CHAPTER 1

# INTRODUCTION TO ASSAY DEVELOPMENT

IN THIS chapter, we first give definitions of the assay and the bioassay. Drug discovery and development processes are then reviewed to show the role bioassay plays in this process. Because drug development is performed in regulated environments, brief discussion of regulations is given. In pharmaceutical research, a drug substance's characterization involves physiochemical characterization and bioassays. Bioassays also play a significant role in screening for potential drug candidates. While physiochemical characterization is a direct measurement, bioassay is an indirect measurement. Because bioassay is indirect, the relevance of the assay to its intended purpose is a significant factor for bioassay development. Finally, common bioassay categories are discussed.



## 1.1 ASSAY AND BIOASSAY

### 1.1.1 Definitions

An assay is a well-defined analytical method that contains the measurement procedure and how the measurement should be interpreted to obtain the properties of a system or object. Assays are very important tools in the pharmaceutical industry and in the medical diagnostics industry. A bioassay is defined as an assay that measures biological activity of a substance based on the response of a biological test system to the test substance. In the pharmaceutical industry, bioassays are commonly applied to characterize a substance's biological properties, to study a biological process, to detect the presence and quantity of a substance in a sample, and to screen for active molecules from a library of molecules. Before a substance is approved for human use, it has to be fully characterized. The characterization of a substance requires the determination of its physiochemical properties by physiochemical assays (characterization) and the determination of its biological activities by bioassays. The physiochemical properties of a drug substances include its chemical composition, chemical structure, solubility, particle size, crystal property, purity, and the like. With biological drug substances (such as proteins), additional physiochemical properties, such as

*Assay Development: Fundamentals and Practices*. By Ge Wu

amino acid sequence, modifications (phosphorylation, glycolation, etc.), and tertiary or higher structure may need to be determined as well. Physiochemical properties of a substance can be directly assayed by studying the drug substance alone using well-established physical and chemical techniques, such as high-performance liquid chromatography (HPLC), mass spectrum, nuclear magnetic resonance (NMR), X-ray crystallography, amino acid sequencing, and so forth. In contrast, the biological activity of a substance by definition is the effect of the substance on a biological test system. Thus, a substance's biological activity cannot be measured by studying the drug substance alone. A biological test system is required for a bioassay. The biological test system can be (1) biochemical, such as the activity of an enzyme or the ability to bind to a predefined protein; (2) cell based, such as isolated primary cells or transformed cell lines; (3) tissue or organ based; and (4) animal based. Due to the diversity of the choices of the biological test system, a variety of a bioassay can be developed for a given project. The most common bioassays are biochemical assays with isolated proteins and cell-based assays. To study a substance, several bioassays can be configured in either biochemical assay format or cell-based format. In biochemical assay, a substance's binding to a protein or the substance's effects on the protein's enzymatic activity can be measured. In cell-based assays, the substance's effects on cell morphology, cell cycle, total number of cells, modification and localization of intracellular proteins, the identity and the quantity of proteins secreted by the cells, transcription activity, or the beating rate and strength of isolated cardiomyocytes can be measured. It is challenging to pick a bioassay system that is best suited for a particular study from many potential bioassays.

### 1.1.2 Comparison of Physiochemical Measurement and Bioassay

Figure 1.1 shows the difference in direct physiochemical measurements and the indirect bioassays. Physiochemical properties of a substance can be measured by directly

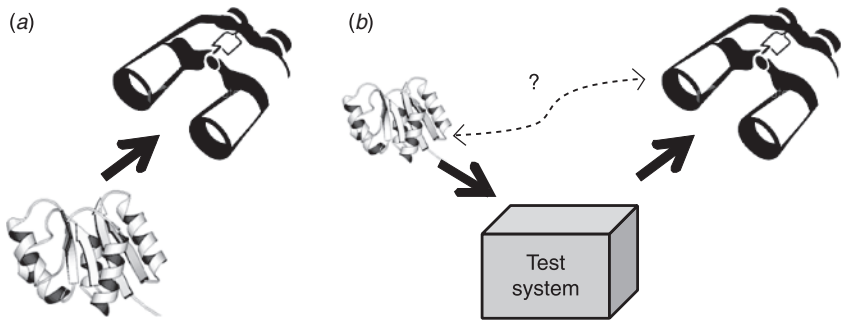


Figure 1.1 Comparison between direct physiochemical measurement and indirect bioassay. (*a*) A substance's physiochemical properties can be directly measured using HPLC, mass spectrum, NMR, IR, etc. (*b*) A substance's bioactivity can only be indirectly measured with a predefined biological test system. In bioassays, observations are made to the test system instead of to the substance. The biological activity of the substance is inferred from the observed changes in the test system based on prior knowledge about the test system.

