

edited by

Richard O'Kennedy | Caroline Murphy

Immunoassays

Development, Applications and Future Trends



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Preface

Immunoassays are analytical tests that utilise antibodies to measure the amount, activity or identity of an analyte. The concept behind this book is to provide a detailed and practical overview of the development and use of immunoassays in many different areas. It is designed to provide a critical and helpful insight into the subject and to give the user practical information that may be of assistance in assay format selection, antibody generation/selection and choice of appropriate detection strategies. This book comprises 12 chapters written by highly experienced researchers in the fields of antibody-based research, immunoassay development, assay validation, diagnostics and microfluidics.

Chapter 1 is a comprehensive survey of antibodies, immunoassay formats and signalling systems. Chapter 2 elucidates key topics related to the development of an antibody-based sensor. Chapter 3 focuses on the important topic of surface modification and explores key parameters in the immobilisation of antibodies onto solid surfaces. Chapter 4 provides a detailed account of what is involved in immunoassay validation. Chapter 5 discusses the move to 'lab-on-a-chip'-based devices and investigates the key parameters necessary for their development. Chapters 6, 7 and 8 are dedicated to the areas of clinical diagnostics, infectious disease monitoring and food security, where immunoassay-based applications have become highly valuable tools. The future of immunoassays and electrochemical-based detection systems are discussed in Chapters 9 and 10. Chapter 11 concentrates on the use of optical detection systems (with a focus on surface plasmon resonance) in immunoassays. Finally, Chapter 12 is a compilation of important, routinely used, immunoassay protocols and addresses problems that may be encountered during assay development.

We would like to sincerely thank all the authors involved, the Applied Biochemistry Group for their help reviewing the chapters and Garvan Doherty, from the Biomedical Diagnostics Institute, for his help in developing the images used in the book.

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Caroline Murphy
Richard O’Kennedy
January 2017

Glossary

If words are not explained at first sight in the book, they are highlighted in bold and are explained in the glossary.

AC impedance Alternating current impedance is a measure of the opposition to the flow in an AC electrical current.

Accuracy Accuracy is a measure of the closeness of agreement of a test result obtained by an analytical method to its theoretical true value, or the accepted reference/standard value.

Affinity The affinity of an antibody refers to its ability to bind to the target antigen. It is a function of how well the antigen fits the binding site of the antibody.

Affinity ceiling Antibody recognition is thought to have an upper boundary for optimal affinity under natural biological conditions. This is called an affinity ceiling. Natural equilibration dissociation constant (K_D) is considered to be in the order of 10^{-8} to 10^{-10} .

Amino acids Amino acids are organic compounds that are composed of an amine ($-\text{NH}_2$) group and carboxyl ($-\text{COOH}$) group. They are the building blocks of proteins. In proteins, specific amino acids are linked together as if in a chain, and when folded, the tertiary structure makes up a protein.

Amperometric Amperometric analytical signalling systems operate when a current is created as a result of redox reactions at a sensor surface. They rely on either the release or consumption of electrons during these reactions.

Analyte Any chemical or substance under analytical scrutiny.

Anthelmintics Anthelmintics are a class of drugs used to treat infections with parasitic worms (helminths) from the body. Tapeworms, roundworms and flukes are classified as helminths.

Antibacterials See **antibiotics**.

Antibiotics Antibiotics are also known as antibacterials and are types of medication that destroy or slow down the growth of bacteria.

Antibody An antibody, also known as immunoglobulin, is an important part of the immune system. Most antibodies are Y-shaped molecules that recognise the presence of invading species such as viruses, bacteria, fungi, parasites and many more. Antibodies recognise these invasive agents through their antigen binding sites and signal to initiate an immune response via their fragment crystallisable (Fc) regions.

Antibody-based latex particle agglutination test (LAT) LAT utilises latex particles coated with antibodies or antigens to detect their counterparts through immunoagglutination of the latex particles to form quantifiable aggregates in the presence of the analyte in the sample.

Anti-idiotypic This is an antibody that binds to the antigen-combining site of another antibody.

Assay An assay is a qualitative or quantitative scientific test to measure the amount, functional activity or identity of an analyte. An **immunoassay** incorporates antibodies into key steps for the quantitation of the target analyte.

