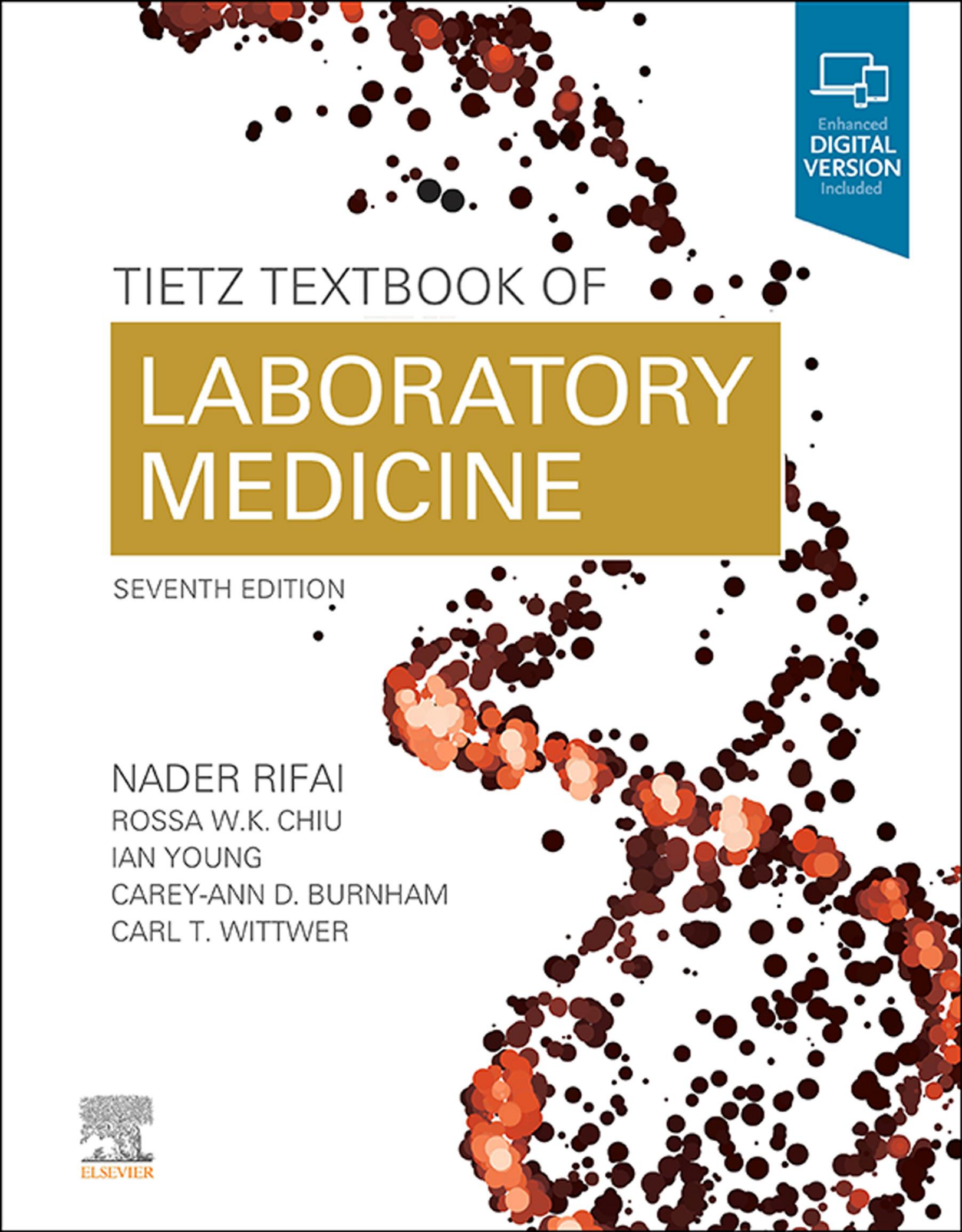

TIETZ TEXTBOOK OF LABORATORY MEDICINE

SEVENTH EDITION

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To our families
To the future generation

PREFACE

We are pleased to introduce the seventh edition of the *Tietz Textbook*, now entitled, *Tietz Textbook of Laboratory Medicine*. We have expanded the scope of chapters from the sixth edition to include various specialties throughout laboratory medicine. In addition, we further refined and enriched the *Platform*, a concept we introduced in the sixth edition, of which the Textbook is only a component.

Although the textbook is available in print for selected chapters, the comprehensive product is only available electronically on the Platform. The chapters in the print version of the textbook are meant to give readers a taste of the entire product and to demonstrate its broad scope. Using Elsevier's Expert Consult electronic system, the Platform encompasses:

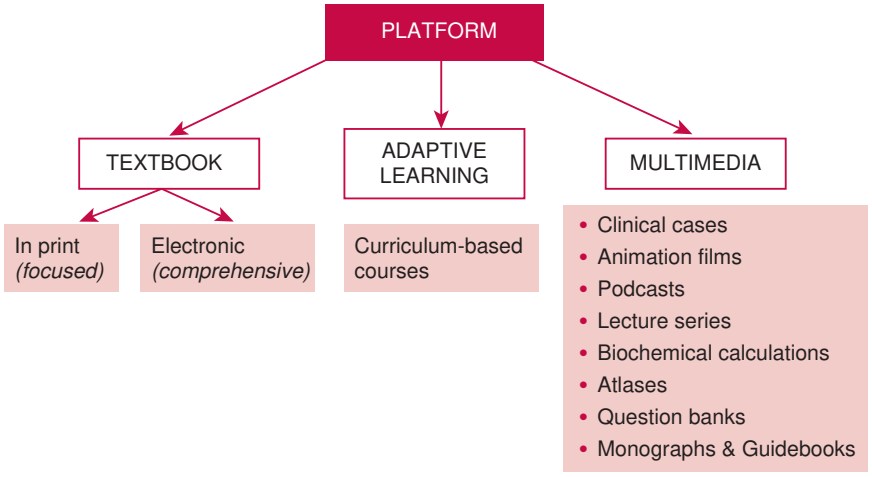
- A textbook covering all major disciplines of laboratory medicine including clinical chemistry, genetic metabolic disorders, molecular diagnostics, hematology and coagulation, clinical microbiology, transfusion medicine, and clinical immunology. Thirty additional chapters are devoted to analytical techniques and basic practices in laboratory medicine, and an extensive compilation of reference intervals is included. Compared to the previous edition, the number of chapters has increased from 81 to 100
- Electronic search capability and a built-in medical dictionary
- Curriculum-based courses utilizing the concept of adaptive learning provide the users with a personalized education experience (https://rhapsode.com/laboratorymedicine/). Over 100 courses, which span across all disciplines of laboratory medicine, encompass more than 15,000 learning objectives and are authored by world-renowned scientists and physicians; almost 50% of these courses were prepared or reviewed by authors participating in this textbook. Courses are linked to the appropriate chapters
- Multimedia and Educational Resources for an enhanced learning experience that include:
  1. The largest compilation ever assembled of clinical cases in laboratory medicine
  2. Animation films to explain complex mechanisms and concepts
  3. Podcasts
  4. Lecture series
  5. Biochemical calculations
  6. Collections of morphologic images and electrophoretic patterns
  7. Banks of multiple-choice and short-answer questions
  8. Important documents, monographs, and guidebooks

The above-described features are linked to the appropriate chapters for the convenience of the reader. These resources were either previously created by prominent laboratory medicine professionals (e.g., Allan Deacon, Michael J. Murphy, Rajeev Srivastava, Allan Gaw, Bobbi Pritt, Ellen F. Foxman, Julie E. Buring, Pamela Rist, Roy Peake, Morayma Reyes Gil, Matthew Diggle, Vera Paulson, Christina Lockwood, Gifford Batstone, Gary Weaving, Kate Shipman, Tamsyn Cromwell, and John Coakley), prestigious journals (e.g., *Journal of Clinical Microbiology*, *Clinical Chemistry*, *Transfusion*, *American Journal of Hematology* and *Blood*) and leading international scientific societies (e.g., the Association for Clinical Biochemistry and Laboratory Medicine-United Kingdom, Association of Clinical Biochemists-Ireland, Royal Society of Chemistry-London, Imperial College-London, Association of Molecular Pathology, and American Association for Clinical Chemistry), or produced de novo by accomplished scientists and physicians using materials from their own institutions (e.g., Mayo Medical Laboratories, ARUP, HudsonAlpha Institute for Biotechnology, Hôpital Universitaire La Pitié Salpêtrière-Paris, Pathology Queensland-Australia, and Boston Children's Hospital).

- A *living product*, where materials are periodically added and information updated as necessary

Our hope for this Platform is to serve as a resource center where important materials in laboratory medicine are deposited for use by and for the benefit of the community at large. Therefore, we encourage those who have similar materials and wish to have them considered for the Platform to contact one of the editors. The Platform can only be enhanced by further efforts.

Unlike most other textbooks, all chapters in this edition were reviewed by three individuals: a reviewer, an associate editor, and a senior editor. We believe that these efforts have



led to a better product. In addition, we made a concerted effort to create an *International* rather than an *American* Platform; about one third of the authors, reviewers, and editors reside outside the United States. We have strongly encouraged authors to include European, Australasian, and other international guidelines in addition to the American ones in order to present different practices and points of view. Furthermore, all measurements are presented both in traditional and SI units.

We aimed to harmonize the presentation of information among chapters while retaining the personality and unique style of each author, hoping for a readable, educational text with enough variety to amuse and occasionally delight.

This ambitious project has been a true group effort and represents the collective intellect, knowledge, and experience of almost 230 leaders in laboratory medicine from 18 countries. We are in debt not only to the authors, reviewers, and editors of the chapters but also to the contributors of the Multimedia and Educational Resources materials and the Adaptive Learning Courses that greatly enriched the Platform. We are grateful to Elsevier, and particularly to Heather Bays-Petrovic, Maria Broeker, and Rachel McMullen and their team for supporting us throughout this project to realize our vision.

We sincerely hope that this product will be a valuable educational and reference resource for the laboratory medicine community worldwide.

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- CR49: Pregnancy and its Disorders, Geralyn Lambert-Messerlian
- CR50: Catecholamines and Serotonin I: Basics, K. Aaron Geno
- CR51: Catecholamines and Serotonin II: Analytical and Clinical Aspects, K. Aaron Geno

GENETIC METABOLIC DISORDERS

- CR52: Inborn Errors of Metabolism I, Roy Peake
- CR53: Inborn Errors of Metabolism II, Roy Peake
- CR54: Inborn Errors of Metabolism III,[a] Roy Peake
- CR54a: Newborn Screening

LABORATORY GENOMICS

- CR55: Foundational Molecular Biology, Jill R Murrell & James A. Trofatter
- CR56: Nucleic Acid Techniques, Christine Schmotzer
- CR57: Nucleic Acid Isolation, Linnea M. Baudhuin
- CR58: Genomes and Variants, Avni Santani, Christine Chung
- CR59: Sequencing-based Techniques, Jillian Buchan, Ozge Birsoy
- CR60: Cell-Free DNA in Prenatal Screening, Christina Lockwood
- CR61: Concepts of Mendelian Inherited Disorders, Ann Moyer
- CR62: Cytogenomics, Anne B.S. Giersch, Heather Mason-Suares
- CR63: Hereditary Arrhythmias, Linnea M. Baudhuin, Katrina Kotzer
- CR64: Hereditary Cardiomyopathies, Linnea M. Baudhuin, Katrina Kotzer

[a]Courses will be released in 2022.

HEMATOLOGY & COAGULATION

CLINICAL MICROBIOLOGY

TRANSFUSION MEDICINE

CLINICAL IMMUNOLOGY

[a]Courses will be released in 2022.

SECTION I

Basics of Laboratory Medicine

Exam questions, case studies, and additional resources are available on ExpertConsult.com.
*Full versions of these chapters are available electronically on www.ExpertConsult.com.

1

Laboratory Medicine

Nader Rifai, Rossa W.K. Chiu, Ian Young, Carey-Ann D. Burnham, and Carl T. Wittwer[a]

ABSTRACT

Background

Laboratory medicine is a complex field that measures biomarkers and microorganisms in bodily specimens or tissues to diagnose and manage diseases. It encompasses multiple disciplines including clinical chemistry, hematology and coagulation, clinical microbiology, clinical immunology, molecular diagnostics, and transfusion medicine. Laboratory medicine is driven by technology that helps define the boundaries among its disciplines. Although laboratory medicine specialists are diverse in terms of their education, training, and career paths, their practice of the profession and their adherence to its guiding principles are similar. The goal is to generate relevant chemical, cellular, and molecular data that can be integrated with clinical and other information and interpreted to aid clinical decision making.

Content

This chapter describes the evolution of laboratory medicine and examines the international practice of the profession, the disciplines it encompasses, academic and postgraduate training, certification, career opportunities, and the skills and roles of laboratory medicine specialists in both clinical laboratory and industry settings. This chapter also discusses the guiding principles of practicing the profession, which include maintaining confidentiality of medical information, using available resources appropriately, abiding by codes of conduct, avoiding conflict of interest, and following ethical publishing rules.

INTRODUCTION

Laboratory medicine is a broad and heterogeneous field that deals with the measurement of chemical, biochemical, cellular, and genetic biomarkers; it encompasses multiple disciplines including clinical chemistry, hematology and coagulation, clinical microbiology (including serology and virology), clinical immunology, molecular diagnostics, and, in certain countries, transfusion medicine. Tissue pathology and cytology, although part of the broad definition of laboratory medicine that includes all testing of human tissue, are not included in this textbook. Although the various fields of laboratory medicine overlap in a continuous dynamic evolution (Fig. 1.1), specific disciplines elicit different images. For clinical chemistry, one thinks of pH measurements or large chemistry analyzers; for hematology or microbiology, microscopic examination is what first comes to mind; and molecular diagnostics conjures up the human genome project, companion diagnostics, and personalized and precision medicine. Whereas clinical chemistry and molecular diagnostics are heavily dependent on technological developments, where the former excels in random access testing and the latter has evolved massively parallel methods, the practice of transfusion medicine and hematology is decidedly clinical. Furthermore, certain disciplines like transfusion medicine are well defined and consistently practiced internationally, while others such as clinical chemistry and clinical microbiology may vary in content depending on the country in which they are practiced. According to the definition of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), "Clinical Chemistry is the largest subdiscipline of Laboratory Medicine which is a multidisciplinary medical and scientific specialty with several interacting subdisciplines, such as hematology, immunology, clinical biochemistry, and others. Through these activities clinical chemists influence the practice of medicine for the benefit of the public."[1]

Hospital-based laboratory medicine departments and commercial clinical laboratories provide in vitro testing of a variety of biomarkers in various fluids or tissues of the human body to screen for a disease, confirm or exclude a diagnosis, help to select or monitor a treatment, or assess prognosis. The popular claim that 60 to 70% of clinical decisions are based on laboratory tests cannot be easily justified by objectively measured data.[2,3] Nevertheless, laboratory testing impacts healthcare delivery to virtually every patient.

