CHAPTER

15

Techniques for Protein Analysis

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15.1 PROTEIN IDENTIFICATION

Proteins are one of the most important macromolecules in nature. Many functions inside the cell are provided with the aid of different types of protein molecules. Despite these different functions, protein structures are similar. Because of their significant roles, it is important to identify the details about their structures, synthesis, functions, and regulations through the lens of molecular biology. The first step in identification of the structure of a protein is to determine its amino acid composition (Murray et al., 2012; Rao et al., 2014; Berg et al., 2002).

15.1.1 Sequencing

The characteristic of each protein depends on its unique amino acid sequence (Garrett and Grisham, 2013). The first protein sequencing was achieved by Frederic Sanger in 1953 bovine insulin for which he was awarded the Nobel Prize in 1958. Protein sequencing is used to identify the amino acid sequence and its conformation. The identification of the structure and function of proteins is important to understand cellular processes.

There are several applications of protein sequencing;

1. Identification of the protein family to which a particular protein belongs and finding the evolutionary history of that protein.
2. Prediction of the cellular localization of the protein based on its target sequence.
3. Prediction of the sequence of the gene encoding the particular protein.

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4. Discovering the structure and function of a protein through various computational methods and experimental methods.

There are two major direct methods for protein sequencing: Edman degradation (Harvey and Ferrier, 2011; Bauer et al., 1997) and mass spectrometry (MS) (Sahukar et al., 2016). A protein of interest is digested by a proteolytic enzyme and subsequently, the enzyme digest is injected into the first mass spectrometer, which separates the oligopeptides according to their mass-to-charge ratios (m/z).

15.1.1.1 Determining Amino Acid Composition With Hydrolysis

Knowledge of the amino acid sequence of proteins is crucial in order to facilitate the discovery of errors during the process of biological information and to distinguish some ambiguous results regarding the process of protein synthesis. To be able to define the number of amino acids in a protein structure, hydrolysis can be used as a general method to assist determination of the number of amino acids that form the protein structure.

The first step is to identify the amino acid composition (Aloisi et al., 2016; Darragh and Moughan, 2005). The peptide bonds, which link amino acids to each other, are broken by hydrolysis. Because peptide bonds are stable at neutral pH, they are hydrolyzed using strong acids and bases; enzymatic catalysis is not effective for a complete hydrolysis. However, it is not possible to hydrolyze proteins without having a partial loss from some amino acid residues. In a typical method for analyzing the amino acid composition of proteins and polypeptides, a protein sample is hydrolyzed in a glass tube at 110°C using 6N HCl. Under these conditions, all tryptophan and most of the cysteine amino acids are disrupted. If there are metals in the structure, methionine and tyrosine are partially disrupted as well. The glutamine and asparagine are delaminated to form glutamate and aspartate, respectively. The serine and threonine contain an OH group in their structure; they are broken down more slowly than the other amino acids. Finally, only 50% of the bonds between neutral residues (Val−Val, Ile−Ile, Val−Ile, Ile−Val) are hydrolyzed after 20 hours. Typically, folded samples are hydrolyzed in 24, 48, 72, and 96-hour time periods. The serine and threonine data are marked on the semilogarithmic paper and are extrapolated backwards through time 0. The valine and isoleucine are calculated from 96-hour data. Dicarboxylic acids and their amides are identified, and they are reported together as either "Glx" or "Asx." Before hydrolysis, cysteine and cystine are converted into a stronger derivative (e.g., cysteic acid). The hydrolysis, which degrades serine, threonine, arginine, and cysteine, is catalyzed with alkalies; it is also used for tryptophan analysis. After hydrolysis, the amino acid composition is determined by either automatic ion-exchange chromatography or by high-performance liquid chromatography (HPLC) (Rombouts et al., 2009; Murray et al., 2012; Garrett and Grisham, 2013).

15.1.1.2 Quantitative Analysis

There are many methods used to determine the amount of protein in a sample; the most important one is the Kjeldahl (Lynch and Barbano, 1999), Warburg (UV absorption method), Coomassie-blue (Bradford method), biuret, and Folin−Lowry methods (Sapan et al., 1999). However, MS can also be used for a precise quantitative analysis of proteins, as it is an important and promising method for protein characterization (refer to Section 15.1.5 for more detail).

15.1.2 Edman Degradation

15.1.2.1 The Edman Degradation Reaction

This reaction is a chemical method for sequencing a whole polypeptide protein from their N-terminus, was developed by Pehr Edman in the early 1950s. This method labels and removes an amino acid residue from the whole sequence without disturbing peptide bonds (Hunkapiller, 1988; Liu et al., 2016).

Edman degradation is a series of chemical reactions that remove amino acids on the N-termini of the proteins. One of the most important reagents in sequence analysis is phenyl isothiocyanate, which was developed by Edman. Moreover, this reagent reacts with N-termini residue of both peptides and proteins. After the reaction with phenyl isothiocyanate, the protein sample is incubated with an anhydrous acid (e.g., trifluoroacetic acid) that breaks the peptide bond between the first and second amino acids of the protein. Thus, the amino acids at the N-termini residue become free as a thiazolinone derivative. This thiazolinone is then extracted using an organic solvent, and dried. Finally, it is converted to a more stable phenylthiohydantoin (PTH) derivative. The PTH derivatives are separated using HPLC and are determined by their order and elution location. In 1967, an automatic amino acid separation and identification method began to be used. Although the automatic Edman method is faster than Sanger method, it is slower and more difficult than other DNA sequencing methods. Because proteins are composed of polypeptides that are bound by noncovalent interactions and disulfide bridges, the first step is to take off these polypeptide structures apart and to make them an individual polypeptide chain. Some denaturation chemicals (urea and guanidine hydrochloride) are used for that purpose. Some oxidative and reductive compounds disrupt the disulfide bonds; the polypeptides are then separated by chromatography (Speicher et al., 2001).

Edman Reactive and Sequencing Methods by Edman Degradation: The automatic sequencing method uses phenyl isothiocyanate (Edman reagent). It involves a series of reactions that end with degradation of the N-termini residue in the form of its PTH derivative (Edman reaction, Fig. 15.1). The instrument consists of a cup-shaped reaction chamber that spins around its axis; the reaction occurs in a thin solution layer on the wall of this cup-shaped chamber making solvent extraction and removal easier. Fully automatic instruments can analyze 30−40 residues, in some cases up to 60 or 80 residues, continuously. This instrument is programmed to the sequential Edman fragmentations on a N-treminal increment of a polypeptide. After the amino acid at N-termini residue is removed, the Edman derivative of the residue is formed. PTH derivatives are separated using HPLC and are defined by their order and elution locations (Nelson and Cox, 2005; Berg et al., 2002; Shively, 2000).

15.1.2.2 Limitations of the Edman Degradation

Edman technique particularly affects proteins at their N-terminus. Accordingly, if this protein is chemically modified, or has stored these proteins in the case of large peptides, this technique does not work as requested to obtain the reliable output (Speicher et al., 2001).

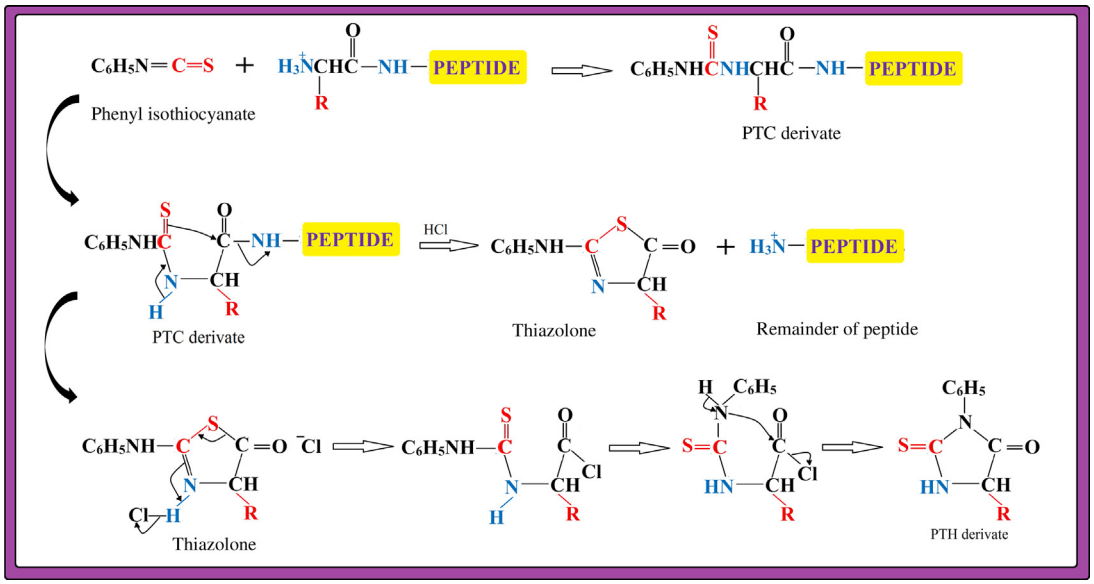

FIGURE 15.1 Schematic illustration of Edman degradation.

Although the main advantage of the Edman technique is to be more easily accessible in many laboratories and so to facilitate the determination of protein sequence and also to improve identification rate in recently developed of gas chromatography and thin layer chromatography method, it is still difficult to realize reliable determination of all the amino acids in single step. Therefore, for discernible results, separate procedure could be required in order to determine the positions of disulfide bridges, and peptide concentrations more than 1 pM (Miyashita et al., 2001).

15.1.3 Gel Electrophoresis

15.1.3.1 Polyacrylamide Gel Electrophoresis

Polyacrylamide gels are based on the free radical polymerization principle of acrylamide and cross-linking *N,N′*-methylene-bis-acrylamide. This material is physically very stable and strong. It is especially used for the electrophoretic separation of small or medium sized (up to about 1×10^6 Da) proteins. Its interaction with the migrating molecules is at the lowest level. The separating power of this gel depends on the dimension of the molecule to be separated as well as the concentration of acrylamide and bis-acrylamide. Low concentration acrylamide and bis-acrylamide polymerization is preferred to prepare gels with larger pores for high molecular weight samples. The difference of polyacrylamide gel electrophoresis (PAGE) from gel permeation chromatography is that small molecules move faster in polyacrylamide gels in comparison with larger molecules (Sadeghi et al., 2006). A standard gel used for the separation of proteins generally contains about 7.5% polyacrylamide.