Autoantibody An antibody produced by the immune system targeting the body's own proteins as opposed to proteins originating from external pathogens. Autoantibodies are involved in the pathogenesis of autoimmune diseases and are also found in sera of cancer patients.

Autoantigen A 'self' antigen; any host component that evokes an immune response by the host.

Batch Synonymous with 'run'. A set of standard curve calibrators, validation samples, and/or quality control samples, and/or study samples that are analysed in a single group.

Bias Systematic difference between a measured test result and the theoretical true value (nominal). Bias is expressed either as a relative error (%RE) or as a ratio (%recovery).

Biomarker The term biomarker refers to a broad subcategory of molecules indicative of the presence of a disease (examples of which include proteins, genes or other molecules). They provide

objective indications of a medical state, which can be measured with a high degree of accuracy and reproducibility. They help to identify the presence or progress of disease or the effects of treatment.

Calibration curve A functional relationship between the analyte concentration in the standards (calibrators) and the measured response. The calibration curve is used to estimate the analyte concentration in test samples by dose interpolation. A calibration curve is synonymous with a standard curve.

Calibrator A volume of matrix spiked with the analyte of interest at a predetermined concentration using a well-characterized reference material.

Carbon nanotubes Are tube shaped molecules composed of carbon atoms that have a diameter on a nanometer scale. They can have either 'metal or semiconductor-like' properties and can be generated as single-walled nanotubes (SWNTs), multi-walled nanotubes (MWNTs) or double-walled nanotubes (DWNTs).

Coefficient of variation (CV) A quantitative measure of precision expressed relative to the mean result (also referred to as the relative standard deviation).

Competitive immunoassay A competitive immunoassay is one in which free analyte competes with immobilised analyte for antibody binding or where antibody in the matrix competes with labelled antibody for binding to an immobilised cognate antigen.

Confirmation assay A secondary assay, following a screening assay, which confirms the result (and may include assessment of **specificity** and/or **titre**).

Cross-validation Validation that utilises the use of two or more bioanalytical methods within the same study.

Cut-off point The cut-off point of the assay is the level of response of the assay at or above which a sample is defined to be positive and below which it is defined to be negative.

Direct antigen testing (DAT) A rapid antibody-based test that utilises direct antigen binding to identify pathogens such as *Mycobacterium tuberculosis* or influenza virus antigens.

Diagnostic A diagnostic is a tool used to identify/characterise a particular disease from its signs and symptoms.

Dialysis/dialyse Dialysis is a diffusion-based process whereby larger soluble molecules are separated from smaller ones. It generally employs a differentially permeable membrane that achieves the separation based on size.

Diffraction grating Can separate light into its constituent wavelengths.

Dilutional linearity A condition in which dilution of a spiked sample does not result in biased measurement of the analyte concentration. For example, when a spiked sample is serially diluted to give a set of samples having analyte concentrations that fall within the quantitative range of the assay, the entire set of dilutions can be measured with acceptable accuracy.

EC50 An abbreviation for the 'concentration necessary to produce a response of 50%'. For a competitive assay, the EC50 is the concentration of the analyte that is necessary to produce a 50% displacement of the tracer. For a non-competitive assay, the EC50 is the concentration of analyte necessary to produce a response of 50% or one-half of the observed maximum of binding.

Electrochemistry/Electrochemical reaction Electrochemistry is the production of an electric current during a chemical reaction. The most common type of electrochemical reaction used in biotechnology is the reduction/oxidation reaction or redox reaction during which electrons are transferred between two interacting species.

Enzyme-linked immunosorbent assay (ELISA) An enzyme-linked immunosorbent assay (ELISA) uses the basic immunological concept of an antigen binding to its specific antibody, allowing for the detection of antigens such as proteins, peptides, hormones or antibodies in a fluid sample or in tissue. ELISAs are commonly used as **diagnostic** tools in medicine and as quality control measures in various industries; they are also used as analytical tools in biomedical research for the detection and quantification of specific antigens or antibodies in a given sample.

Enzyme immunoassay (EIA) Synonymous with ELISA; used for the detection and quantification of specific antigens or antibodies

in a given sample through enzymatic colour or fluorescence changes.