LOOKING BACK

The examination of body fluids for the diagnosis of disease is certainly not a modern concept. The Greeks noticed before 400 BC that ants are attracted to "sweet urine." Laboratory testing, however, was not always appreciated by clinicians; the famous Dublin physician Robert James Graves (1796–1853) once remarked, "Few and scanty, indeed, are the rays of light

[a]The authors gratefully acknowledge the contributions by David E. Bruns, Edward R. Ashwood, Carl A. Burtis, and A. Rita Horvath on which portions of this chapter are based.

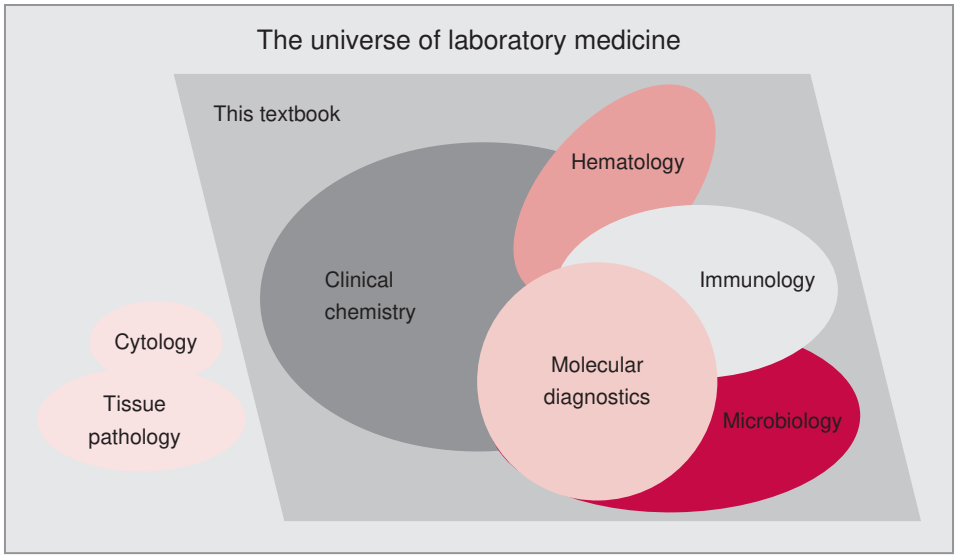

FIGURE 1.1 The interacting disciplines of laboratory medicine. Laboratory medicine encompasses testing and associated activities for the assessment, diagnosis, treatment, management, and prevention of human disease. Although in certain countries tissue pathology and cytology are part of laboratory medicine, their focus on morphology and image analysis sets them apart from other areas of laboratory medicine and they are not considered in this textbook. The largest divisions of laboratory medicine considered within include clinical chemistry, clinical microbiology, clinical immunology, hematology, and molecular diagnostics. These disciplines overlap and evolve over time. *The sizes of the circles are not meant to reflect those of the disciplines.*

which chemistry has flung on the vital mysteries," and the pioneer Max Josef von Pettenkofer (1818–1901) stated that clinicians use their chemistry laboratory services only when needed for "luxurious embellishment for a clinical lecture."[4] Such views have changed throughout the years, and laboratory testing has proven to be a useful tool to clinicians who have grown to depend and rely on the clinical laboratory in the routine management of their patients.

Although it may be difficult to pinpoint the exact date at which the concept of the clinical laboratory was born, a relevant article titled "Hospital Construction" by Francis H. Brown that was published in the *Boston Medical and Surgical Journal*, the precursor of the *New England Journal of Medicine*, in 1861. Dr. Brown stated: "[Every hospital should have] a small room at the end of the ward to serve as a general laboratory … necessary small cooking might be accomplished here; dishes and other articles washed etc.; and it would serve as a general store-room for brooms, pails, and other articles." Although Baron Justus von Liebig (1803–1873) once boasted that his clinical laboratory performed more than 400 tests per annum, the average mid- to large-sized laboratory today performs several million tests yearly; the images presented in Fig. 1.2 depict this striking contrast between the legendary Otto Folin in his biochemistry laboratory at McLean Hospital in Boston in 1905 and the University of Utah Clinical Laboratory/ARUP Laboratories more than a century later.

One of the first laboratories attached to a hospital was established in 1886 in Munich, Germany, by Hugo Wilhelm von Ziemssen.[5] In the United States, the first clinical laboratory recorded was The William Pepper Laboratory of Clinical Medicine, established in 1895 at the University of Pennsylvania in Philadelphia.[6] While there may be some uncertainty about the first hospital laboratory, the concept had become sufficiently well established by the late 1880s to enter popular culture. Arthur Conan Doyle, writing in 1887, set the first meeting of Sherlock Holmes and Dr. Watson in 1881 in the chemical laboratory in St. Bartholomew's Hospital, London, where Holmes had just discovered a reagent that "is precipitated by haemoglobin, and by nothing else" (A Study in Scarlet). Hopefully, the excitement experienced by Holmes at this discovery is still felt by laboratory specialists today.

Basic research usually precedes clinical application. Hematology began with the microscopic observation of red blood cells by Anthony van Leeuwenhoek (1632–1723). The father of microbiology is considered to be Louis Pasteur (1822–1895), who confirmed the germ theory of disease by experimentation. Immunology arose as a combination of the "cellularists," observing phagocytosis, and the "humoralists," who observed that immunity could be transferred as a soluble substance (antibodies and/or complement) in the late nineteenth century.

Molecular diagnostics has more recent origins than the other disciplines of laboratory medicine. "Molecular Diagnosis" was first mentioned in 1968 as the title of a *New England Journal of Medicine* editorial, commenting on a new inborn error of metabolism that overproduced oxalic acid, resulting in kidney stones.[7] "Molecular" referred to an enzymatic pathway and the substrates, not nucleic acid variants. Twenty years later, additional articles describing "molecular diagnostics" began to appear. In 1986, molecular diagnostics was defined as, "…the detection and quantification of specific genes by nucleic acid hybridization procedures," exemplified by speciation of plant nematodes.[8] In 1987, molecular diagnostics was used to describe mapping of antigenic substances by affinity chromatography using immobilized antibodies.[9] In 1988, the term was used to describe methods for detecting gene amplification and rearrangement using Southern blotting.[10] With the advent of polymerase chain reaction (PCR),

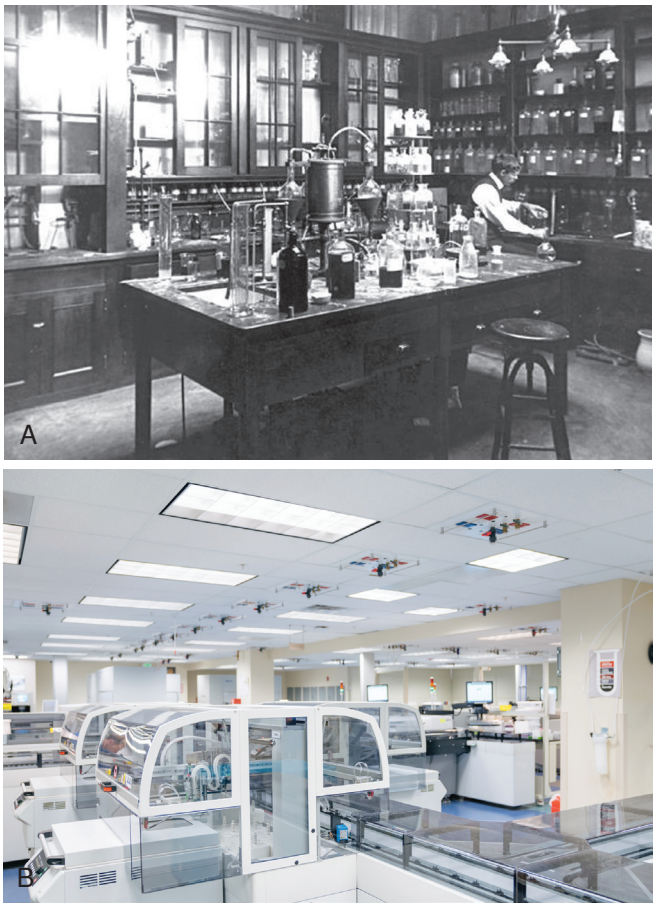

FIGURE 1.2 Early and modern clinical laboratories. The legendary Otto Folin in his biochemistry laboratory at McLean Hospital in Boston in 1905 and the University of Utah Clinical Laboratory/ARUP Laboratories, Salt Lake City, UT, more than a century later. (Image 1 from http://en.wikipedia.org/wiki/File:1905_Otto_Folin_in_biochemistry_lab_at_McLean_Hospital_byAHFolsom_Harvard.png; Image 2 courtesy ARUP Laboratories.)

the term "molecular diagnostics" became more common, its use doubling in the medical literature every 6 to 7 years.[11] By 1997, commercial real-time PCR instruments solidified "molecular diagnostics" as a branch of laboratory medicine.

TRAINING IN LABORATORY MEDICINE

Clinical laboratory professionals are individuals with a medical or a doctoral degree (pharmacy, chemistry, biology, biochemistry, microbiology) who are focused on clinical service. In North America, Australia, and Europe, a minimum of 9 years of academic education (a medical or a doctoral degree) and postgraduate professional training (residency and postdoctoral) is required before an individual becomes an independently practicing specialist (Fig. 1.3).[12] The requirements and training in laboratory medicine to become a specialist differ around the world. For example, in the United States, either those with a medical or a doctoral degree can direct a clinical laboratory after obtaining the appropriate board certification. Those with a medical degree usually do a residency in clinical or clinical/anatomical pathology to direct a general clinical laboratory. However, if they chose to direct a discipline-specific laboratory such as clinical chemistry, microbiology, or transfusion medicine, they may need to complete a fellowship in that specialty. Those with a doctoral degree tend to direct a discipline-specific laboratory and must complete postdoctoral training in that specialty. In the European Union, 40% of laboratory medicine specialists are from medical, 30% are from scientific, and 30% are from pharmacy backgrounds. In some countries such as Austria, Lithuania, Estonia, Malta, and Sweden, only physicians can practice the profession and direct a clinical laboratory. In most other European countries, scientists, pharmacists, and physicians can be laboratory medicine specialists, yet those with a pharmacy degree may not serve as clinical laboratory directors in some of these countries, such as Italy. A pharmacy degree is a "professional" degree (but not equivalent to a PhD) in France.

The curriculum used during the training of a clinical laboratory specialist in the European Union varies depending on the country. In the majority of European countries, trainees get exposed to clinical chemistry (45% of the curriculum), hematology (30%), microbiology (15%), and genetics (10%).[1,12] Molecular diagnostics (nucleic acid testing) is considered a technique and is included in all fields. In contrast, in the United Kingdom and Ireland, chemical pathology training is restricted to the traditional subdiscipline of clinical chemistry. This diversity of subspecialties is reflected in the heterogeneity of postgraduate training across countries.[13]

Postgraduate professional training and certification examinations at the end of the training are not mandated in all countries (see Fig. 1.3). The EFLM Register of Specialists in Laboratory Medicine (EuSpLM) (https://www.eflm.eu/site/page/a/1305) is attempting to standardize the minimum requirements for education and training for laboratory medicine specialists to facilitate the comparability of their professional training within the European Union.[1,12,13] These issues add to the complexity of defining the qualifications of clinical laboratory directors.

EXPANDING BOUNDARIES DEFINED BY TECHNOLOGY

The diversity of background, training, and subspecialization has led to heterogeneity in what the profession is called throughout the world. Name designations include clinical chemistry, clinical biochemistry, chemical pathology, hematology, clinical microbiology, transfusion medicine, clinical pathology, laboratory diagnostics, clinical or medical biology, clinical laboratory, laboratory medicine, clinical analysis, and so on. The EC4 Register (now the EuSpLM) adopted the name "specialist in laboratory medicine" to represent clinical laboratorians in Europe.