Pore size in polyacrylamide gels is determined with the values of $\%T$ [total polyacrylamide percentage (w/v)] and $\%C_{bis}$ [the ratio of bis to monomer (w/w)] using the following formulae:

$$\%\,T = \frac{\text{Acrylamide (g)} + \text{Bis (g)}}{\text{Volume (mL)}} \times 100$$

$$\%\,C_{bis} = \frac{\text{Bis (g)}}{\text{Acrylamide (g)} + \text{Bis (g)}} \times 100$$

PAGE is given different names according to the type of gel (tube and slab gel electrophoresis or continuous and discontinuous gel electrophoresis), the position of the gel (vertical and horizontal gel electrophoresis), the chemical composition of the gel (native and sodiumdodecylsulfate (SDS), gel electrophoresis), and the pore distribution of the gel (homogeneous and gradient gel electrophoresis) (Wenk and Fernandis, 2007).

In tube PAGE, glass tubes (10 cm × 6 mm) are used and the gel material is filled into these tubes and polymerization is attained. Gel tube is placed vertically between two different buffer stocks. Cathode is generally located in the upper stock, whereas anode is located in the lower stock. Since most of the biologic materials are negatively charged, they move toward the anode. Hence, the sample to be analyzed is applied on the upper section of the gel with a tracking dye and electrical current is passed through the system. Since the tracking dye moves faster than the compounds in the sample, the current is stopped when it reaches the end of the gel, the gel is taken out of the tube and dyed.

Slab gels are used more in comparison with the columns since they enable the analysis of many samples in the same support environment (at the same conditions). Polyacrylamide gel is prepared between two glass plates (Fig. 15.2). A plastic comb placed on the top of the gel during polymerization enables the formation of small wells in the gel. The comb is removed after polymerization, the wells are washed with the buffer in order to remove the salts and unpolymerized acrylamide. The gel cassette is placed between two buffers; samples are placed inside the wells and current is passed. The gel is painted accordingly at the end of the electrophoresis for imaging.

SDS anionic detergent is used in SDS-PAGE in order to denaturate the proteins surrounding the main skeleton of the polypeptides as well as to give a negative charge to the molecules (Černý et al., 2013). The movement rate of the polypeptides in this method depends on molecular weight as well as internal electrical loads. Thus, it is a method that is frequently used for determining the molecular weights of the applied samples. A protein sample with unknown molecular weight is applied side by side with a protein of known molecular weight on the same gel and then is separated electrophoretically in different lines. The comparison of the bands on the gel after painting gives an idea about the molecular weight of the protein. In addition, it is also possible to determine the molecular weight by evaluating the results of this separation mathematically (Righetti et al., 2001).

Two different buffer systems, *continuous* and *discontinuous*, can be used in electrophoresis. There is only one separating gel in continuous system; the same buffer is used in the tanks and the gel. Whereas in the discontinuous system two-sided gel preparation with different buffers is used. The "stacking" gel with its large porous structure is located at the upper side of the gel providing the order of the applied sample in terms of its size. Whereas the

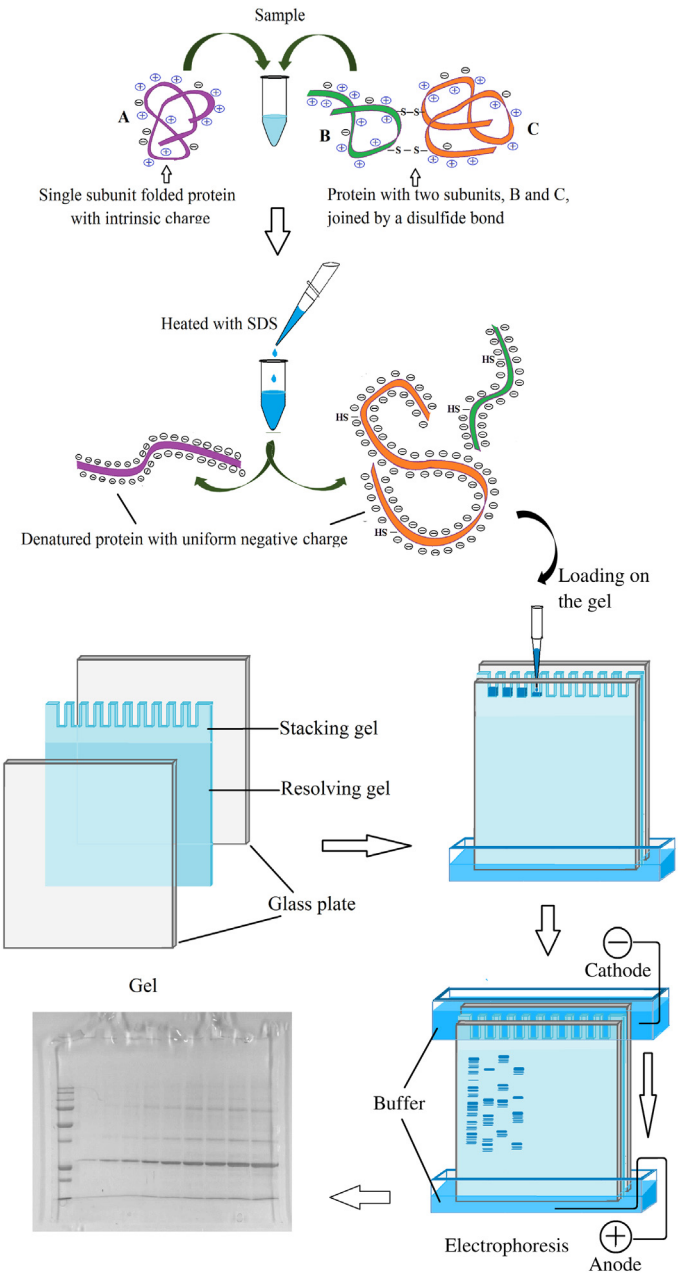

FIGURE 15.2 Schematic diagram of polyacrylamide gel electrophoresis.

"separating" gel with small pores is located at the lower side of the gel and so provides a more sensitive separation of the sample. The buffers used in the preparation of the gels are different from each other. In addition, the tank buffers are prepared differently than the gel buffers so that a better separation can be attained. *Gradient gels* are also used to provide this feature. Acrylamide concentration is gradually increased in gradient gels as we go down from the upper section of the gel. Thus, it is ensured that the pores in the gel decrease in size gradually as we move down. Polypeptides with similar molecular weights are separated more efficiently in this type of gel and form sharper bands (Bolt and Mahoney, 1997).

15.1.3.2 Isoelectric Focusing

Isoelectric focusing (IEF) is an efficient method developed for the electrophoretic analysis of proteins (Mathy and Sluse, 2008). Since the net charge of the proteins depends on pH, electrophoretic is attained by pH changes in this method. The net charge of the proteins is the sum of the negative and positive charges at the amino acid chain regions. This value changes according to pH, and the isoelectric point (pI) is where the net charge is 0. Proteins are charged positively under their isoelectric pH's (pI) and migrate toward the negative charged electrode (cathode) in a medium with fixed pH. Whereas the protein loses a proton at pH values above the isoelectric points therefore becoming negatively charged and migrating toward the positively charged electrode (anode). If the pH of the electrophoresis environment is equal to its pI, the electrophoretic migration stops at its own pI since the net charge of the protein will be 0 (Fig. 15.3). It is theoretically possible to

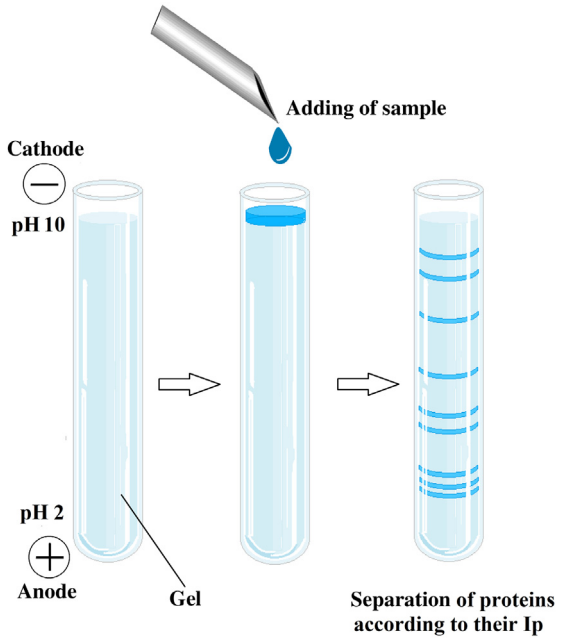

FIGURE 15.3 Representation of isoelectric focusing method.

separate the proteins and to determine the pI value of the protein by monitoring the electrophoretic movements in different experiment series with different environment pH values. For this, the three-dimensional structure of the protein should first be disrupted with chaotropic agents such as urea and the charges should be exposed in the environment (Černý et al., 2013). An acid (generally phosphoric acid) is placed in the anode and a base, such as triethanolamine, is placed in the cathode. The gel medium between the electrodes is adjusted before or during electrophoresis such that the pH will be between 2 and 10. The pI value of the protein is reached by determining the pH value at the point where the protein is focused. The IEF method that enables the separation of polypeptides using the property, that they have different pIs, can be used by itself for analytical or preparative purposes, while it can also form the first dimension of the two-dimensional gel electrophoresis (2DGel) (Curreem et al., 2012). Thus, it can be carried out in both horizontal and vertical (in a capillary tube or column) systems.