Epitope An epitope is an area on an antigen that is recognised by antibodies of the immune system. An epitope can be either conformation-based (on the three-dimensional structure of the antigen's antigenic region) or linear (where an antibody recognises a continuous length of **amino acids** or other constituent components).

Evanescent wave An evanescent wave is an electrical wave that decays exponentially away from the surface interface where it was formed.

Factorial experiments A multivariate approach where two or more variables are varied simultaneously to achieve an optimal solution after taking into account the interaction between these variables. This approach is typically more efficient than one-factor-at-a-time for optimising assays and/or when analysing multiple variables of an assay that are interdependent.

False negative Negative test result from a truly positive sample.

False positive Positive test result from a truly negative sample.

Fluorometric assay An assay that relies on the presence of increased fluorescence or a change in the amount present as a means of quantification.

Four-parameter logistic (4PL) A versatile function that is recognized as the 'reference standard' for function fitting the mean concentration response for immunoassays. The function is defined by the equation $E(Y) = D + (A - D) / [1 + (X/C)^B]$ where $E(Y)$ is the expected response; X , concentration; A , response at zero concentration; D , response at infinite concentration; C , concentration resulting in a response halfway between A and D (ED_{50}); and B , slope parameter that is typically near 1.0.

Fractional factorial experiments A type of multi-factorial experiment in which only a subset of factor-level combinations is tested. These experiments are very useful for screening a large number of factors prior to optimising the most important factors.

Full validation A validation that includes the evaluation of accuracy, precision, curve-fitting (model assessment), sensitivity, specificity, stability, etc.

Functionalisation (surface modification) Functionalisation, also known as surface modification, is the act of changing the surface of a material by altering its chemical characteristics, generally to enhance its ability to bind biomolecules or resist fouling.

Gold nanoparticles Gold nanoparticles (GNPs) or colloidal gold are nano-sized (1–100 nm) particles of gold that are in suspension. Depending on their size, shape, surface chemistry and aggregation properties they produce different optical and electrical properties; as a result of this, they are widely used in sensors.

Gravimetric sensors These are a type of sensor that can detect changes in analyte concentration on the basis of changes in mass.

Hapten A hapten is a small molecule that is of itself incapable of stimulating an immune response (i.e. it is not immunogenic). To overcome this, it is tightly linked to a carrier molecule (most often a protein) by a covalent bond. When used for immunisation purposes, this hapten carrier complex stimulates the immune system and subsequently the production of antibodies.

Hook effect Describes an artefact seen when an analyte is present in very high concentration, beyond the dynamic range of the method. This excess amount cannot be measured accurately and generates spuriously low results.

Hybridoma technology Antibody-producing B-lymphocytes are fused to continuously multiplying cancer cells (myelomas) to generate a hybrid cell or hybridoma. It is used for the continuous production of monoclonal antibodies.

Immunoassay See **assay**.

Immunodepletion Refers to the removal of a target using anti-target antibodies.

Immunodepletion assay A form of confirmation assay wherein the target (anti-product antibody) is specifically depleted by inhibition of reactivity during a pre-incubation step with an excess of product. It demonstrates the presence of antibodies that bind to the product.

Immunosensor An immunosensor is an analytical device that incorporates an antibody as a biological recognition element

and links the binding event that occurs between an antibody and an antigen with a physiochemical detector that signals the presence of a target molecule.

Incoupling This is a resonance phenomenon that occurs at a precise angle of incidence. It is dependent on the refractive index (RI) of the medium covering the surface of a waveguide.

Interference Presence of entities in samples that prevent the target analyte from being detected or accurately quantified. It is a significant bias in the measured analyte concentration due to the effect of another component or properties of the specimen or matrix.

Intermediate precision Precision of repeated measurements within a laboratory, taking into account all relevant sources of variation affecting the results (runs, days, analysts, equipment, reagents, etc.). Intermediate precision is also termed inter-assay or inter-batch precision.

Isoelectric point The isoelectric point of a protein or molecule is the point, where at a certain pH, the protein or molecule has no net charge. In a buffer with a pH less than the pI of the protein or molecule under consideration, the net charge of the protein will be positive. In a buffer with pH greater than the pI, the net charge of the protein will be negative.

