigid polystyrene, polyvinyl and polypropylene are used as the solid phase. The microplates used must be able to appropriately adsorb the antigen and the antibody, but not adsorb the components in the other phases [13,41]. The enzymes that can be employed in ELISA include beta galactosidase, glucose oxidase, peroxidase, and alkaline phosphatase. Alkaline phosphatase can be stored at 4 °C with its conjugate sodium azide. Alkaline phosphatase and P-nitro-phenyl phosphate are used as substrates, are available in safe tablet forms, and produce a yellow color in positive reactions. For the peroxidase conjugate, 5 amino salicylic acid and orthophenylenediamine are used as the substrates and the production of a brown color is considered a positive reaction. If beta galactosidase is used, the sample must be read in a fluorometer. The catabolic effects of enzymes determine both the acceleration and the specificity of the immunological reaction during the enzyme-substrate reaction [12]. The enzyme-substrate reaction is usually completed within 30–60 min. The reaction can be stopped using sodium hydroxide (NaOH), hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) [16]. The results are read on a spectrophotometer and at 400–600 nm depending on the characteristics of the conjugate used.

Types of ELISA

Enzymatic immunoassay methods are considered under two general headings as homogeneous enzymatic immunoassay methods and heterogeneous enzymatic immunoassay methods [27] (Fig. 1). In the homogeneous enzymatic immunoassay methods, enzymes become inactivated when they are bound to the antibody, and thus, there is no stage (washing) where the antigen is separated from the medium. Homogeneous enzymatic immunoassay method is usually employed to measure substances in small quantities, like therapeutic drugs [27]. Homogeneous method is expensive and has low sensitivity. The only advantage it possesses is its ease of use.

As heterogeneous enzymatic immunoassay methods are more commonly used [27], the methods and types of this method are detailed in the following paragraphs. In this method, in order to the prevent interference of any molecule in the medium with it after the binding of the antigen and the antibody, the antigen–antibody complex is bound to the walls of the experiment tubes and anything other than the complex is removed from the medium through washing procedures. In other words, in heterogeneous enzymatic immunoassay methods, it is essential to have a washing stage to separate the bound antigen from the free antigen after the antigen–antibody interaction. Since the heterogeneous method is more sensitive than the homogeneous one, it is more commonly used. ELISA is a heterogeneous immunoassay technique used to detect specific antibodies and soluble antigens, and since the structure and the characteristics of the substances to be measured are not always the same, a variety of ELISA types have been developed to increase the specificity of measurement [27]. Schematic description of the homogeneous enzymatic immunoassay and heterogeneous enzymatic immunoassay methods is presented in Fig. 2.

Direct ELISA (antigen screening)

The technique was simultaneously developed in 1971 by Engvall and Perlmann [13] and by Van Weemen and Schuurs [39], the technique pioneered other ELISA types. Direct ELISA method is suitable 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 removes the unbound antigens or antibodies from the medium. Then the appropriate substrate is added to the medium to produce a signal through coloration. The signal is measured to determine the amount of the antigen or antibody [12,16].

Indirect ELISA

The technique was developed in 1978 Lindström and Wager [25], who were inspired by the direct ELISA method. The researchers reported measuring porcine IgG using this method. The reason why this method is called the indirect method is that what determines and separates the antigen to be measured is not the primary antibody, but another antibody that is placed in the medium. In this method, the diseased serum is added to the antigen-coated wells and the plates are incubated. During this incubation, the antibodies formed against the antigens in the diseased serum plaque produce an antigen–antibody complex. In order to render the antigen–antibody complex visible, a secondary antibody that recognizes the antibody in the serum and that is tagged with the enzyme is added. Then the substrate of the enzyme is added to the medium to produce color and the concentration is determined. This method utilized to identify antigens is used more commonly in endocrinology.

Sandwich ELISA (antibody screening)

The technique was developed in 1977 Kato and his co-workers [20]. In this ELISA method, the wells are coated with a capture antibody and blocked. The sample is added to the microplate 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. In order to reveal the enzyme activity, enzyme substrate is added to the medium and coloration is ensured. Coloration shows a positive result, while lack of coloration indicates 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.

Competitive ELISA (antigen/antibody screening)

The technique was developed in 1976 Yorde and his coworkers [42]. In this method, the surface of the wells is coated with the antigen-specific antibody or antibody-specific antigen. The sample to be measured and the enzyme-tagged antigen or antibody