Everyone, including lay people, knows what a cardiologist is and does; the same is true for an infectious diseases specialist and a surgeon. Within laboratory medicine, the function of certain specialists, such as clinical microbiologists, hematologists, or blood bankers, is also clear. It is more difficult, however, to characterize a clinical chemist. Perhaps, unlike other specialties in laboratory medicine, clinical chemistry is very much influenced and shaped by technology. No discipline in laboratory medicine uses more technologies than clinical chemistry. Technologies that evolved over time not only changed practice but remodeled the boundaries of the traditional clinical chemistry laboratory. For example, with

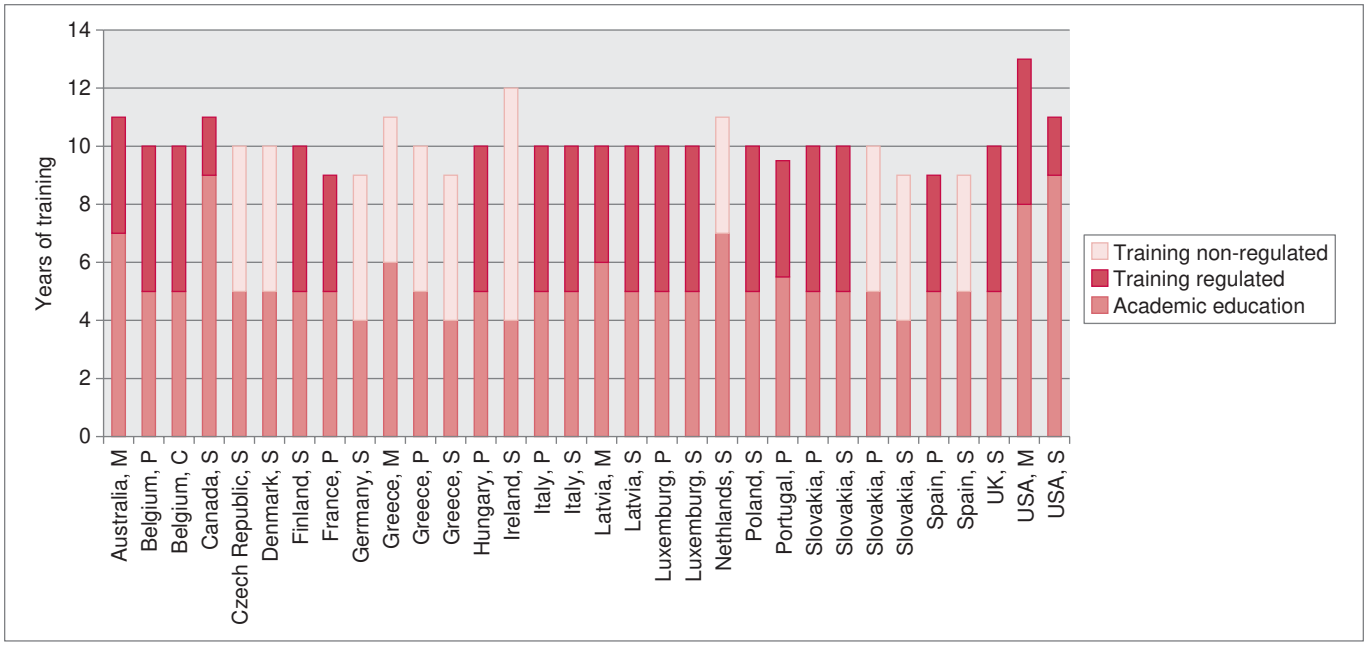

FIGURE 1.3 The number of years of education and training required to practice as clinical laboratory specialist in different countries varies from 9 to 13 years. Different training routes include medical *(M),* pharmacy *(P),* chemistry *(C),* and scientific *(S).* Both academic education *(light red bars)* and postgraduate training are required. Postgraduate training may be regulated *(dark red bars)* or nonregulated *(pink bars)* in different countries and even within the same country. (Modified from EU Directive 2013/55/EU. The recognition of professional qualifications. Proposing a common training framework for specialists in laboratory medicine across the European Union 2013. http://www.ukipg.org.uk/meetings/international_and_european_forum/ctf_e4_bid.)

the emergence of immunochemical techniques in the 1970s, the US Food and Drug Administration approved many tests for the measurement of proteins, small molecule hormones, and drugs, a development that profoundly changed clinical chemistry and its armamentarium of testing at the time. Integrated automated platforms later enabled the measurement of hormones and therapeutic drugs by immunoassays simultaneously with electrolytes, glucose, and other general chemistry tests, thus subsuming the "endocrine lab" and the "drug lab."

Serologic tests for hepatitis and HIV and assays for the evaluation of autoimmune diseases also moved from their traditional home in microbiology and immunology to chemistry analyzers. Immunoglobulin analysis followed a similar path. In certain countries, coagulation is considered part of clinical chemistry because the measurement of coagulation proteins uses similar instruments to those used in the clinical chemistry laboratory. As a result, the typical clinical chemistry laboratory includes testing for general chemistries, specific proteins and immunoglobulins, therapeutic and abused drugs, blood gases, hormones, biogenic amines, porphyrins, vitamins, and trace elements. Testing for inborn errors of metabolism (such as the measurements of amino acids and organic acids), measurements of coagulation factors, general hematologic testing, and serologic assays can belong either to the clinical chemistry laboratory or to another subspecialty, depending on the institution and country. If amino acids and organic acids are measured in the clinical chemistry laboratory, that does not preclude a biochemical geneticist from providing the clinical interpretation. Similar arguments can be made for coagulation, hematology, and serology testing.

Clinical laboratory professionals have embraced technology over the years and used it effectively to derive answers to clinical questions. In modern clinical laboratories, technologies include spectrophotometry, atomic absorption, cytometry, flame emission photometry, nephelometry, electrochemical, and optical sensor technologies, electrophoresis, and chromatography. The influence of automation, information technology, and miniaturization is evident in today's clinical laboratory. Mass spectrometry, once thought of as a research tool, is playing an ever-growing role in clinical chemistry for the measurement of both small molecules and peptides and more recently proteins. In fact, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry is now routinely used in the identification of microorganisms (including bacteria, mycobacteria and fungi), so it is likely that the evolution in this technology will also bring the clinical chemistry and microbiology laboratories closer. In addition, clinical microbiology laboratories are becoming increasingly automated, with total laboratory automation systems, including fluidic handling and high-resolution digital imaging systems, being adopted with increasing frequency.[14] Molecular diagnostics has forever changed virology and microbiology, introducing faster and more sensitive methods based on nucleic acid detection rather than microbial replication. Nanotechnology, microfluidics, electrical impedance, reflectance spectroscopy, and time-resolved fluorescence are only a few of the technologies used in point-of-care testing for proteins, drugs, DNA, and analysis of metabolites in small samples of whole blood. Point-of-care testing is a disruptive innovation that decentralizes laboratory testing and presents the clinical laboratory specialist with many

challenges and opportunities. Molecular diagnostics in particular impacts diverse specialties, including infectious disease, genetics, and oncology, providing new tools for study at a molecular detail never before considered. In summary, the boundaries of laboratory medicine expand with technology, making the profession vibrant, interesting, and ever evolving.

The scope of the profession is constantly changing for the very same reasons. Scientific and technological developments, medical needs, patient demands, and economic pressures bring various disciplines of medicine closer together, and further integration of diagnostic and therapeutic disciplines is envisaged in the pursuit of more integrated and effective healthcare delivery. For example, companion diagnostics, which help predict therapeutic responses and individualize patient treatment options, bring together pharmacy and medical laboratories. Point-of-care testing and use of biomarker measurements in real time with medical interventions break the walls of laboratories and bring the profession closer to clinicians and patients. Integrated diagnostics (a term coined by the medical device industry), whereby in vitro laboratory technology is combined with in vivo imaging technology, intends to provide fully coordinated, interpreted, action-oriented results for managing patient conditions, *and* it places laboratory testing into an integrated patient care pathway (see an example at http://www.healthcare.siemens.com.au/clinical-specialities/reproductive-endocrinology/integrated-diagnostics). New disruptive technologies (e.g., "lab on a chip," nanotechnology, home monitoring) and movement toward patient empowerment and direct-to-consumer testing bring laboratory testing closer to patients. All of these developments present special challenges to the future generations of clinical laboratory specialists both in terms of how they should be trained and how they will have to practice.

Technology alone is not the answer to more effective clinical practice. There must be meaningful, clinically actionable results as a consequence of the data obtained. The generation of more data does not necessarily lead to better patient management. Some technology platforms are useful discovery tools, but seldom provide cost-effective diagnostic or prognostic information that changes patient care. In the 1960s and 1970s, with the advent of automated clinical analyzers, pathologists reported (and charged for) chemistry panels of 10 to 20 results. Many were later sued for excessive production of data that increased their income without commensurate value to patient care. More recently, dense data from expression arrays, genome-wide association studies, epigenomics, and microRNA analyses excel in discovery research, but translation to clinical practice has been slower than anticipated. The promise of greater clinical significance with larger data sets seems intuitive, but history suggests caution.

Clinical laboratorians in this world of "big data" translate high-quality measurement *data* into clinically relevant *information*. This information—when integrated with clinical history and presentation, clinical signs, and an understanding of pathophysiology—becomes *knowledge*. Knowledge, in the context of the experience and judgment of the clinician, is converted to *wisdom* that translates to clinical action for improved patient outcomes. For example, a 2-week-old boy with a suspected inborn error of metabolism had a suppressed thyroid stimulating hormone and increased free T_4 concentrations. Acting on the basis of the data alone would have suggested treatment with methimazole for thyrotoxicosis. However, the patient was receiving biotin as part of his treatment for a metabolic disorder, and the biotin interfered with the immunoassays used in the thyroid function tests. Repeat measurement of these parameters with non–biotin-based immunoassays revealed a normal thyroid profile. Another example: A patient presented with Cushingoid appearance and a markedly decreased serum cortisol concentration. A further examination of his clinical history revealed that he was using topical corticosteroids for a skin condition, a treatment that caused adrenal suppression and thus a low cortisol concentration. Yet another example: A 55-year-old woman complained to her primary care physician about long-standing bony aches and pains. All results to exclude musculoskeletal problems came back normal except for a low alkaline phosphatase (ALP) enzyme activity. After excluding potential preanalytical errors [e.g., contamination of sample by K-EDTA (potassium ethylenediaminetetraacetic acid) anticoagulant], the laboratory proposed the diagnosis of hypophosphatasia, and testing for mutations of the tissue nonspecific ALP gene confirmed the diagnosis both in the patient and in her daughter. The world of laboratory medicine is full of such examples that demonstrate the value of acting on information beyond the generated numbers. Knowledge is what we must provide to clinicians to support informed clinical decision making and for achieving improved patient outcomes.

HOW IS LABORATORY MEDICINE PRACTICED?

Both the training of laboratory medicine professionals and their career paths are heterogeneous. Although the majority of our colleagues choose a career in a clinical laboratory environment, many work in the in vitro diagnostics (IVD) and pharmaceutical industries. Clinical laboratorians, by virtue of their training, are translational researchers who are equipped for and capable of developing, evaluating, and validating biochemical, cellular, and genetic assays for clinical use; they develop skills that are essential for new biomarker assays, reagent kits, and companion diagnostics. Laboratory medicine professionals also provide interfaces between researchers, clinicians, the clinical laboratory, and the IVD industry and help to translate biomarker research into clinically meaningful decisions and actions.

The functions of a clinical laboratorian include:

- Develop and validate de novo laboratory tests to meet clinical needs.
- Evaluate and characterize the analytical and clinical performance of laboratory tests.
- Present laboratory results to clinicians in an effective manner.
- Provide education and advice on the selection and interpretation of laboratory tests as part of the clinical team.
- Determine the cost-effectiveness and intrinsic value of laboratory tests.
- Participate in the development of clinical testing algorithms and clinical practice guidelines.
- Assure compliance with regulatory requirements.
- Participate in quality assurance and improvement of the laboratory service.
- Teach and train future generations of laboratory specialists.
- Participate in basic or clinical research.

Laboratory medicine specialists practicing in the IVD or the pharmaceutical industry may not need to routinely interact with clinicians or interpret laboratory results, but they

understand and appreciate the clinical utility and relevance of the assays and companion diagnostics they are developing and thus contribute more effectively to the development of diagnostics that improve health. The daily practice of the profession has changed over time. In the 1960s and 1970s, clinical chemists, for example, developed laboratory tests. However, as the profession matured and the instrumentation changed from open systems to "black boxes" that relied on manufacturers for assays, the traditional analytical focus of the profession has significantly diminished. At present, de novo assay development is still active only in certain areas such as chromatography, mass spectrometry, and molecular diagnostics.

Laboratory medicine specialists are now more active in the preanalytical and postanalytical phases of testing and in establishing processes such as how best to select the right test for the right patient and to communicate test results to clinicians in a medically meaningful way, how to build laboratory processes that reduce error, and how to continuously improve the quality of laboratory practice. In today's healthcare environment, there is increasing emphasis on clinical impact and cost-effectiveness. Laboratories are expected to demonstrate evidence of improved measurable clinical outcomes and the usefulness and added value of tests to clinical decision making. Proving the fact that laboratory testing contributes to improved patient outcomes is challenging because the relationship between testing and clinical outcomes is mostly indirect. Nevertheless, laboratory medicine specialists should move away from being just providers of high-quality data. Transforming laboratory data to information and knowledge requires more skills in information and information management technology, evidence-based medicine, epidemiology, data mining, and translational research. It also requires a shift of thinking from essentialism to consequentialism and from technology-driven to customer-focused and patient-centered laboratory medicine.[15,16]

To summarize, today's clinical laboratorians are professionals who are trained in pathophysiology and technology. The execution of their daily duties, which are more clinically or technology oriented, is influenced by their training (such as MD vs. PhD), interests, institutional needs, and the country where they practice. Clearly the practice of our profession has evolved over the past half a century, and there are even more challenges on the horizon that will expand and change its scope and role and enhance its diversity.

GUIDING PRINCIPLES OF PRACTICING THE PROFESSION

As in all branches of medicine, practitioners in the clinical laboratory are faced with ethical issues, often on a daily basis; examples are listed in Box 1.1.

BOX 1.1 Ethical Issues in Laboratory Medicine

- Confidentiality of patient medical information
- Allocation of resources
- Codes of conduct
- Publishing issues
- Conflicts of interest

Confidentiality of Patient Information

Safeguarding the confidentiality of a patient's personal and medical information is one of the fundamental ethical principles of the practice of medicine. Upholding of these principles prescribes how some laboratory activities are practiced. The laboratory holds vast amounts of data covering a patient's identifiers and demographics, as well as health and disease status. The patient's morbid state and future risks for illnesses and death are conferred by such information. While laboratory information systems are built to facilitate timely access to the data, the data must be stored in a secure format with measures in place to prevent unwarranted access.

On the other hand, development of new tests requires the use of patient samples and access to patient medical information by the laboratory.[17] Ethical judgments are required regarding the type of informed consent that is needed from patients for use of their samples and clinical information. Clinical laboratory physicians and scientists often serve on institutional review boards that examine proposed research on human subjects. In these discussions, ethical concepts such as clinical equipoise (the genuine uncertainty in the expert medical community over whether a particular treatment or test will be beneficial) and preservation of confidentiality of medical information are central to these decisions.

Broad coverage genetic testing is becoming more of a routine affair. Prominent in the news in the first and second decades of this millennium has been the issue of confidentiality of genetic information. Legislation was considered necessary to prevent denial of health insurance or employment to people found by DNA testing to be at risk of disease. The power of DNA information lies in its heritability. Predictions can be made on the phenotypes and traits of a person's parents, relatives, and offspring based on an individual's DNA profile. In the event of having identified a clinically significant incidental finding, the right to personal confidentiality against the potential duty to disclose the information to at-risk family members is a current subject of debate among stakeholders. Clinical laboratory professionals are actively participating in the development of such disclosure and clinical management guidelines that will need to adapt to the changing standards of information disclosure or nondisclosure.