Lab-on-a-chip Such devices integrate a panel of laboratory functions on a single microfluidic device, permitting for example, immunoassays to be performed using reduced sample volumes, and without the requirement for multiple platforms.

Ligand-binding assay A type of assay format that depends on the specific binding of an analyte to another molecule, usually a macromolecule (biopolymer). This format typically involves reversible non-covalent interactions governed by the laws of mass action.

Limit of detection (LOD) Lowest concentration of analyte for which the response can be reliably distinguished from background noise.

Linearity Ability of the analytical method within a specified concentration range to obtain test results that are proportional to the concentration of the analyte in the test sample.

Lower limit of quantitation (LLOQ) The lowest concentration of analyte that has been demonstrated to be measurable with acceptable levels of accuracy (mean bias) and precision.

Macromolecule Macromolecules are commonly biopolymers that have the potential to provoke an immune response. Due to their inherent molecular complexity, macromolecules are generally more difficult to characterize than conventional small molecules.

Magnetoresistance Refers to a change in electrical resistance in metal or a semiconductor when it is subjected to a magnetic field.

Mass spectrometry (MS) An analytical method used to identify small molecules, proteins, lipids and others substances through changes in their mass-to-charge ratios which are introduced by an ion source and analysed in a mass analysis detector.

Matrix Effect Interference in an assay that is caused by adding the sample matrix. Commonly refers to analytical interference produced by factors other than those that have physicochemical similarity to the analyte.

Matrix The type of environment (e.g. serum, plasma or other biological fluid) in which the target (antibody or analyte) is present.

Microarray Microarrays are two-dimensional high-throughput tests used to identify binding partners to a sample of interest. They are composed of multiple microscopic spots that can be made of, for example, DNA, tissues, cells, proteins or antibodies to formulate the respective microarrays. A biological sample of unknown content is applied to the microarray and binders can be identified using dyed probes or tagged antibodies.

Microfluidics This is a multidisciplinary research field, which integrates biomedical engineering, physics, chemistry, biology and biotechnology. Microfluidic applications focus on the implementation of these disciplines in developing systems accommodating microlitre volumes of fluid as analytical samples.

Nanoarray A nanoarray is an array in which nano-sized objects such as proteins or cells are spotted onto a solid surface. They can use picolitre volumes of liquid. (See **microarray**.)

Nanomaterials These are materials that have at least one dimension in the nanometer scale (1–100 nm). Engineered nanomaterials have unique optical, magnetic and electrical properties. One such example is graphene; it is a lattice-based structure that is composed of carbon atoms. Graphene is being increasingly used in the area of biological sensors.

Nanoparticle A nanoparticle has all three dimensions in the nanoscale range (1–100 nm).

Negative control A negative control is one in which the sample is known to be devoid of a particular substance. By not including this substance it can be shown that the experimental conditions used would generate a negative result.

Nominal concentration A stated or theoretical concentration that may or may not differ from the true concentration.

Nonparametric methods A statistical approach that makes no assumptions about a distribution. Nonparametric methods are a class of methods that are used for a broad non-parameterized set of underlying distributions. Mean, median and percentiles of a sample are nonparametric estimates of the corresponding population quantities.

Nonspecific non-specificity Analytical interference caused by factors other than those that are related physicochemically to the analyte of interest, but which nevertheless affect the in vitro binding reaction. This type of non-specificity is commonly referred to as matrix effects.

Optimisation The process of developing an assay, prior to validation, wherein the variables affecting the assay are elucidated (e.g. antibody concentration, incubation time, wash cycles).

Parallelism A condition in which the dilution of test samples does not result in biased measurements of the analyte concentration. Thus, when a test sample is serially diluted to result in a set of samples having analyte concentrations that fall within the quantitative range of the assay, there is no apparent trend toward increasing or decreasing estimates of analyte concentration over the range of dilutions.

Parametric methods Parametric methods are a class of methods that assume a particular parameterized set of underlying distributions for the data (e.g. normal distribution). These

methods may use mean or robust estimates such as median within the framework of the assumed underlying parametric distribution such as the normal distribution.