15.1.3.3 Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2DGel) is a successful method used for the detection and analysis of proteins. It has been designed as a combination of the 2DGel, IEF and SDS-PAGE methods, and is used in the analysis of complex protein mixtures. In the first step, protein is separated into its charges with IEF, whereas in the second step, the protein is separated according to its mass. The separated protein on the gel with IEF is negatively charged by treatment with SDS, and the electrophoresis is performed by inserting the gel horizontally into the SDS-PAGE gel. (Fig. 15.4). Thus, the proteins that are focused on the pI are separated according to their molecular weights. Generally 20×20 cm large gels are used in SDS-PAGE setup and more than 10,000 proteins can be separated. If the protein amount is around 10 ng, Coomassie dye is used and if the protein amount is around 0.5 ng, silver or fluorescent total-protein satins can be used for detection. Using the system

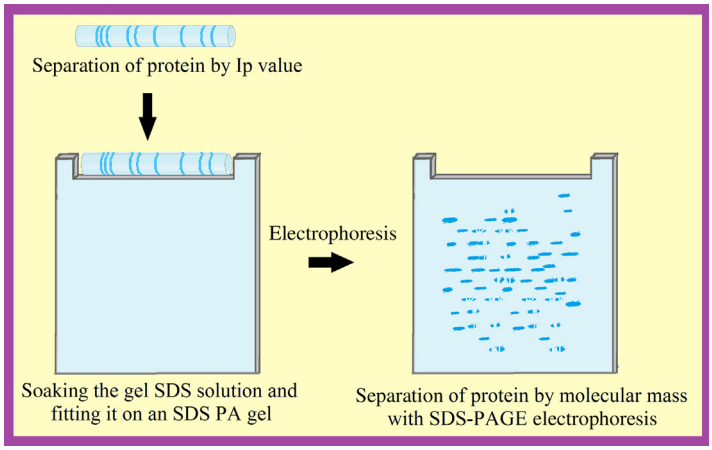

FIGURE 15.4 Steps of two-dimensional gel electrophoresis (2DGel).

known as "ISO-DALT," both IEF and SDS-PAGE can be carried out simultaneously (Chen et al., 2015; Brunelle and Green, 2014; Hanash et al., 1991; Magdeldin et al., 2014).

15.1.4 Isotope Labeling

Quantitative proteomics, which has been performed by 2DGel for over 25 years, is used for analyzing proteins in a cell, tissue, or an organism (Ong et al., 2002; Jungblut, 2014) to obtain quantitative information about all proteins in a sample. Recently, in many research areas, quantitative proteomics methods for relative and absolute quantitation of peptide and proteins are based on MS techniques. Most methods for relative quantification by MS-based techniques use labeling of peptides or proteins with an isotope, but label-free quantification methods are also available (Craft et al., 2013). Labeling of peptides or proteins with an isotope can be done by enzymatic, in vitro (chemical) or in vivo (metabolic) techniques. Labeling stage is an important difference between them; while metabolic labeling requires living cells, chemical labeling can be done on any proteome. Some of enzymatic, chemical (Isotope-Coded Affinity Tag, ICAT) and metabolic (Stable-Isotope Labeling with Amino acids in Cell culture, SILAC) labeling techniques are described later (Craft et al., 2013; Ong et al., 2002).

15.1.4.1 Enzymatic Labeling

Enzymatic labeling, which is achieved by using deuterium and ^{18}O, is one of the most important protein labeling techniques which incorporates functional groups into proteins at specific sites via enzymatic reactions. Enzymatic labeling can be performed during or after the proteolytic digestion with the protease. Isotope labels are introduced into peptides during protein digestion by trypsin or Glu-C-catalyzed incorporation of ^{18}O. Each digested peptide is enzymatically labeled at the C-terminus by ^{18}O which incorporates two heavy oxygen atoms from $H_2{}^{18}O$. Samples are digested with trypsin and either ^{18}O water or ^{16}O water, and then mixed together for MS analysis. Primary amino groups of digested peptides are labeled enzymatically by deuterated (^{2}H) acylating agents such as N-acetoxysuccinimide (NAS) (Bantscheff et al., 2007).

15.1.4.2 Isotope-Coded Affinity Tag

Isotope-coded affinity tag (ICAT) is the first chemical in vitro labeling method that uses a biotin tag to label proteins containing cysteine residues (Craft et al., 2013). Cysteine-containing proteins extracted from the control cells and diseased cells are labeled in vitro by commercial ICAT reagents containing C12 and C13, respectively. After purification of mixed protein samples digested with trypsin by ion-exchange chromatography, cysteine-containing peptides are isolated by affinity chromatography with avidin. Cysteine-containing peptides with C12 and C13 isotope labels are identified and quantified by LC−MS/MS. In chemical labeling, proteomes to be compared have to be purified and fractionated by exactly the same experimental conditions. Protein profiling of two different cell lines can be achieved by this method (Gygi et al., 1999; Yi et al., 2005).

15.1.4.3 Stable-Isotope Labeling in Cell Culture

Stable-isotope labeling in cell culture (SILAC), is a more commonly used metabolic labeling technique for mass spectroscopy-based quantitative proteomics. Nonradioactive isotopic labeling is used for detection of differences in protein abundance between the samples that will be analyzed. SILAC depends on metabolic incorporation of stable isotopes, that is "light" or "heavy" form of the amino acids, into the proteins during normal cell growth and division through the growth medium. Complete substitution of the natural amino acid with a stable isotopic nuclei (e.g., deuterium, 13C, 15N) is needed for successful experimental results. Differently labeled samples can be mixed and analyzed together. In an experiment, different organisms or cell lines are grown in identical culture media, generally containing lysine or arginine with 13C and 15N atoms, except one of them containing a "light" and the other a "heavy" form of a particular amino acid. All natural amino acids in proteins, synthesized after a labeled amino acid has been introduced to a cell, will be replaced by their isotope labeled analog. Isotopically labeled samples are quantified by calculating the ratios of integrated signal intensities for labeled peptides to the signal intensities of the corresponding unlabeled peptides in a chromatogram by mass spectrometer. SILAC data could be analyzed effectively by several software packages like MaxQuant, Mascot Distiller, Xpress, and ASAPratio (Ong and Mann, 2006, 2007; Boumediene et al., 2010; Amanchy et al., 2005; Harsha et al., 2008).

15.1.5 Mass Spectrometry

Mass spectrometry (MS) is a high-throughput analytical detection technique used to get information about the molecular weights and chemical structures of the peptides, proteins, carbohydrates, oligonucleotides, natural products, and drug metabolites (Biemann, 2014).

MS provides some advantages including small amount of sample requirement, label-free detection, fast analysis, capability of defining chemical structures with fragmentation, high sensitivity, and simultaneous detection of multiple analytes (Zhu and Fang, 2013; Glish and Vachet, 2003).

MS is widely used for various molecular biology analysis purposes either alone or combined with other structural proteomics techniques because of its advantages (Pi and Sael, 2013; Wasinger et al., 2013).

Examples of the analysis include molecular weight characterization, posttranslational modifications in proteins, identification of vibrational components in proteins, analysis of protein conformation and dynamics, noncovalent interactions, protein and peptide sequencing, DNA sequencing, protein folding, in vitro drug analysis, and drug discovery (Glish and Vachet, 2003; Benesch and Ruotolo, 2011; Steendam et al., 2013).

15.1.5.1 Principle and Instrumentation

The principle of the spectrometer depends on the separation of the molecules based on their mass-to-charge (m/z) ratio by ionization of the molecules with high energy electrons to break a molecule into fragments. Fragmentation pattern and m/z values provide information about the molecular weights and chemical structures of the peptides and proteins. Each peptide has a specific molecular weight. Molecules from solution or solid phase

should be transferred into gaseous phase, because MS measurements have to be done on ionized molecules in the gaseous phase (Fenn et al., 1989; Ong and Mann, 2005).

15.1.5.2 Components of the Instrument

A spectrometer basically consists of the following components (Dobson, 2003; Oudenhove and Devreese, 2013):

1. Device for sample input into the machine
2. Molecular ionization source
3. Mass analyzer
4. Detector
5. Vacuum system
6. Computer-based data obtaining and processing system

15.1.5.2.1 DEVICE FOR SAMPLE INPUT INTO THE MACHINE

Introduction of a sample into the spectrometer is a changeable process according to sample specifications which can be a solid, liquid, or vapor and the methods of ionization such as direct insertion with a probe or plate with matrix-assisted laser desorption/ionization (MALDI)-MS and direct infusion or injection into the ionization source with electrospray ionization (ESI)-MS (Kang, 2012).

15.1.5.2.2 MOLECULAR IONIZATION SOURCE

Detection by MS requires ionization and transfer of proteins into gaseous phase. Proteins and peptides are usually ionized via protonation in a spectrometer because of their NH_2 groups that accept a $H+$ ion. Different ionization sources are given in Fig. 15.5 (Kang, 2012; Hoffmann and Stroobant, 2007).

In proteomics analysis, the most commonly used two devices for ionization are MALDI and ESI. Both of them are soft ionization techniques, and ions undergo little fragmentation (Guerrera and Kleiner, 2005).

- *Matrix-assisted laser desorption/ionization (MALDI):*
  In MALDI, samples to be analyzed are mixed with crystal matrix material in a solvent and dried on a metal plate. After cocrystallization and absorption of samples on a matrix, the matrix is exposed to the pulse of a nitrogen laser beam, which vaporizes the samples for ionizing the molecules inside the source of the mass spectrometer. MALDI causes not only positive ionization for peptide and protein molecules, but also negative ionization for oligonucleotides and carbohydrate molecules. MALDI is more sensitive and more universal than other laser ionization techniques (Lagarrigue et al., 2012; Gessel et al., 2014).
- *Electrospray ionization (ESI):*
  In ESI, molecules are directly ionized from the solution by applying a high-voltage electric field under atmospheric pressure, to a liquid passing through a capillary tube. The high electrical field creates a charge on the surface of the droplets which become much smaller through vaporization of the solvent. ESI produces multiple charged ionized molecules, and is extremely useful for accurate mass measurement, especially for thermally unstable, high molecular mass substances such as proteins, oligonucleotides, and synthetic polymers (Ho et al., 2003).