Allocation of Resources

Because resources are finite, clinical laboratory professionals must make ethically responsible decisions about allocation of resources. There is often a trade-off between cost and quality and/or speed (turnaround time). What is best for patients generally? How can the most good be done with the available resources?

Codes of Conduct

Most professional organizations publish a code of conduct that requires adherence by their members. For example, the American Association for Clinical Chemistry (AACC) has published ethical guidelines that require AACC members to endorse principles of ethical conduct in their professional activities, including (1) selection and performance of clinical procedures, (2) research and development, (3) teaching, (4) management, (5) administration, and (6) other forms of professional service. A similar code of conduct has been developed and approved by the EC4 Register Commission and the European Federation of Clinical Chemistry and Laboratory Medicine.[18]

Publishing Issues

Publication of documents having high scientific integrity depends on editors, authors, and reviewers all working in concert in an environment governed by high ethical standards.[19]

Editors are responsible for the overall process, including identifying reviewers, evaluating the reviews and the authors' response to them, and making the final decision of whether to accept or reject a manuscript. Editors are also responsible for establishing policies and procedures to assure consistency in the editorial process. Finally, the editor-in-chief is responsible for developing a conflict of interest policy and monitoring it among his or her editors. Publishers, being commercial or scientific societies, should monitor any conflicts of interest of the editor-in-chief.

Authors are responsible for honest and complete reporting of original data produced in ethically conducted research studies. Practices such as fraud, plagiarism (verbatim, mosaic), and falsification or fabrication of data (including image manipulation) are unacceptable. The International Committee of Medical Journal Editors (ICMJE)[20] and the Committee on Publication Ethics (COPE)[21] have published policies that address such behavior. Other practices to be avoided include duplicate publication, redundant publication, and inappropriate authorship credit. In addition, ethical policies require that factors potentially influencing the interpretation of study findings must be revealed, such as (1) the role of the commercial sponsor in the design and conduct of the study, (2) interpretation of results, and (3) preparation of the manuscript. Additional undesirable and harmful practices are publication bias and selective reporting in which only studies with positive findings are reported and authors use "data dredging" and meaningless subanalyses to find positive association rather than reporting the original hypothesis that was negative.[19] These practices inflate the actual value of observations or utility of markers and diminish the quality of meta-analyses. As a result, a comprehensive registry of diagnostic and prognostic studies, similar to the registry of clinical trials, has been advocated.[19,22,23]

To avoid publication of biased study results, reporting guidelines have been published for the main study types on the website of the EQUATOR Network (http://www.equator-network.org). For the laboratory profession, the STARD and TRIPOD statements for diagnostic and prognostic studies are probably the most important,[24,25] but reporting guidelines for randomized controlled trials (CONSORT), observational studies (STROBE), systematic reviews (PRISMA), quality improvement studies (SQUIRE), and economic evaluations (CHEERS) are also relevant for the work of laboratory scientists active in research and publication.

Reviewers must provide a timely, fair, and impartial assessment of manuscripts. They must maintain confidentiality and never contact the authors until after the publication of the report. Finally, reviewers must excuse themselves from the review process if they perceive a conflict of interest.

Most journals now require authors to complete conflict of interest forms and delineate each author's contribution. Some journals, including *Clinical Chemistry,* publish this information along with the article for enhanced transparency.

Conflicts of Interest

The interrelationships between practitioners in the medical field and commercial suppliers of drugs, devices, and equipment can be positive or negative.[26] Concerns led the National Institutes of Health in 1995 to require official institutional review of financial disclosure by researchers and management in situations when disclosure indicates potential or actual conflicts of interest. In 2009, the Institute of Medicine issued a report[27] that questioned inappropriate relationships between pharmaceutical device companies and physicians and other healthcare professionals.[26] Similarly, the relationship between clinical laboratory professionals and manufacturers and providers of diagnostic equipment and supplies has been scrutinized.

As a consequence of these concerns and as a result of the enactment of various laws designed to prevent fraud, abuse, and waste in Medicare, Medicaid, and other federal programs, professional organizations that represent manufacturers of IVD and other device and healthcare companies have published codes of ethics. For example, the Advanced Medical Technology Association (AdvaMed) has published a revised code of ethics that became effective on January 1, 2020.[28] Topics discussed in this revised code include gifts and entertainment, consulting arrangements and royalties, reimbursement for testing, and education. Similarly, MedTech Europe has recently published a code of ethics.[29] In this document, topics include member-sponsored product training and education, support for third-party educational conferences, sales and promotional meetings, arrangements and consultants, gifts, provision of reimbursements and other economic information, and donations for charitable and philanthropic purposes. Both the AdvaMed and the MedTech Europe documents address demands from regulators while nurturing the unique role that clinical chemists and other healthcare professionals play in developing and refining new technology.[26]

WHAT IS IN THIS TEXTBOOK?

In this textbook, we have assembled what is essential to effectively practice laboratory medicine. We begin with introductory chapters that describe the basics of laboratory medicine, including statistics, sample handling, preanalytical processes, reference intervals, quality management, quality control, standardization and harmonization, evidence-based laboratory medicine, biobanking, and biomarker and laboratory support for the pharmaceutical and IVD industries, machine learning, test utilization, and laboratory safety. This is followed by a section on analytical techniques and applications, including mass spectrometry and the specialized topics of microfabrication and microfluidics, cytometry, and point-of-care testing. Next, all the major analytes in clinical chemistry, including enzymes, tumor markers, therapeutic drugs, and many others are discussed. Pathophysiology, covering disease states and malfunction of different organ systems that correlate with abnormal laboratory findings follows. A section on genetic metabolic testing discussing newborn screening and inborn error of metabolism is next. This is followed by a section dedicated to molecular diagnostics, perhaps the fastest growing field in laboratory medicine. Then, there is a section discussing automated hematology and white and red blood cell morphologies, as well as hemostasis and coagulation. Following this is coverage of clinical microbiology including antimicrobial stewardship and infection prevention, infectious disease, antimicrobial susceptibility, bacteriology, virology, mycobacteriology, mycology, and parasitology. A

transfusion medicine section then presents blood groups, blood components, indications for blood transfusion, and transfusion reactions. Finally, our last section focuses on clinical immunology including systemic autoimmune disease, transplantations, immunogenetics, allergy testing, immunogenicity of biologics, and primary and secondary immunodeficiencies. An appendix tabulates reference intervals for the clinical laboratory. The online version includes all of the above topics, whereas the print version is more selective to keep the tome manageable.

In addition to the above-mentioned chapters, the online version contains a wealth of other information including biochemical calculations, animation films to illustrate complex mechanisms, clinical cases, numerous atlases, podcasts, important documents, lecture series, adn adaptive learning courses.

This is an exciting time to be a laboratory medicine professional. Our aim in this book is to provide current scientific and practical knowledge to support laboratory professionals as a knowledge resource and an interface between science and technology on the one hand and the clinician and the patient on the other.

POINTS TO REMEMBER

- Laboratory medicine is a heterogeneous field with multiple disciplines including clinical chemistry, hematology and coagulation, clinical microbiology, molecular diagnostics, clinical immunology, and transfusion medicine.
- Laboratory medicine is a profession that has been shaped and defined by technology.
- Training of laboratory medicine specialists is heterogeneous and includes physicians and doctoral scientists in chemistry, pharmacy, biology, biochemistry, and microbiology.
- The role of clinical laboratory specialists evolved over time from analytically and technology focused to customer and patient centered.
- Clinical laboratory specialists are translational researchers who convert laboratory data to clinical knowledge.
- Career paths of clinical laboratory specialists are heterogeneous and include work in clinical laboratories and IVD and pharmaceutical industries.
- Clinical laboratory specialists must adhere to guiding principles of practicing the profession, which include maintaining confidentiality of medical information, using resources appropriately, abiding by codes of conduct, following ethical publishing rules, and managing and disclosing conflict of interest.

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MULTIPLE CHOICE QUESTIONS

1. In some countries, clinical chemistry encompasses multiple specialties. Which of the following is never included within clinical chemistry?
   a. Hematology
   b. Coagulation
   c. Therapeutic drug monitoring
   d. Cytology
   e. Serology
2. Which of the following statements is not part of the professional role of the clinical laboratory specialist?
   a. Develop and validate de novo laboratory tests to meet clinical needs
   b. Evaluate and characterize the analytical and clinical performance of laboratory tests
   c. Decide the pricing of the test and market laboratory services
   d. Present laboratory results to clinicians in an effective manner
   e. Determine cost-effectiveness and intrinsic value of laboratory tests
3. Each of the following guiding principles of practicing the profession is correct except
   a. Maintaining confidentiality of medical information
   b. Using resources appropriately
   c. Establishing strong ties with manufacturers
   d. Abiding by codes of conduct
   e. Following ethical publishing rules
4. Each of the following statements is correct except:
   a. Reviewer must excuse himself if he has a conflict of interest regarding the manuscript
   b. Reviewer should complete the review in a timely fashion
   c. Reviewer should contact the author if he has a question
   d. Reviewer should provide a thorough examination of the manuscript
   e. Reviewer should provide useful comments to author
5. Molecular diagnostics
   a. Is as old as clinical chemistry
   b. Focuses on long polymers of carbohydrates
   c. Has a long history of providing multiplex assays that translate to clinical practice
   d. Studies the quantity or sequence of nucleic acids
   e. Is none of the above
6. The following statements regarding the laboratory medicine director are correct except:
   a. Must have an MD, PhD, or a pharmacy degree, depending on the country
   b. Usually has undergone a minimum of 9 years of training
   c. Determine the strategic direction of the laboratory
   d. Assist physicians in test utilization
   e. Must be an expert in computer technology
7. Which of the following is not considered part of the role of a journal editor:
   a. Establishing conflict-of interest policy for editors
   b. Determining the direction of the journal
   c. Being responsible for the integrity of the overall review process
   d. Establishing the subscription price
   e. Developing journal policies
8. Which of the following is not considered a recognized discipline in laboratory medicine?
   a. Immunology
   b. Physiology
   c. Microbiology
   d. Hematology
   e. Clinical chemistry
9. Which of the following is not an important driver in transforming laboratory data to information and knowledge?
   a. Application of evidence-based medicine
   b. Application of information management technology
   c. Data mining
   d. Patient-focused laboratory medicine
   e. Technology-driven laboratory medicine
10. With respect to handling of patient information, it is inappropriate to
    a. Store and keep record of patient identifiers
    b. Publicly disclose without obtaining the patient's consent
    c. Store securely in the laboratory information system
    d. Monitor data access by laboratory personnel
    e. Include genetic information

2

Statistical Methodologies in Laboratory Medicine

Analytical and Clinical Evaluation of Laboratory Tests

Kristian Linnet, Karel G.M. Moons, and James Clark Boyd

ABSTRACT

Background

The careful selection and evaluation of laboratory tests are key steps in the process of implementing new measurement procedures in the laboratory for clinical use. Method evaluation in the clinical laboratory is complex and in most countries is a regulated process guided by various professional recommendations and quality standards on best laboratory practice.

Content

This chapter deals with the statistical aspects of both analytical and clinical evaluations of laboratory assays, tests, or markers. After a short overview on basic statistics, aspects such as accuracy, precision, trueness, limit of detection, and selectivity are considered in the first part. After dealing with comparison of assays in detail, including using difference plots and regression analysis, the focus is on quantification of the (added) diagnostic value of laboratory assays or tests. First, the evaluation of tests in isolation is outlined, which corresponds to simple diagnostic scenarios, when only a single test result is decisive (e.g., in the screening context). Subsequently, the chapter addresses the more common clinical situation in which a laboratory assay or test is considered as part of a diagnostic workup and thus a test's added value is at issue. This involves use of receiver operating characteristic (ROC) areas, reclassification measures, predictiveness curves, and decision curve analysis. Finally, principles for considering the clinical impact of diagnostic tests on actual decision making and patient outcomes are discussed.

ASSAY SELECTION OVERVIEW

The introduction of new or revised laboratory tests, markers, or assays is a common occurrence in the clinical laboratory. Test selection and evaluation are key steps in the process of implementing new measurement procedures (Fig. 2.1). A new or revised test must be selected carefully and its analytical and clinical performance evaluated thoroughly before it is adopted for routine use in patient care (see later in this chapter and Chapter 10). Establishment of a new or revised laboratory test may also involve evaluation of the features of the automated analyzer on which the test will be implemented. When a new test is to be introduced to the routine clinical laboratory, a series of technical or analytical evaluations is commonly conducted. Assay imprecision is estimated, and comparison of the new assay versus an existing one is commonly undertaken. The allowable measurement range is assessed with estimation of the lower and upper limits of quantification. Interferences and carryover are evaluated when relevant. Depending on the situation, a limited verification of manufacturer claims may be all that is necessary, or, in the case of a newly developed test or assay, a full validation may be carried out. Subsequent subsections provide details for all these test evaluations. With regard to evaluation of reference intervals or medical decision limits, readers are referred to Chapter 9.

Evaluation of tests, markers, or assays in the clinical laboratory is influenced strongly by guidelines and accreditation or other regulatory standards.[1–3] The Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards [NCCLS]) has published a series of consensus protocols (Clinical Laboratory Improvement Amendments [CLIAs]) for clinical chemistry laboratories and manufacturers to follow when evaluating methods (see the CLSI website at http://www.clsi.org). The International Organization for Standardization (ISO) has also developed several documents related to method evaluation (ISOs). In addition, meeting laboratory accreditation requirements has become an important aspect in the evaluation process with accrediting agencies placing increased focus on the importance of total quality management and assessment of trueness and precision of laboratory measurements. An accompanying trend has been the emergence of an international nomenclature to standardize the terminology used for characterizing laboratory test or assay performance.

This chapter presents an overview of considerations in and methods for the evaluation of laboratory tests. This includes explanation of graphical and statistical methods that are used to aid in the test evaluation process; examples of the application of these methods are provided, and current terminology within the area is summarized. Key terms and abbreviations are listed in Box 2.1.