Pathogen A pathogen is defined as a microorganism that causes, or can cause, disease. That is, a microbe that can cause damage in a host.

Photobleaching Photobleaching (also referred to as fading) is the photochemical alteration of a dye or a fluorophore molecule such that it permanently is unable to fluoresce.

Plasmons A plasmon is a quantum of plasma oscillation. A plasmon is a collective excitation of the electronic 'fluid' in a piece of conducting material and can be considered a quasiparticle (like ripples on the surface of a pond are a collective mode of the water molecules of the liquid).

Point-of-care Point-of-care is a form of medical testing that is performed at or near the site of patient care.

Polymerase chain reaction (PCR) An enzymatic method for the amplification of DNA that relies on melting and enzymatic replication of DNA through a series of thermal cycles.

Positive control Experiments have two major groups: the test subject and the control subject. A positive control is usually what researchers expect from the test, so it gives them something to compare. Hence, positive controls are used to validate the assay and demonstrate legitimacy of the test data.

Potentiometric A potentiometric immunosensor can be defined as a device incorporating a biological sensing element connected to an electrochemical potential transducer.

Precision The closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Pre-study validation Procedures used prior to the analysis of study samples to establish that an analytical method is suitable for its intended application.

Pre-validation Assay development experiments conducted prior to formal validation in order to enhance immunoassay performance.

Quality controls A set of negative and positive controls included in each assay run to monitor, and maintain consistency of assay performance (system suitability).

Radioimmunoassay (RIA) An immunoassay that utilises radioactive substances as markers/labels for measurement. Either an antibody or an antigen may be labelled depending on the assay format. RIA is a very sensitive method, however, it is less frequently used due to safety issues.

Radioreceptor assays Radioreceptor assays are used to quantify receptor proteins present in tissue specimen through the addition of radiolabelled ligands specific to the receptor.

Range The interval between the upper and lower concentrations (amounts) of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of accuracy (mean bias), precision and linearity.

Recovery A measurement of the closeness of an observed result to its theoretical true value. Recovery is generally expressed as the percentage of the observed to the nominal (theoretical) concentration. 'Spike recovery' relates to cases where the theoretical concentration corresponds to the concentration of analyte added to a sample by the analyst.

Reference interval Values set for a clinical test defining a positive or negative meaning of a test result in terms of clinical decision making.

Reflection Reflection is the change in direction of a wavefront (such as light, sound or water waves) at an interface between two different media so that the wavefront returns into the medium from which it originated.

Refraction The change of direction of a wavefront in passing obliquely from one medium into another in which its wave velocity is different.

Regeneration (in terms of its use in surface plasmon resonance (SPR)) Regeneration of the surface of a chip used in SPR is carried out using a solution (which will be identified following a regeneration scout) that will remove all bound analyte while maintaining a fully functional surface.

Regression model A statistical model relating a dependent variable to one or more independent variables.

Relative error (RE) A quantitative measure of the closeness of an observed result to its theoretical true value, expressed as a percent relative difference from the nominal (theoretical) concentration $\%RE = [(Observed/Nominal) - 1] \times 100$.

Relative standard deviation A quantitative measure of precision (also referred to as the coefficient (RSD) of variation) expressed relative to the observed or theoretical (nominal) mean value $\%RSD = (SD/Mean) \times 100$. For repeatability, the SD is computed from replicate analyses within a single validation run. For intermediate precision, the SD is computed from replicate analyses over multiple validation runs within the same laboratory.

Repeatability Repeatability is the precision of repeated measurements within the same analytical run under the same operating conditions over a short interval of time. It is also termed intra-assay precision.

Reproducibility Precision of repeated measurements between laboratories; also termed inter-laboratory precision. Usually applies to collaborative studies that involve the standardisation of a bioanalytical method across multiple laboratories.

Resonant mirror A resonant mirror is an optical sensing system that probes the sensing surface to which an evanescent wave has been applied.

Response error relationship (RER) The relationship between the SD in replicate response values (e.g. counts per minute) and the mean response.

Robust estimates Robust estimates are estimates that down-weight the influence of extreme values in summarising the data via the use of median, trimmed mean, median absolute deviation, etc.