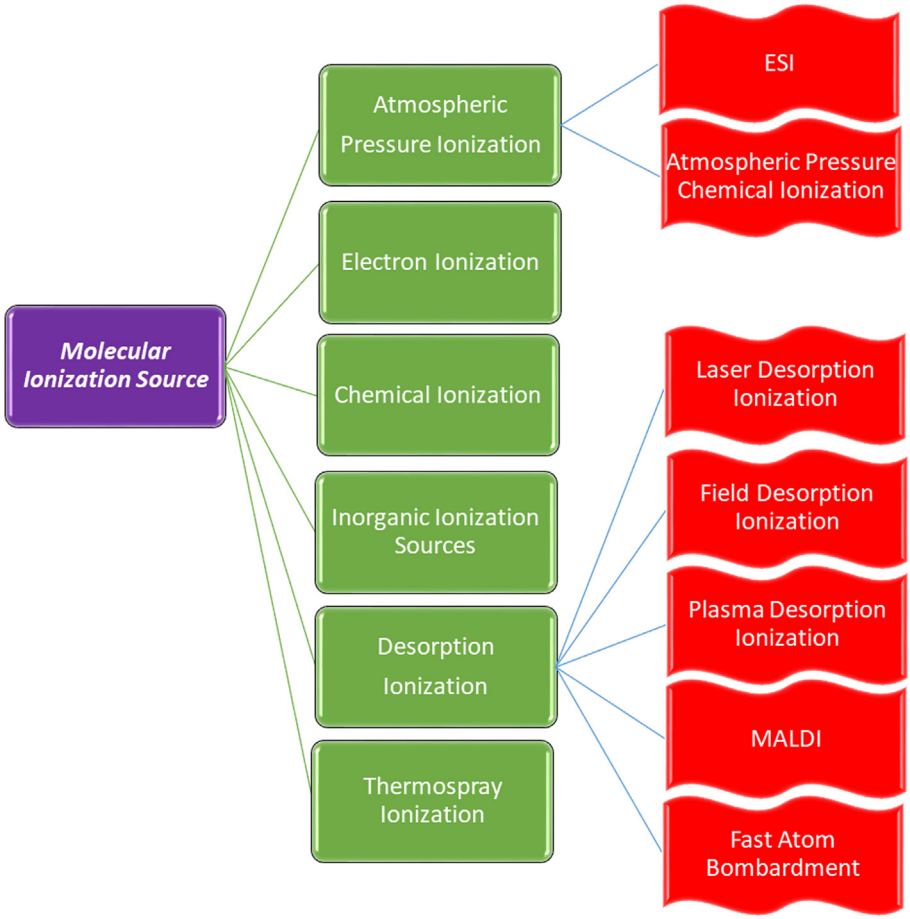

FIGURE 15.5 Types of Ionization.

15.1.5.2.3 MASS ANALYZER

After ionization, the ionized molecules of peptides or proteins enter the mass analyzer section of spectrometer. In the mass analyzer, molecules are separated based on their mass-to-charge ratio by electric and/or magnetic fields or by measuring the time an ion takes to reach a fixed distance from the point of ionization to the detector (Römpp and Spengler, 2013).

For the separation of ionized molecules, different kinds of mass analyzers are available such as quadrupoles, time-of-flight (TOF), magnetic sectors, Fourier transform, and quadrupole ion traps. In proteomics, quadrupole and the TOF analyzers are mostly used. MS with the ESI device usually carries a quadrupole analyzer (El-Aneed et al., 2009).

15.1.5.2.4 DETECTOR

The detector is the final component of a mass spectrometer and is used for monitoring and recording the presence of separated ions coming from the mass analyzer. Depending on the analytical applications and design of the instrument, different detectors can be used such as electron multiplier, Faraday cup, negative-ion detection, postacceleration detector, channel electron multiplier array, photomultiplier conversion dynode, the daly detector, and array detector (Kang, 2012). After detection of ions, the signals are recorded on a graph by plotting the amount of signal versus m/z ratio. MS graphs not only show the presence and abundance of different molecular size peptides and proteins, but also the energy level of the molecules of a particular kind.

15.1.5.2.5 VACUUM SYSTEM AND COMPUTER-BASED DATA OBTAINING AND PROCESSING SYSTEM

MS vacuum pumps are used for free movement of ions within the spectrometer. Computer-Based Data Obtaining and Processing System. Computer-based system is needed for data obtaining and processing.

15.1.5.3 Liquid Chromatography–Mass Spectrometry

Liquid chromatography–Mass spectrometry (LC–MS) technique combines separation and analysis of samples with LC and MS, respectively. LC–MS is a bioanalytical method for quantitative analysis of proteins which has several application areas such as biopharmaceutical drug development, drug metabolism and toxicology studies, quantification of drugs in biological fluids (plasma, urine, tissue, etc.), pharmacokinetic studies, bioavailability studies, doping control, quantification of biogenic amines, and therapeutic drug monitoring. Bioanalytical determination of protein-based biopharmaceuticals in biological matrices can be successfully achieved by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) (Kang, 2012; Irene van den Broek et al., 2013).

15.1.5.4 Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry

MALDI-TOF mass spectrometer is used in conjunction with the MALDI device as an ionization source and TOF as a mass analyzer that is described in Section 15.1.6. It is a simple method with high sensitivity which can be coupled by high resolution analyzers and has different application areas such as detection of cancer biomarkers in various cancers (Rodrigoa et al., 2014), characterization of microorganisms including bacteria, fungi, and viruses (Croxatto et al., 2012), and analysis of glycoproteins, oligonucleotides, carbohydrates, and small biomolecules (Susnea et al., 2013).

15.1.5.5 Tandem Mass Spectrometry

A tandem mass spectrometry (TANDEM MS), also named as MS/MS, is a two-step technique used to analyze a sample either by using two or more mass spectrometers connected to each other or a single mass spectrometer by several analyzers arranged one after another. TANDEM MS (MS/MS) contains two or three quadrupoles and a TOF analyzer

(Broek et al., 2013). The mass analyzer coupled to the MALDI source determines the type of MS/MS analysis that can be carried out (Flatley et al., 2014).

MS/MS is especially useful for analyzing complex mixtures and involves two stages of MS. In the first stage of MS/MS, a predetermined set of m/z ions are isolated from the rest of the ions coming from the ion source and fragmented by a chemical reaction. In the second stage, mass spectra are produced for the fragments. TANDEM MS is generally used for bioanalysis of drugs. Identification and determination of phase I and phase II drug metabolites are achieved by TANDEM MS coupled with HPLC (Glish and Vachet, 2003; Holčapek et al., 2008).

15.1.6 Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) is a rapid, high-throughput, quantitative immunoassay for the selective detection of target antigens. This technique is used as diagnostic tools and as quality control measures in biomedical research or various industries for the detection and quantification of specific antigens or antibodies in a given sample. The general principle behind an ELISA is antibody-mediated capture and detection of an antigen with a measurable substrate. The antigens such as proteins, peptides, hormones, or antibody immobilized on a solid surface and then complexed with an antibody that is linked to an enzyme. Alkaline phosphatase (AP) and horseradish peroxidase (HRP) enzymes are commonly used in ELISA applications. The size of HRP (40 kDa) is smaller than AP (140 kDa) and small size allows more molecules to be coupled to antibodies or avidin. This can boost signal generation. For this reason, HRP can be used with a variety of substrates, most of which are more sensitive than AP equivalents. The addition of substrate giving colored, fluorescent, or luminescent reaction products makes it possible to determine the concentrations of the reactants at very low levels. Colorimetric, chemifluorescent, and chemiluminescent substrates are available for both HRP and AP. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. Colorimetric and chemifluorescent substrates are typically able to detect low- to mid-picogram levels of antigen and chemiluminescent substrates are the most sensitive, with antigen detection possible in the subpicogram. Colorimetric substrates are measured using a standard plate reader with the appropriate filters. Chemifluorescence is measured using a fluorometer with the appropriate excitation and emission filters (Fang and Ramasamy, 2015; Watabea et al., 2016; Akama et al., 2016; Ma et al., 2011).

Various types of ELISAs have been employed with modification to the basic steps described earlier.

15.1.6.1 Indirect ELISA

For indirect detection, a sample that must be analyzed for a specific antigen is adhered to on the solid surface. In order to block any areas of this surface that is not coated with the antigen, a solution of nonreacting protein such as bovine serum albumin is added and incubated. First an unlabeled primary antibody, which is specific for the antigen, is applied. Next, an enzyme-linked secondary antibody which is reactive against the primary

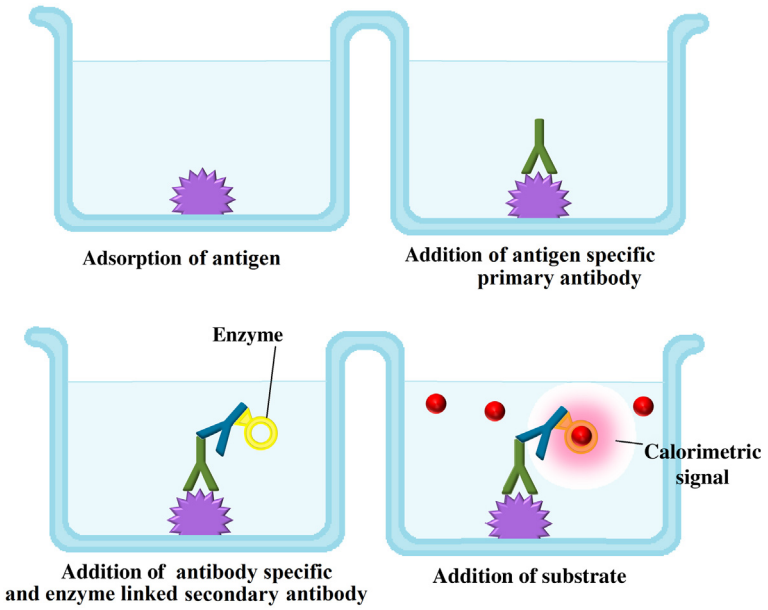

FIGURE 15.6 Schematic illustration of indirect ELISA.

antibody is added. For enzymatic detection and production of a calorimetric signal, the appropriate enzyme substrate is added and this signal is detected with appropriate equipment (Fig. 15.6) (Iannone, 2015).

15.1.6.2 Sandwich ELISA

Sandwich ELISA is also an indirect type of ELISA. The only difference in this ELISA principle is that, just like a sandwich in between two antibodies an antigen is present just a seen in the Fig. 15.7. The sandwich technique is used to identify a specific sample antigen. For this purpose, the well surface is covered with a known quantity of bound antibody (known as capture antibody) to capture the targeted antigen. Nonspecific binding sites of this antibody should be blocked using bovine serum albumin before adding the sample which is including antigen to the plate. A specific primary antibody is then added that "sandwiches" the antigen. Enzyme-linked secondary antibodies (known as detection antibody) are applied that bind to the primary antibody. Unbound antibody–enzyme conjugates are washed off. Substrate is added and the standard colorimetric detection method (described earlier) is used to detect and quantify analyte in the sample (Fig. 15.7) (Goldsby et al., 2000; Iannone, 2015).