Medical Need and Quality Goals

The selection of the appropriate clinical laboratory assays is a vital part of rendering optimal patient care. Advances

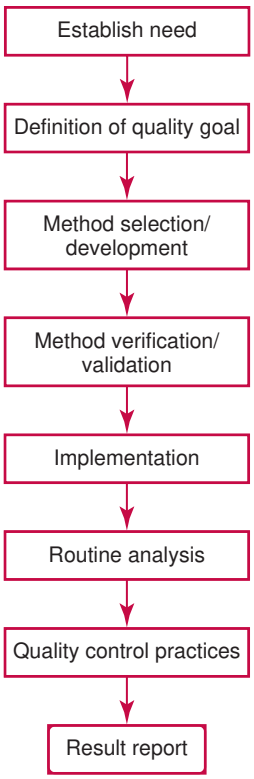

FIGURE 2.1 A flow diagram that illustrates the process of introducing a new assay into routine use.

in patient care are frequently based on the use of new or improved laboratory tests or measurements. Ascertainment of what is necessary clinically from a new or revised laboratory test is the first step in selecting the appropriate candidate test. Key parameters, such as desired turnaround time and necessary clinical utility for an assay, are often derived by discussions between laboratorians and clinicians. When new diagnostic assays are introduced, for example, reliable estimates of its diagnostic performance (e.g., predictive values, sensitivity and specificity) must be considered. With established analytes, a common scenario is the replacement of an older, labor-intensive test with a new, automated assay that is more economical in daily use. In these situations, consideration must be given to whether the candidate assay has sufficient precision, accuracy, analytical measurement range, and freedom from interference to provide clinically useful results (see Fig. 2.1).

Analytical Performance Criteria

In evaluation of a laboratory test, (1) trueness (formerly termed accuracy), (2) precision, (3) analytical range, (4) detection limit, and (5) analytical specificity are of prime importance. The sections in this chapter on laboratory test evaluation and comparison contain detailed outlines of these concepts. Estimated test performance parameters should be related to analytical performance specifications that ensure acceptable clinical use of the test and its results. For more details related to the recommended models for setting analytical performance specifications, readers are referred to Chapters 6 and 8. From a practical point of view, the "ruggedness" of the test in routine use is of importance and reliable performance, when used by different operators and with different batches of reagents over long time periods, is essential.

When a new laboratory analyzer is at issue, various instrumental parameters require evaluation, including (1) pipetting, (2) specimen-to-specimen carryover, (3) reagent lot-to-lot variation, (4) detector imprecision, (5) time to first reportable result, (6) onboard reagent stability, (7) overall throughput, (8) mean time between instrument failures, and (9) mean time to repair. Information on most of these parameters should be available from the instrument manufacturer; the manufacturer should also be able to furnish information on what studies should be conducted in estimating these parameters for an individual analyzer. Assessment of reagent lot-to-lot variation is especially difficult for a user, and the manufacturer should provide this information.

Other Criteria

Various categories of laboratory tests may be considered. New tests may require "in-house" development. (Note: Such a test is also referred to as a laboratory-developed test [LDT].) Commercial kit assays, on the other hand, are ready for implementation in the laboratory, often in a "closed" analytical system on a dedicated instrument. When prospective assays are reviewed, attention should be given to the following:

1. Principle of the test or assay, with original references
2. Detailed protocol for performing the test
3. Composition of reagents and reference materials, the quantities provided, and their storage requirements (e.g., space, temperature, light, humidity restrictions) applicable both before and after the original containers are opened
4. Stability of reagents and reference materials (e.g., their shelf lives)
5. Technologist time and required skills
6. Possible hazards and appropriate safety precautions according to relevant guidelines and legislation
7. Type, quantity, and disposal of waste generated
8. Specimen requirements (e.g., conditions for collection and transportation, specimen volume requirements, the necessity for anticoagulants and preservatives, necessary storage conditions)
9. Reference interval of the test and its results, including information on how such interval was derived, typical values obtained in both healthy and diseased individuals, and the necessity of determining a reference interval for one's own institution (see Chapter 9 for details on how to generate a reference interval of a laboratory test.)
10. Instrumental requirements and limitations
11. Cost-effectiveness
12. Computer platforms and interfacing with the laboratory information system
13. Availability of technical support, supplies, and service

Other questions concerning placement of the new or revised test in the laboratory should be taken into account. They include:

1. Does the laboratory possess the necessary measuring equipment? If not, is there sufficient space for a new instrument?
2. Does the projected workload match the capacity of a new instrument?
3. Is the test repertoire of a new instrument sufficient?
4. What is the method and frequency of (re)calibration?
5. Is staffing of the laboratory sufficient for the new technology?
6. If training the entire staff in a new technique is required, is such training worth the possible benefits?

BOX 2.1 Abbreviations and Vocabulary Concerning Technical Validation of Assays

Abbreviations

CI	Confidence interval
CV	Coefficient of variation (=SD/x, where x is the concentration)
CV%	= CV × 00%
CV_A	Analytical coefficient of variation
CV_G	Between-subject biological variation
CV_I	Within-subject biological variation
CV_{RB}	Sample-related random bias coefficient of variation
DoD	Distribution of differences (plot)
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
OLR	Ordinary least-squares regression analysis
SD	Standard deviation
SEM	Standard error of the mean ($5SD/\sqrt{N}$)
SD_A	Analytical standard deviation
SD_{RB}	Sample-related random bias standard deviation
x_m	Mean
x_{mv}	Weighted mean
WLR	Weighted least-squares regression analysis

Vocabulary[a]

Analyte Compound that is measured.

Bias Difference between the average (strictly the expectation) of the test results and an accepted reference value (ISO 3534-1). Bias is a measure of trueness.[11]

Certified reference material (CRM) is a reference material, one or more of whose property values are certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation that is issued by a certifying body.

Commutability Ability of a material to yield the same results of measurement by a given set of measurement procedures.

Limit of detection The lowest amount of analyte in a sample that can be detected but not quantified as an exact value. Also called lower limit of detection or minimum detectable concentration (or dose or value).[23]

Lower limit of quantification (LLOQ) The lowest concentration at which the measurement procedure fulfills specifications for imprecision and bias (corresponds to the *lower limit of determination* mentioned under *Measuring interval*).

Matrix All components of a material system except the analyte.

Measurand The "quantity" that is actually measured (e.g., the concentration of the analyte). For example, if the analyte is glucose, the measurand is the concentration of glucose. For an enzyme, the measurand may be the enzyme *activity* or the *mass concentration* of enzyme.

Measuring interval Closed interval of possible values allowed by a measurement procedure and delimited by the *lower limit of determination* and the *higher limit of determination*. For this interval, the total error of the measurements is within specified limits for the method. Also called the *analytical measurement range.*

Primary measurement standard Standard that is designated or widely acknowledged as having the highest metrologic qualities and whose value is accepted without reference to other standards of the same quantity.[73]

Quantity The amount of substance (e.g., the concentration of substance).

Random error Arises from unpredictable variations in influence quantities. These random effects give rise to variations in repeated observations of the measurand.

Reference material (RM) A material or substance, one or more properties of which are sufficiently well established to be used for the calibration of a method or for assigning values to materials.

Reference measurement procedure Thoroughly investigated measurement procedure shown to yield values having an uncertainty of measurement commensurate with its intended use, especially in assessing the trueness of other measurement procedures for the same quantity and in characterizing reference materials.

Selectivity or specificity Degree to which a method responds uniquely to the required analyte.

Systematic error A component of error that, in the course of a number of analyses of the same measurand, remains constant or varies in a predictable way.

Traceability "The property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties."[43] This is achieved by establishing a chain of calibrations leading to primary national or international standards, ideally (for long-term consistency) the Système International (SI) units of measurement.

Uncertainty A parameter associated with the result of a measurement that characterizes the dispersion of values that could reasonably be attributed to the measurand. More briefly, *uncertainty* is a parameter characterizing the range of values within which the value of the quantity being measured is expected to lie.

Upper limit of quantification (ULOQ) The highest concentration at which the measurement procedure fulfills specifications for imprecision and bias (corresponds to the *upper limit of determination* mentioned under *Measuring interval*).

[a]A listing of terms of relevance in relation to analytical methods is displayed. Many of the definitions originate from Dybkær[12] with statement of original source where relevant (e.g., International Organization for Standardization document number). Others are derived from the Eurachem/Citac guideline on uncertainty.[79] In some cases, slight modifications have been performed for the sake of simplicity.

7. How frequently will quality control (QC) samples be run?
8. What materials will be used to ensure QC?
9. What approach will be used for proficiency testing?
10. What is the estimated cost of performing an assay using the proposed method, including the costs of calibrators, QC specimens, and technologists' time? Questions applicable to implementation of new instrumentation in a particular laboratory may also be relevant. Does the instrument satisfy local electrical safety guidelines? What are the power, water, drainage, and air conditioning requirements of the instrument? If the instrument is large, does the floor have sufficient load-bearing capacity?

A qualitative assessment of all these factors is often completed, but it is possible to use a value scale to assign points to the various features weighted according to their relative importance; the latter approach allows a more quantitative test evaluation process. Decisions are then made regarding the assays that best fit the laboratory's requirements and that have the potential for achieving the necessary analytical quality for clinical use.

BASIC STATISTICS

In this section, fundamental statistical concepts and techniques are introduced in the context of typical analytical investigations. The basic concepts of (1) populations, (2) samples, (3) parameters, (4) statistics, and (5) probability distributions are defined and illustrated. Two important

probability distributions—Gaussian and Student *t*—are introduced and discussed.

Frequency Distribution

A graphical device for displaying a large set of laboratory test results is the *frequency distribution,* also called a *histogram*. Fig. 2.2 shows a frequency distribution displaying the results of serum gamma-glutamyltransferase (GGT) measurements of 100 apparently healthy 20- to 29-year-old men. The frequency distribution is constructed by dividing the measurement scale into cells of equal width; counting the number, n_i, of values that fall within each cell; and drawing a rectangle above each cell whose area (and height because the cell widths are all equal) is proportional to n_i. In this example, the selected cells were 5 to 9, 10 to 14, 15 to 19, 20 to 24, 25 to 29, and so on, with 60 to 64 being the last cell (range of values, 5 to 64 U/L). The ordinate axis of the frequency distribution gives the number of values falling within each cell. When this number is divided by the total number of values in the data set, the relative frequency in each cell is obtained.

Often, the position of the value for an individual within a distribution of values is useful medically. The *nonparametric* approach can be used to directly determine the *percentile* of a given subject. Having ranked N subjects according to their values, the n-percentile, Perc_n, may be estimated as the value of the $[N(n/100) + 0.5]$ ordered observation.[4] In the case of a noninteger value, interpolation is carried out between neighbor values. The 50th percentile is the median of the distribution.

Population and Sample

It is useful to obtain information and draw conclusions about the characteristics of the test results for one or more target populations. In the GGT example, interest is focused on the location and spread of the population of GGT values for 20- to 29-year-old healthy men. Thus a working definition of a *population* is the complete set of all observations that might occur as a result of performing a particular procedure according to specified conditions.

Most target populations of interest in clinical chemistry are in principle very large (millions of individuals) and so are impossible to study in their entirety. Usually a subgroup of observations is taken from the population as a basis for forming conclusions about population characteristics. The group of observations that has actually been selected from the population is called a *sample*. For example, the 100 GGT values make up a sample from a respective target population. However, a sample is used to study the characteristics of a population only if it has been properly selected. For instance, if the analyst is interested in the population of GGT values over various lots of materials and some time period, the sample must be selected to be representative of these factors, as well as of age, sex, and health factors of the individuals in the targeted population. Consequently, exact specification of the target population(s) is necessary before a plan for obtaining the sample(s) can be designed. In this chapter, a sample is also used as a specimen, depending on the context.

Probability and Probability Distributions

Consider again the frequency distribution in Fig. 2.2. In addition to the general location and spread of the GGT determinations, other useful information can be easily extracted from this frequency distribution. For instance, 96% (96 of 100) of the determinations are less than 55 U/L, and 91% (91 of 100) are greater than or equal to 10 but less than 50 U/L. Because the cell interval is 5 U/L in this example, statements such as these can be made only to the nearest 5 U/L. A larger sample would allow a smaller cell interval and more refined statements. For a sufficiently large sample, the cell interval can be made so small that the frequency distribution can be approximated by a continuous, smooth curve, similar to that shown in Fig. 2.3. In fact, if the sample is large enough, we can consider this a close representation of the "true" target *population frequency distribution*. In general, the functional form of the population frequency distribution curve of a variable x is denoted by $f(x)$.

The population frequency distribution allows us to make probability statements about the GGT of a randomly selected member of the population of healthy 20- to 29-year-old men. For example, the probability $\Pr(x > x_a)$ that the GGT value x of a randomly selected 20- to 29-year-old healthy man is greater than some particular value x_a is equal to the area under the population frequency distribution to the right of x_a. If $x_a = 58$, then from Fig. 2.3, $\Pr(x > 58) = 0.05$. Similarly, the probability $\Pr(x_a < x < x_b)$ that x is greater than x_a but less than x_b is equal to the area under the population frequency distribution between x_a and x_b. For example, if $x_a = 9$ and $x_b = 58$, then from Fig. 2.3, $\Pr(9 < x < 58) = 0.90$. Because the population frequency distribution provides all information related to probabilities of a randomly selected member of the population, it is called the probability distribution of the population. Although the true probability distribution is never exactly known in practice, it can be approximated with a large sample of observations, that is, test results.

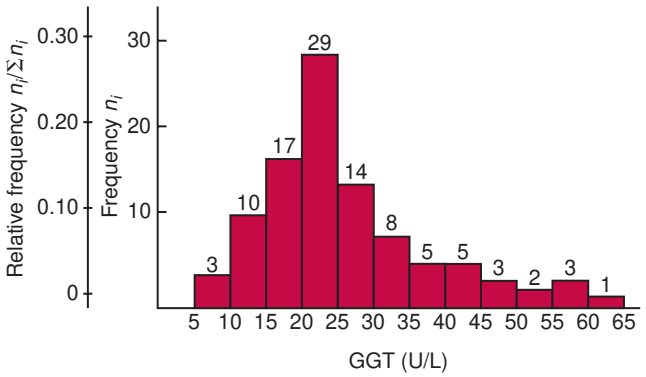

FIGURE 2.2 Frequency distribution of 100 gamma-glutamyltransferase *(GGT)* values.

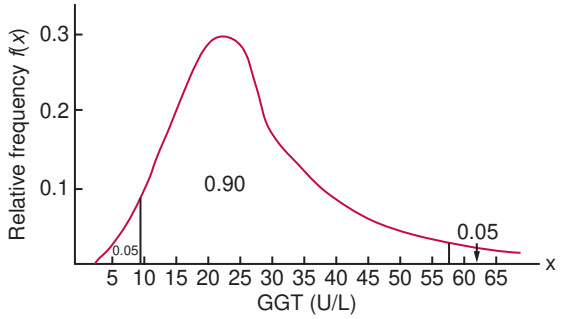

FIGURE 2.3 Population frequency distribution of gamma-glutamyltransferase *(GGT)* values.