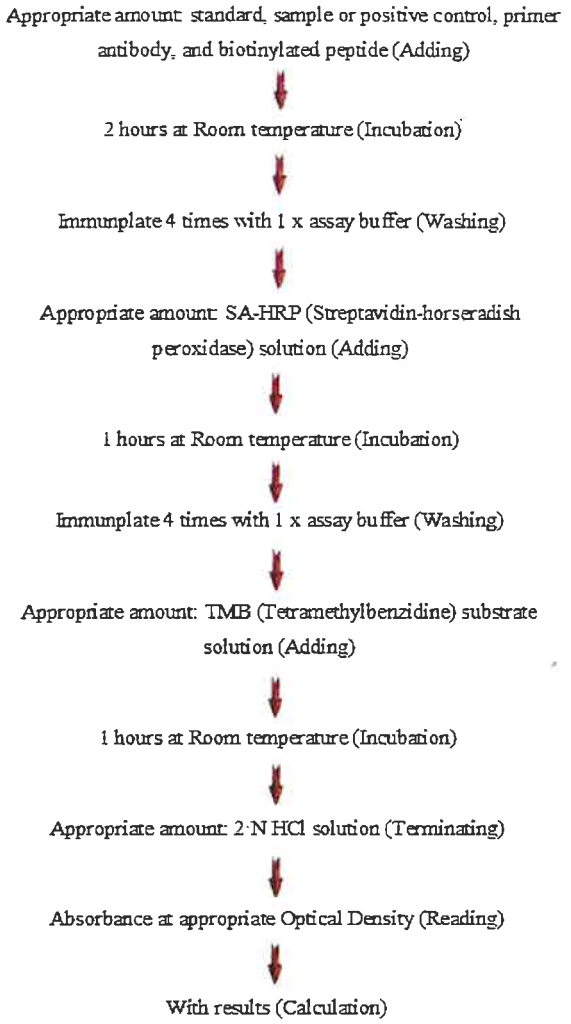

Fig. 3. General steps for performing ELISA (For interpretation of the references to color in this text, the reader is referred to the web version of the article.). Adapted and modified from resource number [19].

acids. This last form is called desacyl ghrelin [32]. Ghrelins with fatty acids, on the other hand, are called acylated ghrelins. In order to enhance the stability of acylated ghrelins, the sample should be added HCl at a volume ratio of 1/10 per milliliter from a 1 mL normal HCl [2,17]. Besides, the researchers must indicate which form of the ghrelin they studied in their reports. Variety of form is seen not only in ghrelins [17], but in a number of peptides, including apelines, among others [4]. Therefore, it is important for peptide researchers to state which form they examined for the purposes of standardization.

Table 3
The list of minimum materials needed for an ELISA analysis.

Materials required but not supplied	KIT materials
Automatic pipettes (10,100,1000 μL)	Acetate plate sealer, 2 N HCl
Biologic material collection tubes	Assay diagram (sheet), protocol book
Distilled water, dry paper, vortex,	Biotinylated peptide
ELISA Microplate Reader, Washer, Writer	Concentrate assay buffer
Flask (50–500 mL), eppendorf tubes, Benmari	Standard peptide, positive control
Multi-channel pipette (50–100 μL)	Streptavidin-horseradish peroxidase
Plate Shaker, Protease inhibitor	Substrate solution (TMB)
Solution reservoir, Buffers, incubator	96 well immunoplate

Another important issue encountered in peptide analyses is that some peptides may remain below the detection limit of the kit or stay low because of certain diseases. In this case, standard addition–subtraction methods should be used [36]. That is, a known quantity of peptide must be added to all biological samples. In order to identify the actual concentration of peptides, the added concentration should be subtracted from the concentration found at the end of the experiment. Thus, it becomes possible to measure peptides found in very low concentrations in biological samples.

As peptides are found in a number of biological fluids, ELISA method has been used recently to identify peptide amounts in the supernatants of biological tissues, as well as the biological fluids. If the peptides are to be quantified in the supernatants of biological tissues, the wet weight of the tissue should be determined immediately after it is removed from the organism (200–300 mg of tissue will be adequate) and the tissue should be boiled in boiling water for 5 min [21]. Thus, proteases are inactivated and peptides are protected against proteases. Besides, when the tissues are to be homogenized, 20–30 μl of aprotinin out of 500 KIU aprotinin should be added [17]. If possible, the supernatant obtained after the centrifuge must be studied simultaneously with other biological samples. If the supernatant will not be studied shortly, then it must be stored at −20 °C or −80 °C until the time of analysis. Our laboratory experience shows that samples stored as such will stay intact for 3 years at −20 °C and for 7 years at −80 °C.

Other possible pre-analytical errors in peptide analyses

The patient's position while the blood is collected is important and can be one of the pre-analytical errors [1,29]. For instance, the blood volume of a healthy adult in standing position is 600–700 mL (10%) lower than the volume in lying position. In an erectly sitting position, the liquid without protein is transferred to the tissues through capillaries and this causes a significant difference in the

Fig. 4. Washer (a), reader (b) and printer (c) used in ELISA procedures.

plasma volume. As a result, peptides and proteins, hormones with protein structure, drugs carried by binding to proteins, calcium, enzymes and bilirubin concentrations will be elevated. Exercise also causes pre-analytical errors. Exercise-associated increases have been reported in aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatinine kinase (CK), urea, creatinine, transferring [24,28,33,37] and irisin levels [9].