15.1.6.3 Competitive ELISA

Competitive ELISA also known as inhibition ELISA is often used to indicate the presence of the antigen in the sample such as blood serum. Targeted antigen-specific antibody is immobilized on the solid surface. The enzyme-labeled antigen and the clinical sample are

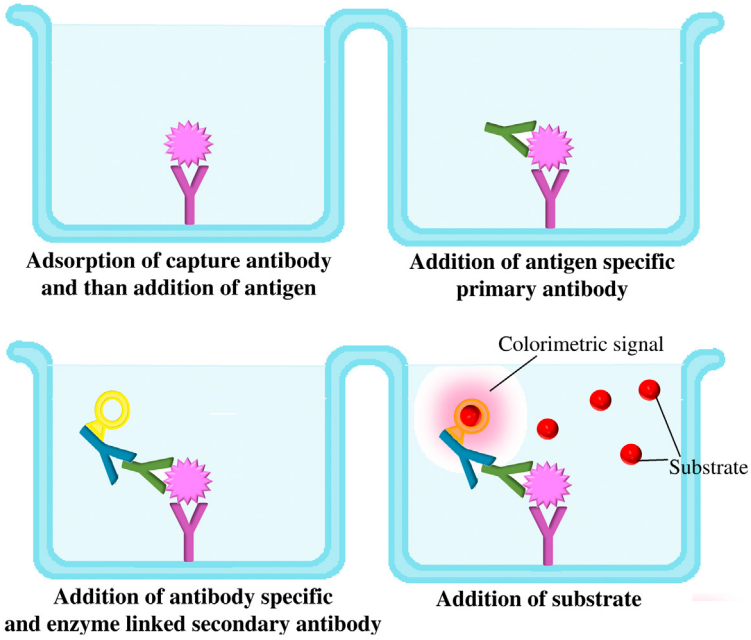

FIGURE 15.7 Schematic illustration of sandwich ELISA.

added at the same time into well and incubated in order to compete for binding with the immobilized antibodies. After an incubation period, any unbound antibody is removed by washing the plate and substrates are added (Aydin, 2015). The more antigen in the sample, the less antibody will be able to bind to the antigen in the well, hence "competition." After that, a standard colorimetric detection method that is related with substrates is used to detect and quantify analyte in the sample (Fig. 15.8). The hydrolyzed substrate amounts are inversely proportional to the amount of antigen in the sample. For this reason, the presence of antigen in the sample does not change the color (Ma et al., 2011).

15.1.6.4 Reverse ELISA

The solid phase of the Reverse ELISA consists of polystyrene pins protruding rod with 8–12 protruding ogives. The solid phase is immersed into primary antibody solution and incubated. After washing procedure of these pins, bovine serum albumin is used for blocking step. After each washing, pins were dried on absorbent paper and immersed directly in the collected sample (tube) or the sample dispensed in a microplate wells. After an incubation period, pins are removed and washed. The pins are then immersed into the wells prefilled with specific conjugate(s) antibodies and incubated. After the color development step the pins are removed from the wells and the plate can be immediately analyzed by a microplate reader if necessary (Fig. 15.9). Further developments of the test could foresee the simultaneous detection of an increased number of targets since each pin

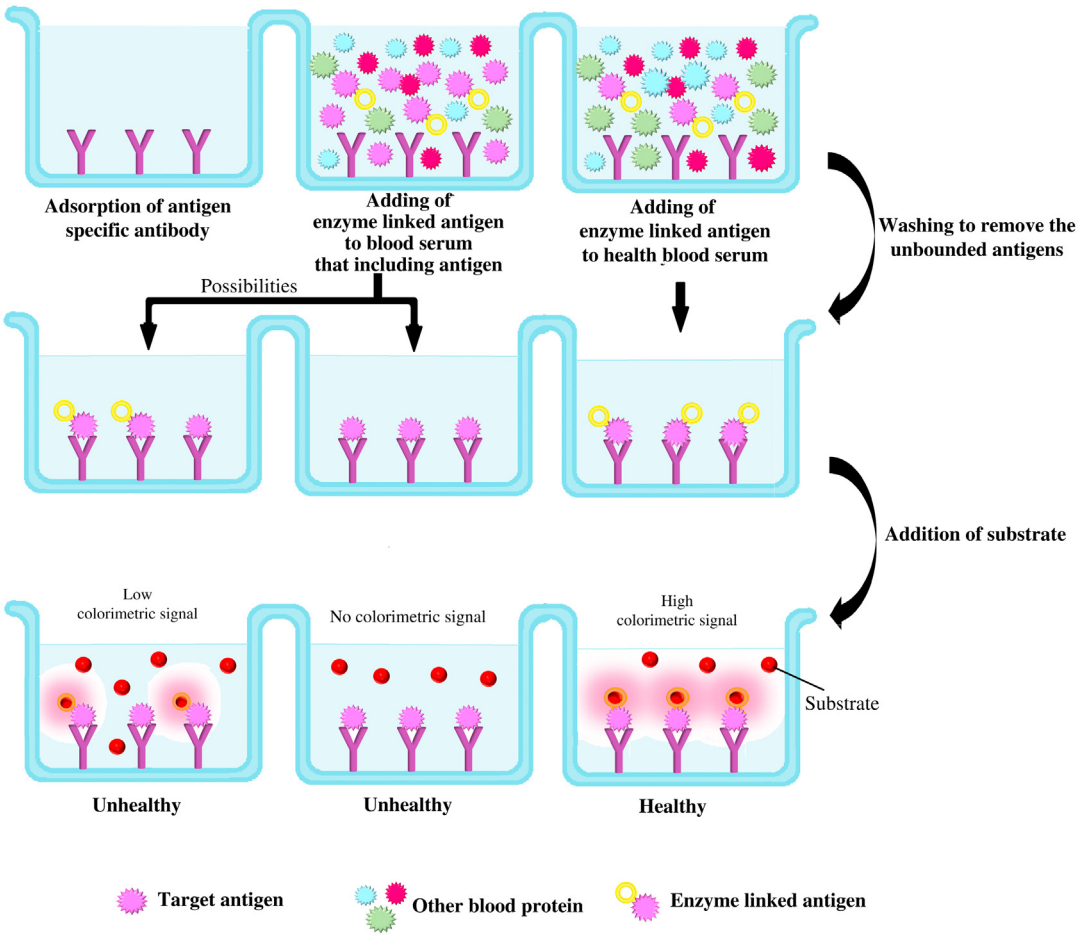

FIGURE 15.8 Representation of competitive ELISA.

could be dedicated to a specific target sensitizing the pin with an appropriate monoclonal antibody (Wilson et al., 2015; Folloni et al., 2011).

15.1.7 Immunohistochemistry

Protein analysis by immunohistochemistry (IHC) is based on the detection of antigens in organ or tissue sections by binding of specific antibodies and visualization of antigen–antibody complexes microscopically after immunohistochemical staining via an appropriate detection system (Ramos-Vara, 2005). Fluorescent dyes, enzymes, colloidal gold particles, and radioactive elements are the markers used for detection. Respectively, fixing and embedding the specimen, sectioning, deparaffinizing and rehydrating the section, antigen retrieval, immunohistochemical staining and visualization under the microscope are the basic steps of the IHC (Renshaw, 2017).

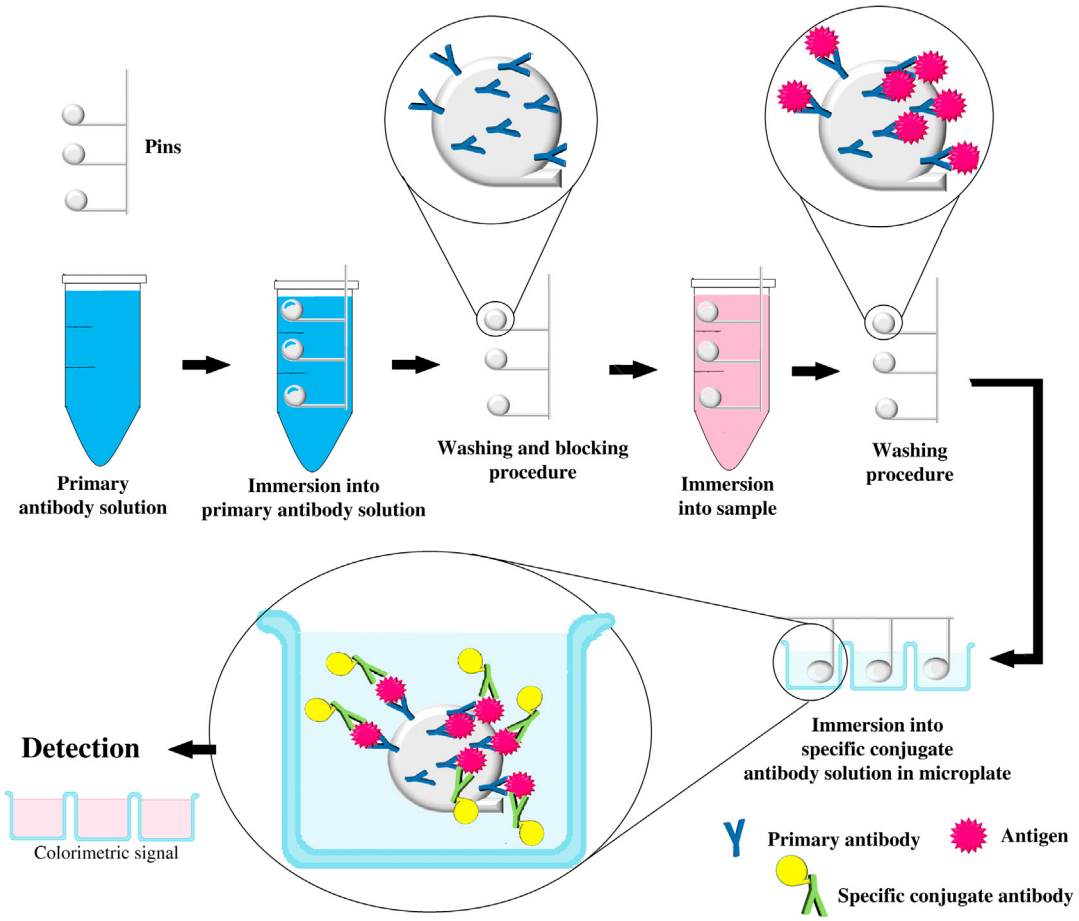

FIGURE 15.9 Schematic illustration of reverse ELISA.

15.1.7.1 Sample Preparation

Sample preparation is a critical step to maintain cellular components and special feature of antigenicity for IHC analysis. The specimen must be properly collected, fixed, embedded, and sectioned in the shortest time to prevent damage on the cell or tissue.