Parameters: Descriptive Measures of a Population

Any population of values can be described by measures of its characteristics. A *parameter* is a constant that describes some particular characteristic of a population. Although most populations of interest in analytical work are infinite in size, for the following definitions, we shall consider the population to be of finite size N, where N is very large.

One important characteristic of a population is its *central location*. The parameter most commonly used to describe the central location of a population of N values is the *population mean* (μ):

$$\mu = \frac{\sum x_i}{N}$$

An alternative parameter that indicates the central tendency of a population is the *median,* which is defined as the 50th percentile, $Perc_{50}$.

Another important characteristic is the *dispersion* of values about the population mean. A parameter very useful in describing this dispersion of a population of N values is the *population variance* σ^2 (sigma squared):

$$\sigma^2 = \frac{\sum (x_i - \mu)^2}{N}$$

The *population standard deviation (SD)* σ, the positive square root of the population variance, is a parameter frequently used to describe the population dispersion in the same units (e.g., mg/dL) as the population values. For a Gaussian distribution, 95% of the population of values are located within the mean $\pm 1.96\ \sigma$. If a distribution is non-Gaussian (e.g., asymmetric), an alternative measure of dispersion based on the percentiles may be more appropriate, such as the distance between the 25th and 75th percentiles (the interquartile interval).

Statistics: Descriptive Measures of the Sample

As noted earlier, clinical chemists usually have at hand only a sample of observations (i.e., test results) from the overarching targeted population. A *statistic* is a value calculated from the observations in a sample to estimate a particular characteristic of the target population. As introduced earlier, the sample mean x_m is the arithmetical average of a sample, which is an estimate of μ. Likewise, the sample SD is an estimate of σ, and the coefficient of variation (CV) is the ratio of the SD to the mean multiplied by 100%. The equations used to calculate x_m, SD, and CV, respectively, are as follows:

$$x_m = \frac{\sum x_i}{N}$$

$$SD = \sqrt{\frac{\sum (x_i - x_m)^2}{N-1}} = \sqrt{\frac{\sum x_i^2 - \frac{(\sum x_i)^2}{N}}{N-1}}$$

$$CV = \frac{SD}{x_m} \times 100\%$$

where x_i is an individual measurement and N is the number of sample measurements.

The SD is an estimate of the dispersion of the distribution. Additionally, from the SD, we can derive an estimate of the uncertainty of x_m as an estimate of μ (see later discussion).

Random Sampling

A random sample of individuals from a target population is one in which each member of the population has an equal chance of being selected. A *random sample* is one in which each member of the sample can be considered to be a random selection from the target population. Although much of statistical analysis and interpretation depends on the assumption of a random sample from some population, actual data collection often does not satisfy this assumption. In particular, for sequentially generated data, it is often true that observations adjacent to each other tend to be more alike than observations separated in time.

The Gaussian Probability Distribution

The *Gaussian* probability distribution, illustrated in Fig. 2.4, is of fundamental importance in statistics for several reasons. As mentioned earlier, a particular test result x will not usually be equal to the true value μ of the specimen being measured. Rather, associated with this particular test result x will be a particular measurement error $\varepsilon = x - \mu$, which is the result of many contributing sources of error. Pure measurement errors tend to follow a probability distribution similar to that shown in Fig. 2.4, where the errors are symmetrically distributed, with smaller errors occurring more frequently than larger ones, and with an expected value of 0. This important fact is known as the central limit effect for distribution of errors: if a measurement error ε is the sum of many independent sources of error, such as $\varepsilon_1, \varepsilon_2, \ldots, \varepsilon_k$, several of which are major contributors, the probability distribution of the measurement error ε will tend to be Gaussian as the number of sources of error becomes large.

Another reason for the importance of the Gaussian probability distribution is that many statistical procedures are based on the assumption of a Gaussian distribution of values; this approach is commonly referred to as *parametric.* Furthermore, these procedures usually are not seriously invalidated by departures from this assumption. Finally, the magnitude of the uncertainty associated with sample statistics can be ascertained based on the fact that many sample statistics computed from large samples have a Gaussian probability distribution.

The Gaussian probability distribution is completely characterized by its mean μ and its variance σ^2. The notation

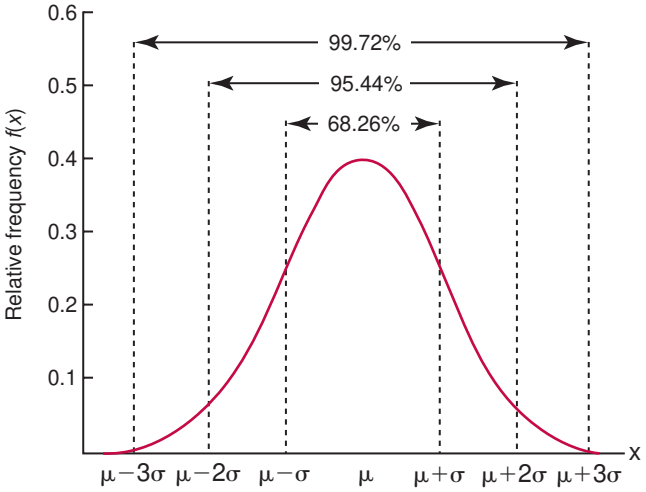

FIGURE 2.4 The Gaussian probability distribution.

$N(\mu, \sigma^2)$ is often used for the distribution of a variable that is Gaussian with mean μ and variance σ^2. Probability statements about a variable x that follows an $N(\mu, \sigma^2)$ distribution are usually made by considering the variable z,

$$z = \frac{x - \mu}{\sigma}$$

which is called the *standard Gaussian variable*. The variable z has a Gaussian probability distribution with $\mu = 0$ and $\sigma^2 = 1$, that is, z is $N(0, 1)$. The probably that x is within 2 σ of μ [i.e., $\Pr(|x - \mu| < 2\sigma) =$] is 0.9544. Most computer spreadsheet programs can calculate probabilities for all values of z.

Student *t* Probability Distribution

To determine probabilities associated with a Gaussian distribution, it is necessary to know the population SD σ. In actual practice, σ is often unknown, so we cannot calculate z. However, if a random sample can be taken from the Gaussian population, we can calculate the sample SD, substitute SD for σ, and compute the value *t:*

$$t = \frac{x - \mu}{\text{SD}}$$

Under these conditions, the variable t has a probability distribution called the *Student* t *distribution*. The t distribution is really a family of distributions depending on the degrees of freedom (df) ν $(= N - 1)$ for the sample SD. Several t distributions from this family are shown in Fig. 2.5. When the size of the sample and the df for SD are infinite, there is no uncertainty in SD, so the t distribution is identical to the standard Gaussian distribution. However, when the sample size is small, the uncertainty in SD causes the t distribution to have greater dispersion and heavier tails than the standard Gaussian distribution, as illustrated in Fig. 2.5. At sample sizes above 30, the difference between the t-distribution and the Gaussian distribution becomes relatively small and can usually be neglected. Most computer spreadsheet programs can calculate probabilities for all values of t, given the df for SD.

The Student t distribution is commonly used in significance tests, such as comparison of sample means, or in testing conducted if a regression slope differs significantly from 1. Descriptions of these tests can be found in statistics textbooks.[5] Another important application is the estimation of confidence intervals (CIs). CIs are intervals that indicate the uncertainty of a given sample estimate. For example, it can be proved that $X_m \pm t_{alpha}$ (SD/$N^{0.5}$) provides an approximate 2*alpha*-CI for the mean. A common value for *alpha* is 0.025 or 2.5%, which thus results in a 0.95% or 95% CI. Given sample sizes of 30 or higher, t_{alpha} is ca. 2. (SD/$N^{0.5}$) is called the standard error (SE) of the mean. A CI should be interpreted as follows. Suppose a sampling experiment of drawing 30 observations from a Gaussian population of values is repeated 100 times, and in each case, the 95% CI of the mean is calculated as described. Then, in 95% of the drawings, the true mean μ is included in the 95% CI. The popular interpretation is that for an estimated 95% CI, there is 95% chance that the true mean is within the interval. According to the central limit theorem, distributions of mean values converge toward the Gaussian distribution irrespective of the primary type of distribution of x. This means that the 95% CI is a robust estimate only minimally influenced by deviations from the Gaussian distribution. In the same way, the t-test is robust toward deviations from normality.

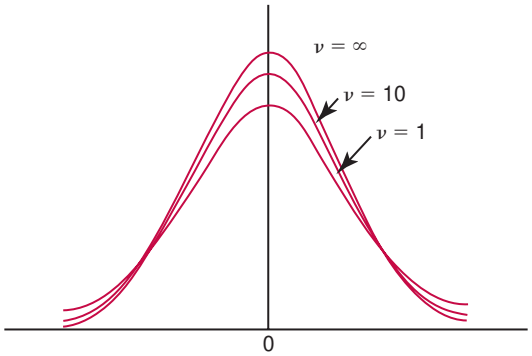

FIGURE 2.5 The t distribution for $\nu = 1$, 10, and ∞∞.

Nonparametric Statistics

Distribution-free statistics, often called nonparametric statistics, provides an alternative to parametric statistical procedures that assume data to have Gaussian distributions. For example, distributions of reference values are often skewed and so do not conform to the Gaussian distribution (see Chapter 9 on reference intervals). Formally, one can carry out a goodness of fit test to judge whether a distribution is Gaussian or not.[5] A commonly used test is the Kolmogorov-Smirnov test, in which the shape of the sample distribution is compared with the shape presumed for a Gaussian distribution. If the difference exceeds a given critical value, the hypothesis of a Gaussian distribution is rejected, and it is then appropriate to apply nonparametric statistics. A special problem is the occurrence of outliers (i.e., single measurements highly deviating from the remaining measurements). Outliers may rely on biological factors and so be of real significance (e.g., in the context of estimating reference intervals or be related to clerical errors). Special tests exist for handling outliers.[5]

Given that a distribution is non-Gaussian, it is appropriate to apply nonparametric descriptive statistics based on the percentile or quantile concept. As stated under the earlier section Frequency Distribution, the n-percentile, Perc_n, of a sample of N values may be estimated as the value of the $[N(n/100) + 0.5]$ ordered observation.[4] In the case of a noninteger value, interpolation is carried out between neighbor values. The median is the 50th percentile, which is used as a measure of the center of the distribution. For the GGT example mentioned previously, we would order the $N = 100$ values according to size. The median or 50th percentile is then the value of the $[100(50/100) + 0.5 = 50.5]$ ordered observation (the interpolated value between the 50th and 51st ordered values). The 2.5th and 97.5th percentiles are values of the $[100(2.5/100) + 0.5 = 3]$ and $[100(97.5/100) + 0.5 = 98]$ ordered observations, respectively. When a 95% reference interval is estimated, a nonparametric approach is often preferable because many distributions of reference values are asymmetric. Generally, distributions based on the many biological sources of variation are often non-Gaussian compared with distributions of pure measurement errors that usually are Gaussian.

The nonparametric counterpart to the t-test is the Mann-Whitney test, which provides a significance test for the difference

between median values of the two groups to be compared.[5] When there are more than two groups, the Kruskal-Wallis test can be applied.[5]

Categorical Variables

Hitherto focus has been on quantitative variables. When dealing with qualitative tests and in the context of evaluating diagnostic testing, categorical variables that only take the value positive or negative come into play. The performance is here given as proportions or percentages, which are proportions multiplied by 100. For example, the diagnostic sensitivity of a test is the proportion of diseased subjects who have a positive result. Having tested, for example, 100 patients, 80 might have had a positive test result. The sensitivity then is 0.8 or 80%. We are then interested in judging how precise this estimate is. Exact estimates of the uncertainty can be derived from the so-called binomial distribution, but for practical purposes, an approximate expression for the 95% CI is usually applied as the estimated proportion $P \pm 2\text{SE}$, where the SE in this context is derived as:

$$\text{SE} = [P(1 - P)/N]^{0.5}$$

where P is here a proportion and not a percentage.[5] In the example, the SE equals 0.0016 and so the 95% CI is 0.77 to 0.83 or 77 to 83%. The applied approximate formula for the SE is regarded as reasonably valid when NP and $N(1 - P)$ both are equal to or higher than 5.

POINTS TO REMEMBER

- Statistics as means, SDs, percentiles, proportions, and so on are computed from a sample of values drawn from a population and provide *estimates* of the unknown population characteristics.
- Whereas parametric statistics rely on the assumption of a Gaussian population of values, which typically applies for measurement errors, nonparametric statistics is a distribution-free approach that apply to, for example, asymmetric distributions often observed for biologic variables.
- The Gaussian distribution is characterized by the mean and the SD, and other types of distributions are described by the median and the percentile (quantile) values.
- Distributions of categorical variables are characterized by proportions or percentages and their SEs.

TECHNICAL VALIDITY OF ANALYTICAL ASSAYS

This section defines the basic concepts used in this chapter: (1) calibration, (2) trueness and accuracy, (3) precision, (4) linearity, (5) limit of detection (LOD), (6) limit of quantification, (7) specificity, and (8) others (see Box 2.1 for definitions).

Calibration

The calibration function is the relation between instrument signal *(y)* and concentration of analyte *(x)*, that is,

$$y = f(x)$$

The inverse of this function, also called the measuring function, yields the concentration from response:

$$x = f^{-1}(y)$$

This relationship is established by measurement of samples with known quantities of analyte[6] (calibrators). One may distinguish between solutions of pure chemical standards and samples with known quantities of analyte present in the typical matrix that is to be measured (e.g., human serum). The first situation applies typically to a reference measurement procedure that is not influenced by matrix effects; the second case corresponds typically to a routine method that often is influenced by matrix components and so preferably is calibrated using the relevant matrix.[7] Calibration functions may be linear or curved and, in the case of immunoassays, may often take a special form (e.g., modeled by the four-parameter logistic curve).[8] This model (logistic in log x) has been used for immunoassay techniques and is written in several forms (Table 2.1). An alternative, model-free approach is to estimate a smoothed spline curve, which often is performed for immunoassays; however, a disadvantage of the spline curve approach is that it is insensitive to aberrant calibration values, fitting these just as well as the correct values. If the assumed calibration function does not correctly reflect the true relationship between instrument response and analyte concentration, a systematic error or bias is likely to be associated with the analytical method. A common problem with some immunoassays is the "hook effect," which is a deviation from the expected calibration algorithm in the high-concentration range. (The hook effect is discussed in more detail in Chapter 26.)