Robustness of the assay A measure of the capacity of an assay to remain unaffected by small changes in test conditions. Robustness provides an indication of the ability of an assay to perform under normal usage. Studies of the robustness of an assay measure the effect on the assay output of deliberate changes in assay inputs (incubation time, temperature, sample preparation, buffer pH) that can be controlled through specifications in the assay protocol.

Ruggedness The robustness/ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Run A run represents a set consisting of standard curve (calibrators) and validation samples and/or quality control samples and/or study samples that are analysed together as a single group using the method to be validated. Run is synonymous with 'batch'.

Screening assay The primary assay that differentiates study samples into potential negatives or positives for the presence of anti-product antibodies.

Sedatives Sedatives are central nervous system (CNS) depressants, a category of drugs that work by slowing down brain activity resulting in drowsiness or relaxation.

Selectivity The extent to which a bioanalytical method can measure particular analyte(s) in a complex mixture without interference from other components of the mixture.

Sensitivity The ability of an assay to discriminate small changes in analyte concentration.

Solid-phase extraction (SPE) SPE is a basic sample preparation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties.

Specificity The ability to unequivocally measure the analyte in the presence of other components (often very closely related in terms of structure) that may be expected to be present in the biological specimen, including impurities, metabolites and endogenous matrix components.

Specific nonspecificity Analytical interference that is caused by substances in the test sample that have physicochemical similarity to the analyte of interest. Examples of such substances include metabolites, degraded forms of the analyte, isoforms, precursors and structural variants that differ with regard to post-translational modification.

System suitability System suitability comprises quantitative or qualitative criteria which ensures that a validated assay performs as expected in day-to-day assay runs. System suitability tests are used to verify that the reliability of the system is

adequate (in other words, remains in a validated state) for the analysis to be performed. This quality control system alerts the analyst when the assay is out of specification and ensures that the results obtained in a given run are meaningful.

Titre It is the reciprocal of the highest dilution of the sample that tests positive in the method. It is a common practice to express titer as the common logarithm of the highest dilution of the sample that tests positive in the method. This dilution must be clearly discernible above background.

Total error A concept that expresses the closeness of agreement between a measured test result and its theoretical true value. The term total error describes a combination of systematic (mean bias) and random (precision) error components. In other publications, the term total error is also referred to as accuracy, e.g. ISO definition.

Total internal reflection (TIR) When polarised light is shone through a prism onto a metal surface at a certain angle, light is reflected. By altering the angle at which the light passes through the prism, at a certain angle, total internal reflection (TIR) occurs.

Toxin A toxin is a poisonous substance. It is a specific product of the metabolic activities of a living organism and is harmful when introduced into biological tissues.

Tracer A tracer molecule is a labelled version of the target analyte. The tracer is frequently labelled with an enzyme or fluorophore which is capable of generating a detectable signal.

Upper limit of quantitation The highest concentration of analyte that has been demonstrated to be measurable with stated levels of accuracy (mean bias) and precision.

Validated range The interval of analyte concentrations over which the assay method has been validated. This interval includes concentrations from **LLOQ** to **ULOQ**.

Validation It is the confirmation, via extensive laboratory investigations, that the performance characteristics of an assay are suitable and reliable for its intended analytical use. It describes in mathematical and quantifiable terms the performance characteristics of an assay.

Validation samples Biological matrix samples spiked with the analyte of interest at predetermined concentrations. Alternatively, the analyte may be present endogenously in the biological matrix. A set of validation samples is used during pre-study validation to assess accuracy (mean bias) and precision.

4-6-20 QC rule A batch (run) acceptance criterion widely used in the pharmaceutical industry, which requires that 4 out of 6 QC results be within $\pm 20\%$ of their respective nominal value. Recently, this rule was modified for small-molecule chromatographic-based assays to require 67% (4 out of 6) of QC results to be within 20% of their respective nominal values; 33% of the results (not all replicates at the same concentration) may be outside the $\pm 20\%$ of the nominal value. In this book, this rule has been modified for the bioanalysis of macromolecules to require 4 out of 6 of QC results to be within $\pm 30\%$ of their respective nominal value with at least 50% of the QC results in range at each concentration level.