Fixation of the specimen is an important parameter for preserving and detecting cellular morphology and antigenic properties. Depending on the size and type of the specimen and fixative agent, the time for ideal fixation changes. Specimen fixation can be done by air drying, chemical fixation with cross-linking fixatives, and coagulating fixatives (Buchwalow and Böcker, 2010). Cross-linking fixatives stop enzymatic degradation more quickly than coagulating fixatives. Aldehydes such as formalin, paraformaldehyde, and glutaraldehyde are the most common used cross-linking fixatives for specimens that should be embedded in paraffin. Acetone and alcohols are used as coagulating fixatives for cryosections, cell smears, and cell monolayers.

After fixation, specimens are either frozen in liquid nitrogen or dehydrated and embedded in paraffin wax or synthetic resin prior to cutting by microtome to the desired thickness and mounted onto slides.

Prior to immunohistochemical staining, antigenicity of tissue sections must be recovered by antigen retrieval (Hyatt, 2002). Conformational changes of protein macromolecules affected by formaldehyde fixation and paraffin embedding can be reestablished by antigen retrieval, which can be achieved by either heat treatment (90–110°C) or enzymatic (trypsin, pepsin, and proteinase K) digestion. After antigen retrieval, samples are treated with antibodies for detection of antigenic properties of the tissue by antigen–antibody complex reaction (Renshaw, 2017).

15.1.7.2 Sample Labeling

Antigen–antibody immunoreaction can be detected by labeling the antibody before visualization by a microscope. Immunolabeling methods can be classified as direct or indirect according to the kind of procedure. Direct labeling is one step method in which the antigen directly binds to its specific labeled primary antibody in contrast to indirect labeling which could be two, three, or multi step and involves the labeled antibody reacting with the unlabeled primary antibody that will bind to the antigen. Although indirect labeling is a time consuming and complex method and has a potential for cross reactivity, it is more sensitive than the other. Direct labeling has lower signal, higher cost, and less flexibility. Polyclonal antibodies, obtained from many species such as rabbit, goat, pig, sheep, guinea pig, and horse, and monoclonal antibodies, generally obtained from mouse or rabbit hybridoma cells, are used for immunolabeling. Incubation time and antibody titers are important parameters for the antibodies affinity to the antigen (Miller, 2001).

Labeling the antibody by immunofluorescence, immunoenzymological (with HRP), calf intestinal AP, glucose oxidase, and beta-galactosidase) and immunoaffinity methods (with Avidin–Biotin complex) are done by using enzymes, biotin, fluorophore, and colloidal gold particles as labeling agents (Giorno, 1984; Hsu et al., 1981).

15.1.7.3 Sample Visualization

Sample visualization can be achieved by microscopic techniques such as light, fluorescent, and electron microscopes. In order to detect antigen–antibody immunoreaction by the microscope, antibodies should be labeled as described earlier. If the enzyme label is used for antibody labeling, immunoreaction could be visualized by light microscope using the brightfield illumination modus which is the most widely used observation mode in optical microscopy. Fluorophore labeling agents can be visualized in a fluorescent microscope. Electron microscope is needed for visualizing the colloidal gold particles. Depending on the labels, the Avidin–Biotin complex system can be visualized by light, fluorescence, and electron microscopy (Miller, 2001).

15.1.7.4 Applications

IHC has different application areas such as drug development, molecular biology, and diagnosis. Major changes in the expression pattern of antigen, specific cell, or tissue expression pattern of antigen, tissue or cellular localization of antigen can be determined by IHC. IHC is also used for diagnosis of diseases by specific tumor markers that can

determine the origin of tumor and grade of tumor cells, identify the cell type, classify the tumor type as malign or benign, and determine the localization. Membrane antigens, antigens localized in the nucleus and structural antigens in the cytoplasm, could be identified by IHC. Quantitative analysis of IHC can be achieved by computer-based programs designed for IHC such as BLISS, ACIS, iVision, GenoMx, ScanScope, LSC, and AQUA (Cregger et al., 2006).

15.2 PROTEIN STRUCTURAL ANALYSIS

Proteins are complex macromolecules that are composed of amino acid residues covalently bonded together by peptide bonds. Four levels of protein structure, such as primary, secondary, tertiary, and quaternary structure, are defined. Primary structure is the amino acid sequence of the specific protein. In a protein chain, the number, chemical structure, and order of amino acid sequences determine the structure and chemical behavior of the protein. Secondary structure is the regularly repeated local structure, stabilized by hydrogen bonds. Alpha helix and beta sheets are two main types of secondary structure. Tertiary structure, the three-dimensional structure of a protein molecule, is the intramolecular arrangement of the secondary structure. The alpha-helixes and beta sheets are folded into a compact structure by the nonspecific hydrophobic interactions. Three-dimensional structure analysis is important for understanding the functions of proteins at molecular level. Quaternary structure is the three-dimensional structure of a single protein complex which is formed by several protein molecules, such as dimers, trimers, tetramers, or even high order aggregates of identical polypeptide chains. Protein structures can be analyzed by some methods such as circular dichroism (CD), nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography, and electron microscopy which are discussed later (Kamp et al., 1997; Jiskoot and Crommelin, 2005).

15.2.1 Circular Dichroism

Circular Dichroism (CD), which is the difference in the absorption of left-handed circularly polarized light and right-handed circularly polarized light that arise due to structural asymmetry, is the technique used for analyzing secondary and tertiary structures and folding properties of proteins in solutions, which could be changed due to its environmental changes such as temperature or pH.

Folding properties of proteins, characterization of either secondary structure or tertiary structure in the far-UV and near-UV, respectively, comparing the structures of proteins obtained from different sources, determining thermal stability of the proteins, comparing thermal stability of proteins after changes in manufacturing processes or formulations, conformational stability of proteins under different environmental conditions, and kinetics of conformational changes could be done by CD analysis.

Molecules should contain one or more chiral molecules (light-absorbing groups, chromophores) for CD analysis. CD is measured with a CD spectropolarimeter that measures in the far-UV spectral region at 190−250 nm and near-UV spectral region at 250−350 nm.

Chromophores are the peptide bonds at 190−250 nm wavelengths and the aromatic amino acids and disulfide bonds at 250−350 nm wavelengths, which have specific CD signals.

15.2.2 Nuclear Magnetic Resonance Spectroscopy

Three-dimensional structure and conformational dynamics of the macromolecules affect the biological activity. Nuclear Magnetic Resonance (NMR) spectroscopy is generally used for analyzing small-to-medium sized flexible proteins, with molecular weights up to approximately 30 kDa, which could not be crystallized, and detailed information can be obtained about topology, dynamics, and three-dimensional structure of molecules in solutions and the solid state (Dötsch and Wagner, 1998; Arora and Tamm, 2001; Castellani et al., 2002, Loquet et al., 2008). The principle of the method is based on the magnetic properties of the nuclei of certain atoms. When the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second magnetic field, NMR occurs between these nuclei through bond (scalar coupling) or through space (dipolar coupling) interactions (Dyson and Wright, 1996). The nuclei of many isotopes with odd electron numbers such as ^{1}H, ^{13}C, ^{15}N, ^{19}F, and ^{31}P carry magnetic dipoles, and NMR measures the energy levels of magnetic atoms that are orientated differently and have a different energy in a magnetic field (Kwan et al., 2011).

An image of a protein cannot be obtained directly by NMR. Protein structure is calculated from the NMR spectra as a result of interactions between pairs of atoms by extensive data analysis and computer calculations (Wider, 2000).

15.2.3 X-Ray Crystallography

X-ray crystallography is a method used for various materials in the crystallized state to determine the arrangement of atoms within a crystal. Three-dimensional structure and function of many biological molecules, including proteins and nucleic acids, can be discovered by this method. The crystals of a pure protein are exposed to X-ray beam and X-ray is diffracted by atoms present in a protein crystal. Depending on the organization of atoms within a crystal and the number of electrons in the atoms, an X-ray beam is diffracted into many specific directions. From the angles and intensities of these diffracted beams, diffraction pattern is obtained and the electron density map is produced by a crystallographer. From this electron density map, the mean positions of the atoms in a crystallized protein and three-dimensional structure of the protein can be determined (Drent, 1994).

15.2.4 Electron Microscopy

Electron microscopy, in combination with image analysis, is used to determine the shape and three-dimensional structures of large proteins and large macromolecular complexes, with molecular weights greater than 150 kDa, that could not be investigated by conventional X-ray crystallography or NMR methods because of their large size or heterogeneous structure. Direct images of the molecules in their physiological environment can be obtained with the help of electron beams however, resolution of the images is low

(5–15A°) and it usually requires additional information from X-ray crystallography and/or NMR (Topf and Sali, 2005).

Transmission electron microscopy (TEM) is the original form of electron microscopy and produces two-dimensional, black and white images. Unlike the light microscopes that use glass lenses, electromagnetic and/or electrostatic lenses are used in all electron microscopes to control the path of electrons. TEM requires a high-voltage electron beam which is formed by electromagnetic lenses. The structure of the sample is determined by the electron beam that has been partially transmitted through the sample.

In the scanning electron microscope, the electron beam is scanned across the surface of the sample and image is detected by mapping the detected signals with detectors (Zhou et al., 2006).

15.3 PROTEIN PURIFICATION

The protein source to be purified can be plant, animal (organs and blood samples of animals such as rabbit, cow, pig), human (blood and placenta samples), and microorganism based (bacteria, fungus, yeasts and organisms that produce recombinant protein). The existence of proteins other than the one that is targeted as well as contaminations such as bacteria, virus, and nucleic acids and progens in the extracts obtained from these sources may lead to problems in purification (Tan and Yiap, 2009). Hence, all molecules and structures apart from the proteins should be removed on by one during the first stage of purification. Precipitation of the DNA molecules by adding chemical substances (streptomycin sulfate) to the raw extract, dissolving of the dissoluble proteins in a proper environment, and extraction from other proteins by way of centrifuging may be given as examples to these means of separation.

A purification method is selected afterward taking into account the characteristic properties such as the size and shape of the proteins, the total charge, the hydrophobic groups on the surface as well as the binding capacity with the stable phases used. The most important points when applying these methods are environment temperature and pH. Working temperature should generally be 4°C since changes in temperature and pH are effective in the denaturation and inactivation of proteins and the pH should be kept at the desired value (Takeda and Moriyama, 2015; Nelson and Cox, 2005).