The precision of the analytical method depends on the stability of the instrument response for a given quantity of analyte. In principle, a random dispersion of instrument signal (vertical direction) at a given true concentration transforms into dispersion on the measurement scale (horizontal direction), as is shown schematically (Fig. 2.6). The detailed statistical aspects of calibration are complex,[5,9] but in the following sections, some approximate relations are outlined. If the calibration function is linear and the imprecision of the signal response is the same over the analytical measurement range, the analytical SD (SD_A) of the method tends to be constant over the analytical measurement range (see Fig. 2.6). If the imprecision increases proportionally to the signal response, the analytical SD of the method tends to increase proportionally to the concentration *(x)*, which means that the *relative* imprecision ($\text{CV} = \text{SD}/x$) may be constant over the analytical measurement range if it is assumed that the intercept of the calibration line is zero.

With modern, automated clinical chemistry instruments, the relation between analyte concentration and signal can in some cases be very stable, and where this is the case, calibration is necessary relatively infrequently[10] (e.g., at intervals of

TABLE 2.1 The Four-Parameter Logistic Model Expressed in Three Different Forms

Algebraic Form	Variables[a]	Parameters[b]
$y = (a - d)/[1 + (x/c)^b] + d$	(x, y)	a, b, c, d
$R = R_0 + K_c/[1 + \exp(-\{a + b \log[C]\})]$	(C, R)	R_0, K_c, a, b
$y = y_0 + (y_\infty - y_0)(x^d)/(b + x^d)$	(x, y)	y_0, y_∞, b, d

[a]Concentration and instrument response variables shown in parentheses.
[b]Equivalent letters do not necessarily denote equivalent parameters.

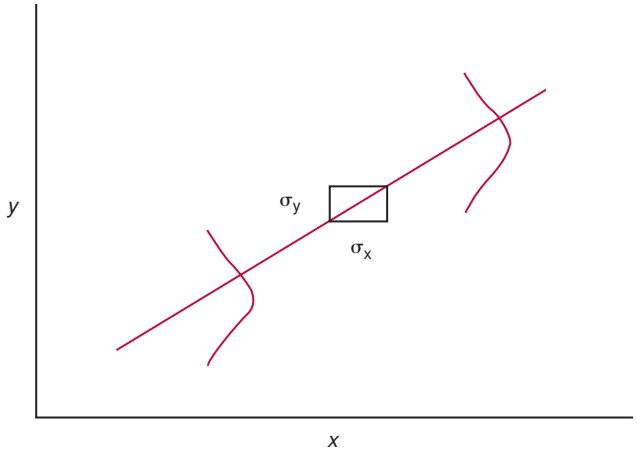

FIGURE 2.6 Relation between concentration *(x)* and signal response *(y)* for a linear calibration function. The dispersion in signal response (σ_y) is projected onto the *x*-axis and is called assay imprecision [σ_x ($=\sigma_A$)].

several months). Built-in process control mechanisms may help ensure that the relationship remains stable and may indicate when recalibration is necessary. In traditional chromatographic analysis (e.g., high-performance liquid chromatography [HPLC]), on the other hand, it is customary to calibrate each analytical series (run), which means that calibration is carried out daily.

Trueness and Accuracy

Trueness of measurements is defined as closeness of agreement between the average value obtained from a large series of results of measurements and the true value.[11]

The difference between the average value (strictly, the mathematical expectation) and the true value is the *bias,* which is expressed numerically and so is inversely related to the trueness. *Trueness* in itself is a qualitative term that can be expressed, for example, as low, medium, or high. From a theoretical point of view, the exact true value for a clinical sample is not available; instead, an "accepted reference value" is used, which is the "true" value that can be determined in practice.[12] Trueness can be evaluated by comparison of measurements by the new test and by some preselected reference measurement procedure, both on the same sample or individuals.

The ISO has introduced the trueness expression as a replacement for the term *accuracy,* which now has gained a slightly different meaning. *Accuracy* is the closeness of agreement between the result of a measurement and a true concentration of the analyte.[11] Accuracy thus is influenced by both bias and imprecision and in this way reflects the total error. Accuracy, which in itself is a qualitative term, is inversely related to the "uncertainty" of measurement, which can be quantified as described later (Table 2.2).

In relation to trueness, the concepts *recovery, drift,* and *carryover* may also be considered. *Recovery* is the fraction or percentage increase in concentration that is measured in relation to the amount added. Recovery experiments are typically carried out in the field of drug analysis. One may distinguish between *extraction recovery,* which often is interpreted as the fraction of compound that is carried through an extraction process, and the recovery measured by the entire analytical procedure, in which the addition of an internal standard

TABLE 2.2 An Overview of Qualitative Terms and Quantitative Measures Related to Method Performance

Qualitative Concept	Quantitative Measure
Trueness	Bias
Closeness of agreement of mean value with "true value"	A measure of the systematic error
Precision	Imprecision (SD)
Repeatability (within run)	A measure of the dispersion of random errors
Intermediate precision (long term)	
Reproducibility (inter-laboratory)	
Accuracy	*Error of measurement*
Closeness of agreement of a single measurement with "true value"	Comprises both random and systematic influences

SD, Standard deviation.

compensates for losses in the extraction procedure. A recovery close to 100% is a prerequisite for a high degree of trueness, but it does not ensure unbiased results because possible nonspecificity against matrix components (e.g., an interfering substance) is not detected in a recovery experiment. *Drift* is caused by instrument or reagent instability over time, so that calibration becomes gradually biased. Assay *carryover* also must be close to zero to ensure unbiased results. Carryover can be assessed by placing a sample with a known, low value after a pathological sample with a high value, and an observed increase can be stated as a percentage of the high value.[13] Drift or carryover or both may be conveniently estimated by multifactorial evaluation protocols (EPs).[14,15]

Precision

Precision has been defined as the closeness of agreement between independent replicate measurements obtained under stipulated conditions.[12] The degree of precision is usually expressed on the basis of statistical measures of imprecision, such as SD or CV (CV = SD/*x*, where *x* is the measurement concentration), which is inversely related to precision. Imprecision of measurements is solely related to the random error of measurements and has no relation to the trueness of measurements.

Precision is specified as follows[11,12]:

Repeatability: closeness of agreement between results of successive measurements carried out under the same conditions (i.e., corresponding to within-run precision)

Reproducibility: closeness of agreement between results of measurements performed under changed conditions of measurements (e.g., time, operators, calibrators, reagent lots). Two specifications of reproducibility are often used: total or between-run precision in the laboratory, often termed *intermediate precision,* and interlaboratory precision (e.g., as observed in external quality assessment schemes [EQAS]) (see Table 2.2).

The total SD (σ_T) may be divided into within-run and between-run components using the principle of analysis of variance of components[5] (variance is the squared SD):

$$\sigma^2 T = \sigma^2_{\text{Within-run}} + \sigma^2_{\text{Between-run}}$$

It is not always clear in clinical chemistry publications what is meant by "between-run" variation. Some authors use

the term to refer to the total variation of an assay, but others apply the term *between-run variance component* as defined earlier. The distinction between these definitions is important but is not always explicitly stated.

In laboratory studies of analytical variation, estimates of imprecision are obtained. The more observations, the more certain are the estimates. It is important to have an adequate number so that that analytical variation is not underestimated. Commonly, the number 20 is given as a reasonable number of observations (e.g., suggested in the CLSI guideline for manufacturers).[16] To verify method precision by users, it has been recommended to run internal QC samples for five consecutive days in five replicates.[17] If too few replications are applied, it is likely that the analytical variation will be underestimated.

To estimate both the within-run imprecision and the total imprecision, a common approach is to measure duplicate control samples in a series of runs. Suppose, for example, that a control is measured in duplicate for 20 runs, in which case 20 observations are present with respect to both components. The dispersion of the means (x_m) of the duplicates is given as follows:

$$\sigma^2_{x_m} = \sigma^2_{\text{Within-run}} / 2 + \sigma^2_{\text{Between-run}}$$

From the 20 sets of duplicates, we may derive the within-run SD using the following formula:

$$\text{SD}_{\text{Within-run}} = \left[\sum d_i^2 / (2 \times 20)^{0.5} \right]$$

where d_i refers to the difference between the ith set of duplicates. When SDs are estimated, the concept df is used. In a simple situation, the number of df equals $N - 1$. For N duplicates, the number of df is $N(2 - 1) = N$. Thus both variance components are derived in this way. The advantage of this approach is that the within-run estimate is based on several runs, so that an average estimate is obtained rather than only an estimate for one particular run if all 20 observations had been obtained in the same run. The described approach is a simple example of a *variance component analysis*. The principle can be extended to more components of variation. For example, in the CLSI EP05-A3 guideline,[16] a procedure is outlined that is based on the assumption of two analytical runs per day, in which case within-run, between-run, and between-day components of variance are estimated by a *nested* component of variance analysis approach.

Nothing definitive can be stated about the selected number of 20. Generally, the estimate of the imprecision improves as more observations become available. Exact confidence limits for the SD can be derived from the χ^2 distribution. Estimates of the variance, SD^2, are distributed according to the χ^2 distribution (tabulated in most statistics textbooks) as follows: $(N - 1)\,\text{SD}^2/\sigma^2 \approx \chi^2_{(N-1)}$, where $(N - 1)$ is the df.[5] Then the two-sided 95% CI is derived from the following relation:

$$\Pr\left[\chi^2_{97.5\%(N-1)} < (N-1)\text{SD}^2 / \sigma^2 < \chi^2_{2.5\%(N-1)} \right] = 0.95$$

which yields this 95% CI expression:

$$\text{SD} \times \left[(N-1) / \chi^2_{2.5\%(N-1)} \right]^{0.5} < \sigma < \text{SD} \times \left[(N-1) / \chi^2_{97.5\%(N-1)} \right]^{0.5}$$

Example

Suppose we have estimated the imprecision as an SD of 5.0 on the basis of $N = 20$ observations. From a table of the χ^2 distribution, we obtain the following 2.5 and 97.5 percentiles:

$$\chi^2_{2.5\%(19)} = 32.9 \text{ and } \chi^2_{97.5\%(19)} = 8.91$$

where 19 within the parentheses refers to the number of df. Substituting in the equation, we get

$$5.0 \times (19/32.9)^{0.5} < \sigma < 5.0 \times (19/8.91)^{0.5}$$

or

$$3.8 < \sigma < 7.3$$

A graphical display of 95% CIs at various sample sizes is shown in Fig. 2.7. For individual variance components, the relations are more complicated.

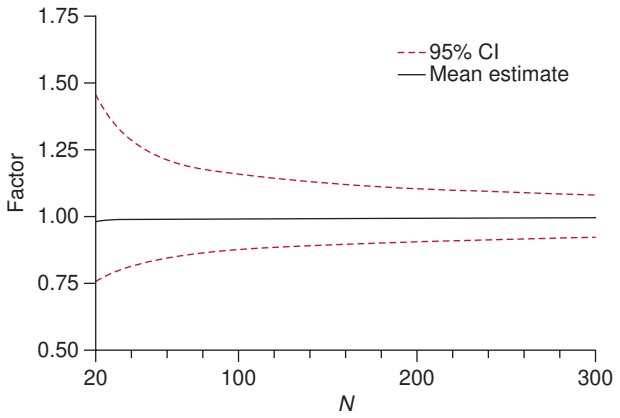

FIGURE 2.7 Relation between factors indicating the 95% confidence intervals *(CIs)* of standard deviations (SDs) and the sample size. The true SD is 1, and the solid line indicates the mean estimate, which is slightly downward biased at small sample sizes.

Precision Profile

Precision often depends on the concentration of analyte being considered. A presentation of precision as a function of analyte concentration is the precision profile, which usually is plotted in terms of the SD or the CV as a function of analyte concentration (Fig. 2.8). Some typical examples may be considered. First, the SD may be constant (i.e., independent of the concentration), as it often is for analytes with a limited range of values (e.g., electrolytes). When the SD is constant, the CV varies inversely with the concentration (i.e., it is high in the lower part of the range and low in the high range). For analytes with extended ranges (e.g., hormones), the SD frequently increases as the analyte concentration increases. If a proportional relationship exists, the CV is constant. This may often apply approximately over a large part of the analytical measurement range. Actually, this relationship is anticipated for measurement error that arises because of imprecise volume dispensing. Often a more complex relationship exists. Not infrequently, the SD is relatively constant in the low range, so that the CV increases in the area approaching the lower limit of quantification (LLOQ). At intermediate concentrations, the CV may be relatively constant and perhaps may decline somewhat at increasing concentrations. A square root relationship can be used to model the relationship in some situations as an intermediate form of relation between the constant and the proportional case. The relationship between the SD and the concentration is of importance (1) when method specifications over the analytical measurement

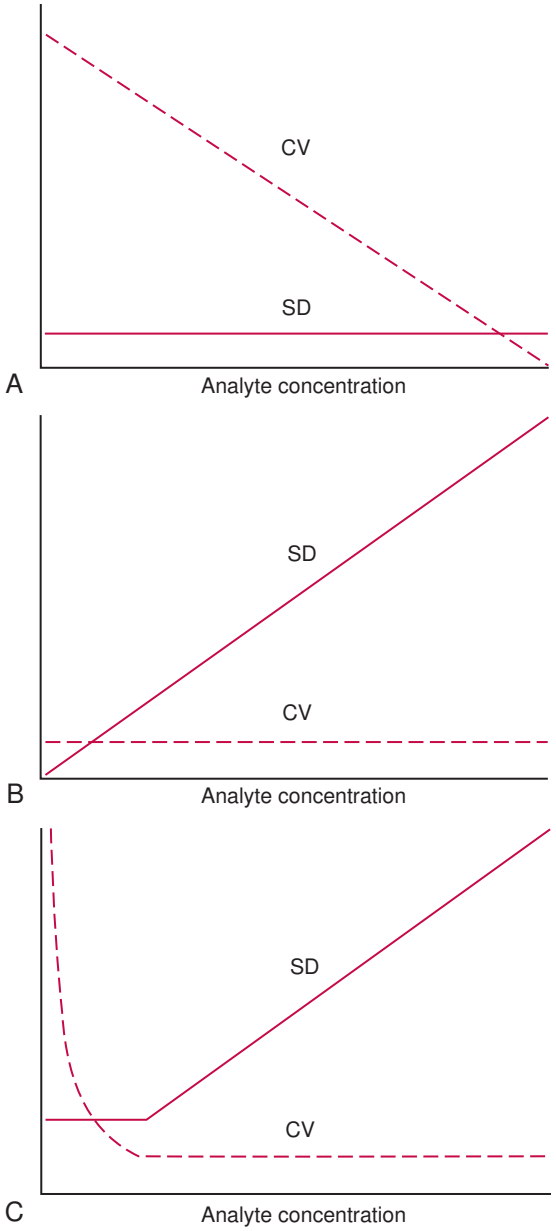

FIGURE 2.8 Relations between analyte concentration and standard deviation *(SD)*/coefficient of variation *(CV)*. **A,** The SD is constant, so that the CV varies inversely with the analyte concentration. **B,** The CV is constant because of a proportional relationship between concentration and SD. **C,** A mixed situation with constant SD in the low range and a proportional relationship in the rest of the analytical measurement range.

range are considered, (2) when limits of quantification are determined, and (3) in the context of selecting appropriate statistical methods for method comparison (e.g., whether a difference or a relative difference plot should be applied, whether a simple or a weighted regression analysis procedure should be used) (see the "Relative Distribution of Differences Plot" and "Regression Analysis" sections later).