The patient's condition [fasting (fasting for about 10–12 h is appropriate for taking samples), satiety, drug use, pre-examination, etc.], incorrect sample collection (from the arm of the infusion), collecting the blood sample into the wrong tube, collecting more or less than the appropriate amount, effects of food (drinks containing caffeine like tea, coffee, coke, fat, protein and carbohydrate rates), alcohol consumption (changes many analytes depending on short-term or long-term effects), effects of fever, age and sex (cause changes in reference values in biochemical and hematological tests), pregnancy, diurnal rhythm (the release and metabolism of some analytes change over the day), seasonal changes (for instance, higher vitamin D levels in summer and higher triglyceride and total cholesterol levels during summer, in comparison to winter; therefore, this should be considered, when a correlation between vitamin D and peptides is explored), altitude (when blood is collected from individuals living in higher altitudes, hemoglobin, hematocrite and CRP will be higher; this should be considered when a correlation is examined between these molecules and peptides), different body mass indices (relationships have been reported between many peptides and BMI), use of tobacco and tobacco products, inappropriate transfer (samples which kept waiting for too long), freezing and thawing, and the measurement interval of the kits being sensitive enough are among the most common causes of pre-analytical errors [1,24,28,29,37]. All these conditions should be taken into consideration in sound scientific research.

Possible analytical errors

Using kits from different firms in the same study

Use of kits from different firms in the same study is among the most common analytical errors. There are several ELISA manufacturers. The ELISAs of these different companies can measure the same sample in different amounts. For instance, ELISA kit of the Phoenix firm measures ghrelin amounts 10 times lower than that of the Linco firm, although linearity was reported [14]. Accordingly, for the purposes of standardization, it is important for a laboratory to use the kits of the same firm. It is all the better if the kits of the same firm and with the same lot number can be used in the same study.

Research laboratories depend on grants to continue operating. The institution which offers the grant requires that the products of the firm which gives the best offer be bought or asks for an explanation as to why certain products are not preferred (this is the common procedure). If the ELISA kit you have already been using in your laboratory gives the best offer in the bid, then there is no problem. Otherwise, you have to conduct the assay validity experiments for the new ELISA kit. The assay validity test procedures for an ELISA kit were used in our laboratory and the relevant details can be found in the article cited here. Although firms manufacturing ELISA kits provide the assay validity parameters on their kit catalogs, it is sometimes difficult to obtain these data. Therefore, laboratories must test the assay validity parameters when they use different ELISA kits. Furthermore, the kits are usually designed to measure peptides in blood. Consequently, if the kit is going to be used for another purpose, for instance to detect peptides in tissue supernatants, ELISA assay validity parameters must be certainly tested to ensure the reliability of results, as briefly explained as follows [3].

Precision assay

The intra-assay (within-day) and inter-assay (between-days) variation should be determined for biological samples. The coefficient of variation (CV) can be calculated as: CV = Standard Deviation (SD)/Mean. The CV values used in testing are generally less than 15% for clinical use.

Linearity assay

Biological samples should be diluted with distilled water and assayed. After biological sample dilution, concentration of biological sample constituents must indicate perfect linearity on serial dilution.

Recovery assay

Biological samples should be enriched with increasing amounts of standard samples. Then, the percentage recovery can be calculated as follows: observed value-baseline value/amount added 100×. The obtained results would verify whether or not the used kit would detect peptides/proteins quantitatively in other biological fluid, beside serum [3].

Inexperienced staff error

A laboratory director cannot do all the work on their own. However, they must not reveal the title of the research in question to the research assistant and lab technician to avoid biases; that is, the staff should be blinded to the study [17]. In addition, if a new staff member is hired, the same sample should be studied by several people. Thus, it can be checked whether the same sample is measured in the same way.

Other possible analytical errors in peptide analyses

Among the most common analytical errors are the degradation of reagents, equipment errors, and pipettes that do not measure correctly, and not enough biological samples. Serum/plasma is a yellow liquid that is around 55% total volume of blood. 100 μl of serum/plasma is necessary for ELISA. So that some one can estimate how much blood they need for ELISA test. Another error is the failure to follow the washing procedures and to keep to the time reactions must be ended. Errors are also encountered when the antigen and the antibody used to coat the solid phase is not at the right concentration or when planting the standards, using only one plate to analyze a number of samples at the same time (7–8 standards prepared for each plate should be added at all times, because this is the most important step showing whether the experiment was correctly conducted. Theoretically expected concentrations of the standards should more or less coincide with the concentrations found in the experiment). Failure to arrange for the optimum incubation duration or temperature may cause errors. Other errors may include the following: failure to choose the most appropriate substrate, using reagents with different lot numbers during the study, accidental manual contact with the bottom of microplates, not maintaining the room temperature (below or over 20–23 °C), not determining the appropriate dilution factors, presence of unacceptably high levels of interfering proteins in the samples, the tubes having air bubbles in them and neglecting to change the tubes at every step, differences in pipette techniques (to ensure standardization, the same analysis must be carried by one person; more than one person should not be involved in the analysis), making an error in incubation time (longer or shorter than the appropriate duration) and differences in washing techniques.