15.3.1 Chromatography

Chromatography is the separation and purification of the substances in a mixture using a two phase system comprised of a stationary phase and a mobile phase that do not mix. There is a stationary phase (matrix) and a mobile phase in all chromatographic applications. The separation of proteins via chromatographic methods is based on driving the protein over the matrix via the mobile phase. Since the migration speeds of different proteins will be different, it is possible that they will group on the stationary phase and separate in the mixture. The factors that lead to this separation are the adsorption, partition, ion-exchange, and affinity properties of molecules or the differences between their

molecular weights (Ly and Wasinger, 2011). Various chromatographic methods have been explained later based on these properties.

15.3.1.1 Column Chromatography

The separation of proteins with this method depends on the differential separation of proteins between the mobile phase and the stationary phase (chromatographic medium or adsorbent) and it is one of the most effective methods. In general, the stationary phase is packed in vertical glass, plastic, or stainless steel columns and the mobile phase is pumped to the column. Protein extract is placed on the top part of the column and it is ensured that it moves downward with the mobile phase. The proteins that make up the mixture move at varying speeds in the column according to the differences between their adsorption or dispersion properties. Different proteins can be separated by collecting in fractions the liquid phase that comes out of the column (Carta and Jungbauer, 2010).

The primary materials that are used as stationary phase in column chromatography are classified as inorganic (porous silica, controlled pore glass, and hydroxyapatite), synthetic organic polymers (polyacrylamide, polymethacrylate, and polystyrene), and polysaccharides (cellulose, dextran, and agarose). These materials can be used to pack the column by themselves or in combination. The particle size in the restraining material is expressed in Mesh size (Janson and Jönsson, 2011).

15.3.1.2 Size-Exclusion (Gel-Filtration) Chromatography

Size-exclusion chromatography (SEC) method is also known as gel-filtration chromatography and ensures the separation of biological molecules according to their molecular size. When compared with other chromatographic methods, SEC is not an adsorption method. The column is packed with a solid-phase matrix made up of beads with pores of 100−250 μm and the mobile phase is passed through this structure. The mobile phase fills both the pores of the beads as well as their exterior. A matrix of high porosity generally covers more than 95% of the total liquid in the column. The protein extract applied to the top part of the column moves down in the column with the aid of the mobile phase passing around the beads as well as flowing through the pores of the beads. Since proteins with sizes greater than the pore size cannot go inside the pores, they move around the beads with the help of the mobile phase and reach the lower side of the column thus easily separating. Whereas the movement of the small molecules that can spread inside the pores is slower. Since the small molecules inside the pores can separate more easily than the beads, they are sorted according to their sizes inside the column (Lodish et al., 2000).

The pore size of the matrix used, the physical and chemical stability, its inertness and hydrophilic properties as well as its chemical interactions with the proteins are important. The most commonly used ones are dextran, dextran/bis-acrylamide, agarose, agarose/dextran, polyacrylamide, methacrylate, acrylamide, acrylamide/agarose, and cellulose. Dextran is a natural polysaccharide and is the first gel type that has been developed. Dextran-based gels are suited for the separation of molecules with molecular weights ranging between 1000 and 600,000 Da. The addition of bis-acrylamide can reach up to 107 Da. Whereas the separation limits of polyacrylamide gels range between 100 and 100,000 Da. The agarose gels have the highest separation limit with (1000−5,000,000 Da) (Phillips, 1992).

15.3.1.3 Ion-Exchange Chromatography

This method depends on the principle of the adsorption of charged proteins due to electrostatic interaction with oppositely charged proteins in the column as well as the matrix known as ion exchanger. The adsorption of the proteins to the matrix by way of electrostatic interaction is a reversible process. While the charge loads on the protein arise from different amino acids in the protein, the net charge of the protein depends on the combination of negatively and positively charged amino acids. The hydrogen ion concentration (acidity) of the mobile phase, that is its pH, causes variations to occur in the charges of amino acids. A highly acidic mobile phase will cause many groups to become positively charged thereby making the protein charge positive, whereas high amounts of alkaline mobile phase will form negatively charged proteins. Since the proteins are positively charged at pH values below their isoelectric points and negatively charged at pH values above their isoelectric points, it is important for this method to know their isoelectric points beforehand (Fanali et al., 2017).

The column is packed with the matrix in the first stage. Ion exchangers consist of a matrix with either acidic or basic groups. If the ion exchanger contains positive groups, it is called anion exchanger and if it contains negative groups, it is called as cation exchanger. These matrixes can be nonporous synthetic hydrophilic polymers that have been designed so as to not diffuse inside (Zou et al., 2001) or hydrophobic polystyrene based or partially hydrophobic polymethacrylate-based various polymers or hydrophilic and macroporous synthetic or natural polymers such as polyacrylamide, cellulose, dextran, and agarose (Černý et al., 2013; Ly and Wasinger, 2011).

In the second stage; the mobile phase is added to the positively or negatively charged matrix inside the column and it is ensured that the matrix is surrounded with ions in the buffer that are oppositely charged (Luqman and Inamuddin, 2012).

Whereas, in the third stage, negatively, positively charged or neutral proteins separated are packed in the column. It is ensured that proteins with charges opposite to that of the matrix are bound tightly to the stationary phase. Neutral molecules and molecules that have the same charge with the matrix have either no affinity to the stationary phase or the affinity is very low and thus they move together with the mobile phase and are removed from the column. Whereas the molecules that have been electrostatically bound to the matrix can be taken back from the column using another mobile phase with increased ionic force or pH. The increase in the ionic force of the mobile phase results in the separation of bound molecules; whereas the increase in pH results in the reduction of the charge of the molecule or matrix and the decrease of the electrostatic interaction power (Zou et al., 2001).

15.3.1.4 Affinity Chromatography

The aforementioned chromatographic separations are based on nonspecific physicochemical interactions between the matrix and molecules in the column. Molecular properties of proteins such as charge, size, and polarity do not provide a high selectivity in separation, purification, and isolation. On the other hand, the affinity chromatography is a method based on biological interactions which enables strong-specific separations. The interactions between the protein and the matrix depend not on general properties such as isoelectric point or hydrophobicity, but on selective properties such as the interactions

between antigen and antibody, enzyme and substrate analog, nucleic acid and binding proteins as well as hormone and receptors (Fanali et al., 2017).

The biological functions that are carried out by macromolecules such as proteins in biological systems are the results of the interaction with specific molecules known as ligands. These ligands can bind strongly with their protein of interest. That is why ligands are used in affinity chromatography. Ligands bond covalently with the water-insoluble matrix and are immobilized. The matrix is packed to the column and the column is packed with the mobile phase. Afterward, the extract of the protein that will be separated is added and it is ensured that it passes through the column with the mobile phase. There is a slowing down in the movement of the macromolecules that know and bind to the ligand during the passage through the column. Unbound molecules are removed from the column via washing. Whereas the protein bound to the matrix via ligands is recycled after the complex it forms with the ligand is decomposed via various methods.

This method is used in the isolation and purification of almost all biological macromolecules. However, there are various points that should be taken into account; if the interaction between the ligand and the protein is low, there is no adsorption and if the interaction is high, it is difficult to remove the protein from the ligand. The selection of detergents or other chemical substances to strengthen pH, salt concentration, and interaction are important to provide the proper environment for protein−ligand interaction. The most important criteria is that the substrates used for the binding of protein to the ligand or the removal of the protein from the ligand following chromatography will not harm the protein structure in any way (Bailon et al., 2000; Hage et al., 2012; Lodish et al., 2000).

15.3.1.5 Reverse Phase High-Performance Liquid Chromatography

This method takes advantage of the large-scale protein separations. Reverse phase high-performance liquid chromatography (RP-HPLC) is a more sensitive, relatively rapid, and accurate method for the purification of peptides, small polypeptides, and related compounds of pharmaceutical interest have not been replicated to the same extent for larger polypeptides and globular proteins. Additionally, this method has been applied on the nano, micro, and analytical scale, and has also been scaled up for preparative purifications, to large industrial scale for proteins.

RP-HPLC has a nonpolar stationary phase and an aqueous, moderately polar mobile phase. The separation of proteins depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase. Adding more water to the mobile phase can increase retention times, thereby the affinity of the hydrophobic properties of proteins for the hydrophobic stationary phase gets stronger relative to the more hydrophilic mobile phase. The proteins are, therefore, eluted in order of increasing molecular hydrophobicity. Acetonitrile containing an ionic modifier such as trifluoroacetic acid is used as a common solvent, although other organic solvents such as ethanol also may be used. This acidic solvent has increased solubility at pH values further removed from isoelectric point of protein. This *technique has advantage* for excellent separation of complex mixtures of peptides and proteins with easy experimentation via changes in mobile phase characteristics, temperature. However, these changes can cause the irreversible denaturation of protein samples thereby reducing the potential recovery of material in a biologically active form .

The most commonly employed experimental procedure for the RP-HPLC analysis of peptides and proteins generally involves the use of the more hydrophobic C18 ligands. Peptides and proteins behave as hydrophobic molecules because of their size and most often bind very strongly to C18 ligands. Whereas C4 (*n*-butyl) and C8 (*n*-octyl), phenyl and cyanopropyl ligands can provide different selectivities for peptides and proteins. Increases in column length can increase the resolution of small peptides and proteins. Thus, column lengths between 15 and 25 cm and id of 4.6 mm are generally preferred for applications such as tryptic mapping. However, for larger proteins, low mass recovery and loss of biological activity such as irreversible binding and denaturation may result with these columns. For this reason, shorter columns of between 2 and 20 cm in length are preferred (Aguilar, 2004; Sundaram et al., 2009; Bird, 1989).

15.4 PROTEIN QUANTITATION WITH WESTERN BLOTTING

15.4.1 Tissue Preparation

The original location of the protein inside the cell should be known before the protocol is set. If a tissue sample is used in the study, the most commonly used method is the SDS lysis method, but for cellular studies, ultrasonication is used. Thus, either cells in the tissue structure or cell cultures are homogenized. At the end of the homogenization, the undissolved material in the homogenate is discarded by centrifugation. The protein of interest stays in the liquid phase (supernatant). The cytoplasmic proteins and nuclear proteins are both obtained by disrupting the cell membrane: there are thousands of proteins in the whole cell extract (Kurien et al., 2015).