Linearity

Linearity refers to the relationship between measured and expected values over the analytical measurement range. Linearity may be considered in relation to actual or relative analyte concentrations. In the latter case, a dilution series of a sample may be examined. This dilution series examines whether the measured concentration changes as expected according to the proportional relationship between samples introduced by the dilution factor. Dilution is usually carried out with an appropriate sample matrix (e.g., human serum [individual or pooled serum] or a verified sample diluent).

Evaluation of linearity may be conducted in various ways. A simple, but subjective, approach is to visually assess whether the relationship between measured and expected concentrations is linear. A more formal evaluation may be carried out on the basis of statistical tests. Various principles may be applied here. When repeated measurements are available at each concentration, the random variation between measurements and the variation around an estimated regression line may be evaluated statistically[18] (by an *F*-test). This approach has been criticized because it relates only the magnitudes of random and systematic error without taking the absolute deviations from linearity into account. For example, if the random variation among measurements is large, a given deviation from linearity may not be declared statistically significant. On the other hand, if the random measurement variation is small, even a very small deviation from linearity that may be clinically unimportant is declared significant. When significant nonlinearity is found, it may be useful to explore nonlinear alternatives to the linear regression line (i.e., polynomials of higher degrees).[19]

Another commonly applied approach for detecting nonlinearity is to assess the residuals of an estimated regression line and test whether positive and negative deviations are randomly distributed. This can be carried out by a runs test (see "Regression Analysis" section).[20] An additional consideration for evaluating proportional concentration relationships is whether an estimated regression line passes through zero or not. The presence of linearity is a prerequisite for a high degree of trueness. A CLSI guideline suggests procedure(s) for assessment of linearity.[21]

Analytical Measurement Range and Limits of Quantification

The analytical measurement range (measuring interval, reportable range) is the analyte concentration range over which measurements are within the declared tolerances for imprecision and bias of the method.[12] Taking drug assays as an example, there exist (arbitrary) requirements of a CV% of less than 15% and a bias of less than 15%.[22] The measurement range then extends from the lowest concentration (LLOQ) to the highest concentration (upper limit of quantification [ULOQ]) for which these performance specifications are fulfilled.

The LLOQ is medically important for many analytes. Thyroid-stimulating hormone (TSH) is a good example. As assay methods improved, lowering the LLOQ, low TSH results could be increasingly distinguished from the lower limit of the reference interval, making the test increasingly useful for the diagnosis of hyperthyroidism.

The LOD is another characteristic of an assay. The LOD may be defined as the lowest value that confidently exceeds the measurements of a blank sample. Thus the limit has been estimated on the basis of repeated measurements of a blank sample and has been *reported* as the mean plus 2 or 3 SDs of the blank measurements. In the interval from LOD up to LLOQ, one should report a result as "detected" but not

provide a quantitative result. More complicated approaches for estimation of the LOD have been suggested.[23]

Analytical Sensitivity

The LLOQ of an assay should not be confused with analytical sensitivity. That is defined as ability of an analytical method to assess small differences in the concentration of analyte.[6] The smaller the random variation of the instrument response and the steeper the slope of the calibration function at a given point, the better is the ability to distinguish small differences in analyte concentrations. In reality, analytical sensitivity depends on the precision of the method. The smallest difference that will be statistically significant equals $2\sqrt{2}\ SD_A$ at a 5% significance level. Historically, the meaning of the term *analytical sensitivity* has been the subject of much discussion.

Analytical Specificity and Interference

Analytical specificity is the ability of an assay procedure to determine the concentration of the target analyte without influence from potentially interfering substances or factors in the sample matrix (e.g., hyperlipemia, hemolysis, bilirubin, antibodies, other metabolic molecules, degradation products of the analyte, exogenous substances, anticoagulants). Interferences from hyperlipemia, hemolysis, and bilirubin are generally concentration dependent and can be quantified as a function of the concentration of the interfering compound.[24] In the context of a drug assay, specificity in relation to drug metabolites is relevant, and in some cases, it is desirable to measure the parent drug, as well as metabolites. A detailed protocol for evaluation of interference has been published by the CLSI.[25]

POINTS TO REMEMBER

- Technical validation of analytical methods focuses on (1) calibration, (2) trueness and accuracy, (3) precision, (4) linearity, (5) LOD, (6) limit of quantification, (7) specificity, and (8) others.
- The difference between the average measured value and the true value is the *bias*, which can be evaluated by comparison of measurements by the new test and by some preselected reference measurement procedure, both on the same sample or individuals.
- The degree of precision is usually expressed on the basis of statistical measures of imprecision, such as SD or CV (CV = SD/x, where x is the measurement concentration).
- The measurement range extends from the lowest concentration (LLOQ) to the highest concentration (ULOQ) for which the analytical performance specifications are fulfilled (imprecision, bias).
- Analytical specificity is the ability of an assay procedure to determine the concentration of the target analyte without influence from potentially interfering substances or factors in the sample matrix.

QUALITATIVE METHODS

Qualitative methods, which currently are gaining increased use in the form of point-of-care testing (POCT), are designed to distinguish between results below and above a predefined cutoff value. Note that the cutoff point should not be confused with the detection limit. These tests are assessed primarily on the basis of their ability to correctly classify results in relation to the cutoff value.

Diagnostic Accuracy Measures

The probability of classifying a result as positive (exceeding the cutoff) when the true value indeed exceeds the cutoff is called *sensitivity*. The probability of classifying a result as negative (below the cutoff) when the true value indeed is below the cutoff is termed *specificity*. Determination of sensitivity and specificity is based on comparison of test results with a gold standard. The gold standard may be an independent test that measures the same analyte, but it may also be a clinical diagnosis determined by definitive clinical methods (e.g., radiographic testing, follow-up, outcomes analysis). Determination of these performance measures is covered later on in the diagnostic testing part. Sensitivity and specificity may be given as a fraction or as a percentage after multiplication by 100. SEs of estimates are derived as described for categorical variables. The performance of two qualitative tests applied in the same groups of nondiseased and diseased subjects can be compared using the McNemar's test, which is based on a comparison of paired values of true and false-positive (FP) or false-negative (FN) results.[26]

One approach for determining the recorded performance of a test in terms of sensitivity and specificity is to determine the true concentration of analyte using an independent reference method. The closer the concentration is to the cutoff point, the larger the error frequencies are expected to be. Actually, the cutoff point is defined in such a way that for samples having a true concentration exactly equal to the cutoff point, 50% of results will be positive, and 50% will be negative.[27] Concentrations above and below the cutoff point at which repeated results are 95% positive or 95% negative, respectively, have been called the "95% interval" for the cutoff point for that method, which indicates a grey zone where the test does not provide reliable results (Fig. 2.9).[27,28]

Agreement Between Qualitative Tests

As outlined previously, if the outcome of a qualitative test can be related to a true analyte concentration or a definitive

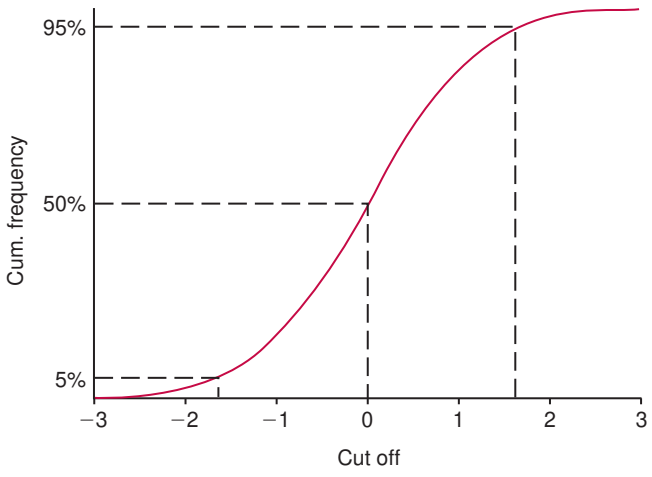

FIGURE 2.9 Cumulative frequency distribution of positive results. The x-axis indicates concentrations standardized to zero at the cutoff point (50% positive results) with unit standard deviation.

TABLE 2.3 2 × 2 Table for Assessing Agreement Between Two Qualitative Tests

		TEST 1	
		+	−
Test 2	+	a	b
	−	c	d
Total		a + c	b + d

TABLE 2.4 2 × 2 Table With Example of Agreement of Data for Two Qualitative Tests

		TEST 1		
		+	−	Total
Test 2	+	60	20	80
	−	15	40	55
Total		75	60	135

clinical diagnosis, it is relatively straightforward to express the performance in terms of clinical specificity and sensitivity. In the absence of a definitive reference or "gold standard," one should be cautious with regard to judgments on performance. In this situation, it is primarily *agreement* with another test that can be assessed. When replacement of an old or expensive routine assay with a new or less expensive assay is considered, it is of interest to know whether similar test results are likely to be obtained. If both assays are imperfect, however, it is not possible to judge which test has the better performance unless additional testing by a reference procedure is carried out.

In a comparison study, the same individuals are tested by both methods to prevent bias associated with selection of patients. Basically, the outcome of the comparison study should be presented in the form of a 2 × 2 table, from which various measures of agreement may be derived (Table 2.3). An obvious measure of agreement is the overall fraction or percentage of subjects tested who have the same test result (i.e., both results negative or positive):

$$\text{Overall percent agreement} = (a + d)/(a + b + c + d) \times 100\%$$

If agreement differs with respect to diseased and healthy individuals, the overall percent agreement measure becomes dependent on disease prevalence in the studied group of subjects. This is a common situation; accordingly, it may be desirable to separate this overall agreement measure into agreement concerning negative and positive results:

$$\text{Percent agreement given test 1 positive: } a/(a + c)$$

$$\text{Percent agreement given test 1 negative: } b/(b + d)$$

For example, if there is a close agreement with regard to positive results, overall agreement will be high when the fraction of diseased subjects is high; however, in a screening situation with very low disease prevalence, overall agreement will mainly depend on agreement with regard to negative results.

A problem with the simple agreement measures is that they do not take agreement by chance into account. Given independence, expected proportions observed in fields of the 2 × 2 table are obtained by multiplication of the fraction's negative and positive results for each test. Concerning agreement, it is excess agreement beyond chance that is of interest. More sophisticated measures have been introduced to account for this aspect. The most well-known measure is kappa, which is defined generally as the ratio of observed excess agreement beyond chance to maximum possible excess agreement beyond chance.[29] We have the following:

$$\text{Kappa} = (I_o - I_e)/(1 - I_e)$$

where I_o is the observed index of agreement and I_e is the expected agreement from chance. Given complete agreement, kappa equals +1. If observed agreement is greater than or equal to chance agreement, kappa is larger than or equal to zero. Observed agreement less than chance yields a negative kappa value.

Example

Table 2.4 shows a hypothetical example of observed numbers in a 2 × 2 table. The proportion of positive results for test 1 is 75/(75 + 60) = 0.555, and for test 2, it is 80/(80 + 55) = 0.593. Thus by chance, we expect the ++ pattern in 0.555 × 0.593 × 135 = 44.44 cases. Analogously, the —pattern is expected in (1 − 0.555) × (1 − 0.593) × 135 = 24.45 cases. The expected overall agreement percent by chance I_e is (44.44 + 24.45)/135 = 0.51. The observed overall percent agreement is I_o = (60 + 40)/135 = 0.74. Thus we have

$$\text{Kappa} = (0.74 - 0.51)/(1 - 0.51) = 0.47$$

Generally, kappa values greater than 0.75 are taken to indicate excellent agreement beyond chance, values from 0.40 to 0.75 are regarded as showing fair to good agreement beyond chance, and values below 0.40 indicate poor agreement beyond chance. An SE for the kappa estimate can be computed.[29] Kappa is related to the intraclass correlation coefficient, which is a widely used measure of interrater reliability for quantitative measurements.[29] The considered agreement measures, percent agreement, and kappa can also be applied to assess the reproducibility of a qualitative test when the test is applied twice in a given context.

Various methodological problems are encountered in studies on qualitative tests. An obvious mistake is to let the result of the test being evaluated contribute to the diagnostic classification of subjects being tested (circular argument). This is also termed *incorporation bias*.[30,31] Another problem is partial as opposed to complete verification. When a new test is compared with an existing, imperfect test, a partial verification is sometimes undertaken, in which only discrepant results are subjected to further testing by a perfect test procedure. On this basis, sensitivity and specificity are reported for the new test. This procedure (called *discrepant resolution*) leads to biased estimates and should not be accepted.[30–33] The problem is that for cases with agreement, both the existing (imperfect) test and the new test may be wrong. Thus only a measure of agreement should be reported, not specificity and sensitivity values. In the biostatistical literature, various procedures have been suggested to correct for bias caused by imperfect reference tests, but unrealistic assumptions concerning the independence of test results are usually put forward.