Because protein-degrading protease enzymes appear when the cell is disrupted, proteins should be protected from these proteases. Therefore protease inhibitors such as diisopropylfluorophosphate, aprotinin, leupeptin, sodium orthovanadate, or phenyl methyl sulfonyl fluoride are used. Thereby, the degradation of proteins is blocked by the inhibition of protease activity. Because proteins are affected by physical conditions, such as pH and temperature, these conditions need to be tightly controlled (Xiong and Gendelman, 2013; Iannone, 2015).

15.4.2 Gel Electrophoresis

This section was provided in detail in Section 15.1.3.

15.4.3 Transfer Methods

A nitrocellulose membrane is the first choice for protein blotting. However, in recent years, other membrane types have also been developed for different protein sizes. The physical features and physical characteristics of the membrane should be selected according to different transfer conditions. Membranes that have pores between 0.45 and 0.2 μm are commonly used. The membrane with 0.2-μm pore size is usually used for small proteins (<15,000 kDa). The nitrocellulose and supported nitrocellulose membranes can warm

up easily, making protein transfer easier. Protein binding to nitrocellulose is instantaneous, nearly irreversible, and quantitative up to 80–100 $\mu g/cm^2$. Supported nitrocellulose is an inert support structure with nitrocellulose applied to it. This support structure makes the membrane more flexible. It warms up more quickly than a nitrocellulose membrane, but its protein binding capacity is higher, and it can be autoclaved (121°C) (Mahmood and Yang, 2012).

Polyvinylidene difluoride (PVDF) membrane is an ideal support for N-terminal sequencing, amino acid analysis, and immunoassay of blotted proteins. The nitrocellulose membrane protects proteins that are exposed to acidic–basic conditions or organic solvents. It is effective for detecting low amounts of proteins and also provides an opportunity to determine sequencing manipulations of high molecular weight proteins (Young and Hongbao, 2006; Shively, 2000).

In addition, these membranes can bind proteins even in the presence of the SDS that comes from the transfer buffer. Of note, 1–2 minutes before using a PVDF membrane, it should be wetted with 100% methanol and then incubated in ice-cold transfer buffer. The transfer of proteins from gel to membrane can be done in two ways: wet and semidry transfer. The semidry transfer is usually quicker than wet transfer, but wet transfer keeps membrane from drying out. For both kinds of transfer, the membrane is placed next to the gel. The two are sandwiched between blotting filter paper, and the sandwich is clamped between solid supports to maintain tight contact between the gel and membrane. Blotting filter paper, which is made of 100% cotton fiber, provides a uniform flow of buffer through the gel (Liu et al., 2014).

In wet transfer, the gel and membrane are sandwiched between sponge and paper (sponge/paper/gel/membrane/paper/sponge) and all are clamped tightly together after ensuring that no air bubbles have been formed between the gel and membrane. The sandwich is submerged in transfer buffer to which an electrical field is applied. The negatively charged proteins travel toward the positively charged electrode, but the membrane stops them, binds them, and prevents them from continuing on (Iannone, 2015).

15.4.4 Blocking Buffers

After proteins are transferred onto the membrane, it is blocked with the primary antibody and is washed. After this step, the membrane is incubated with the secondary antibody and is washed once more. Before using antibodies to detect proteins that have been transferred to a membrane, the remaining binding surface must be blocked to prevent the nonspecific binding of the antibodies. Otherwise, the antibodies or other detection reagents will bind to any remaining sites that initially served to immobilize the proteins of interest. A variety of blocking buffers ranging from milk or normal serum to highly purified proteins have been used to block free sites on a membrane. The blocking buffer should improve the sensitivity of the assay by reducing background interference and improving the signal-to-noise ratio.

The primary antibody is specific for the protein of interest, and the secondary antibody enables its detection (Fig. 4.8). The secondary antibody can usually be radiolabeled, labeled with a fluorescent compound, or conjugated to an enzyme-like AP or HRP.

Available detection methods now include colorimetric, chemiluminescent, radioactive, and fluorescent detection (Young and Hongbao, 2006; Thieman and Palladino, 2004; Ghosh et al., 2014; Taylor and Posch, 2014; Taylor et al., 2013).

15.4.5 Detection

After proteins have been transferred to a membrane, they can be visualized using a variety of specialized detection reagents. Total-protein stains allow visualization of the protein pattern on the blot and immunological detection methods, employing antibody or ligand conjugates, and allow visualization of specific proteins of interest. This chapter will provide a brief summary of the immunological detections.

15.4.5.1 Colorimetric Detection

Because chromogenic or precipitating substrates are inexpensive and easily detectable, they are commonly used as a detection method. When these substrates bind to appropriate substrates (e.g., AP and HRP), they become colorful and insoluble products that precipitate onto the membrane. There is no need for an instrument for the detection of this colorful precipitation band. Colorimetric detection is typically considered a medium-sensitivity method, compared to radioactive or chemiluminescent detection (Iannone, 2015).

15.4.5.2 Chemiluminescent Detection

Chemiluminescence is a chemical reaction in which a chemical substrate is catalyzed by an enzyme, such as AP or HRP, and produces detectable light. The light signal can be captured on X-ray film, or by a charge-coupled device (CCD) imager. Faster and more precise measurements can be made with CCD than with other systems such as colorimetric systems and radioisotopic detection. The detection sensitivity is dependent on the affinity of the protein, primary antibody, and secondary antibody, and can vary from one sample to another. In addition, this method does not have negative outcomes such as radioactive exposure, environmental concerns, and high cost (Burgess and Deutscher, 2009).

15.4.5.3 Radioactive Detection

Radioactive detection system does not require enzyme substrates. The antibody itself can be labeled with a radioactive marker such as ^{125}I or ^{35}S. These labeled antibodies are placed in direct contact with X-ray film. After exposure of the membrane to the film for a suitable period, the film is developed and a photographic negative is made of the location of radioactivity on the membrane and dark regions are created which correspond to the protein bands of interest.

The importance of radioactive detections methods is declining, because it is very expensive, health and safety risks are high. Additionally, the signal produced by ^{125}I and ^{35}S is unaffected by enzyme, metal salts, pH, or temperature. Therefore, using the other detection methods, it has become more common in recent years (Walker, 2002).

15.4.5.4 Fluorescent Detection

Fluorescent detection is different from the methods explained previously in this chapter (those that work with an enzyme-substrate system). In this system, the secondary antibody is labeled with a fluorophore such as fluorescein (FITC), Texas Red, rhodamine (TRITC), or R-phycoerythrin. Fluorescent detection methods involve detection of light emitted transiently by a fluorescent molecule after it has absorbed light (excitation) and then releases photons (emission) as it returns to its normal state. Fluorescent western blot detection can allow multiplexing and provide better linearity and better quantitation within the detection limits. Application of fluorophores in different channels on the same blot can detect many target proteins at the same time (Walker, 2002).

15.4.6 Protein Microarray

Microarrays constitute a new platform which allows the discovery and characterization of proteins. This technology is also known as protein chips that are miniaturized and parallel assay systems containing small amounts of purified proteins (Hall et al., 2007). These properties allow the simultaneous analysis of thousands of parameters and simultaneously perform high-throughput studies of thousands of proteins within a single experiment. Protein microarrays are typically prepared by immobilizing proteins onto a microscope slide using a standard contact spotter or noncontact microarrayer. Different type slide surfaces can be used such as aldehyde and epoxy-derivatized glass surfaces, nitrocellulose, or gel-coated slides, and nickel-coated slides for different types of protein. After proteins are immobilized on the slides, they can be recognized for a variety of functions/activities. Finally, the resulting signals are usually measured by detecting fluorescent or radioisotope labels.

Three types of protein microarrays are currently used to study the biochemical activities of proteins: analytical microarrays, functional microarrays, and reverse phase microarrays. Additionally, microspheres bead-based systems were noted with different size or color beads as a support of the capture agent to analyze the sample (Sahukar et al., 2016).

15.4.6.1 Analytical Microarray

Analytical microarrays are typically used to determine parameters such as the binding affinity, specificities, and protein expression levels of the proteins in the mixture. In this method, antigens or antibodies are immobilized on a glass microscope slide and the test sample, which including proteins dropped on the slide to interact with them. These types of microarrays can be used to monitor differential expression profiles and for clinical diagnostics. Moreover, only selected target proteins can be analyzed by antibody microarrays.

15.4.6.2 Functional Protein Microarray

Functional protein microarrays have recently been applied to many aspects of discovery-based biology, including protein−protein, protein−DNA, protein−RNA, protein−phospholipid, protein−drug, and protein−peptide interactions. This technology holds great potential for basic molecular biology research, disease marker identification, toxicological response profiling, and pharmaceutical target screening. Productivities of functional protein

microarray are related with creation of a detailed expression clone library, high-throughput protein expression, isolation and purification, adaptation of DNA microarray technology to accommodate protein substrates, and ensuring the stability of arrayed proteins. These allow studying on different areas such as cell-free expression systems (*Escherichia coli*), wheat germ extracts, vaccine development, early detection of biomarkers biochemical activity protein–protein interaction studies.

15.4.6.3 Reverse Phase Protein Array

Reverse phase protein array (RPPA): In this technique, target proteins such as cellular or tissue lysate or serum samples are immobilized onto a nitrocellulose slide using a contact pin microarrayer and interacted with an antibody. Fluorochrome-conjugated secondary antibody is added to the first one to achieve a higher fluorescent signal to be detected with chemiluminescent, fluorescent, or colorimetric assays. The fluorescent signal intensity is related with the binding affinity, the specificity, and the steric accessibility of the antibody against the target protein. Reference peptides are printed on the slides to allow for protein quantification of the sample lysates (Krishnan and Davidovitch, 2015; Hall et al., 2007).

15.5 CONCLUSION

Proteins play crucial roles in nearly all biological processes. Moreover, they emerge as an important tool in scientific studies and pharmaceutical industry. Protein analysis aims to explore how amino acid sequences are specified in the structure of proteins and how these proteins bind to substrates and perform their functions. The true diagnosis of some diseases, the determination of where to start to identify the type of mutation and inheritance, the development of animal models for gene therapy, and the production and purification of drugs in peptide/protein structure are within the scope of protein analysis purposes. These analyses should be regarded as indispensable steps in determining the activity of proteins depending on the understanding of their structures.

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