analyzing the substance with one or more analytical instruments. An expert in a particular analytical method usually can make accurate conclusions on the aspect of the particular physiochemical measurement. For example, the composition of an organic substance can be readily obtained using well-established elemental analysis for carbon, hydrogen, nitrogen, and oxygen. A crystallographer armed with well-established analysis software can obtain three-dimensional structure of a substance with high-quality crystals. Physiochemical measurement is usually performed on a well-defined substance either in its pure form or in a formulation. It is an absolute measurement and a reference material usually is not required. Physiochemical measurements of a substance are commonly used in the quality control of drug manufacturing process and in the monitoring of drug distribution in the body. In contrast to physiochemical measurement, bioassay measures the responses of a test system to an external stimulation. A substance's bioactivity can only be indirectly measured by its effect on a predefined biological test system. The observations are made to the test system instead of to the substance. The biological activity of the substance is then inferred from the observed changes in the test system based on prior knowledge about the test system. Bioassay is commonly used in screening unknown molecules to discover active molecules and in accessing the known biological activity of the drug. To develop a bioassay, the assay developer has to first establish a test system and decide which response from the test system is relevant and how to interpret the response. Because the values of biological responses are relative, a control, which is a substance known to have an effect on the system, is required in bioassays. One characteristic of bioassay is the high sensitivity that substance at subnanomolar concentration can be readily detected with many well-designed bioassays. This is due to the fact that (1) the assay designer can choose a measurement among many responses in a bioassay system; (2) the test system can be manipulated to employ chemical/biological amplification (different from electronic amplification that raise the signal and background with the same amplitude); and (3) the most sensitive detection technology can be used. In comparison, the measurement of a substance's physiochemical properties in physiochemical assay usually requires the substance at a concentration of more than tens of micromolar (with the exception of mass spectrum technology).

### 1.1.3 Biological Relevance versus Experimental Control, Complexity, and Data Quality

For any type of measurement, it is most desirable to have full control of the system so that the system can be set at any predefined experimental conditions. A response from a system can only be measured after perturbations to the system are made. To obtain reliable interpretation about the responses from a system, bioassay scientists usually only vary one parameter at a time. All the responses from individual perturbation of the system are then recorded and synthesized to reach a conclusion. If more than two conditions are varied simultaneously, the interpretation of the experimental results will be difficult. Full control of a test system is not always possible. Physical scientists, such as physicists and chemists, have the luxury to vary experimental conditions in their system almost at will (only limited by existing technology and imagination). In contrast, sociology is at the other extreme: No changes can be made to a society

in order to study a hypothesis. Sociologists can only observe the existing society or study its past to draw conclusions. Between the two extremes is life science in terms of experimental control. We can pick a system over which we have almost full control (purified stable proteins), a system over which we have some control (live cells and animals), or a system over which we have almost no control (human). The "biological relevance" limits full control over cells or animals used in the biological assays. Biological relevance means how much the test system resembles the system in its native state. Dramatically exerting controls over live entities (such as cells or animals) may make the measurement irrelevant. Protein kinase A (PKA) is used below as an example to illustrate different test systems. The hypothetical goal is to find a molecule that will inhibit PKA in humans.

PKA is a tetramer that comprises two regulatory subunits that are regulated by adenosine cyclic 3′,5′-phosphate (cAMP) and two catalytic subunits that catalyze the transfer of the phosphate group from adenosine 5′-triphosphate (ATP) to its protein (or peptide) substrate. The tetramer is inactive. The regulatory subunits dissociate from the catalytic subunits upon cAMP binding. The free catalytic subunit is active. For the purpose of finding an inhibitor for PKA, the simplest study system is the isolated catalytic subunits of PKA. The catalytic subunit of PKA has two binding pockets, one for ATP and one for a protein substrate. The simplest assay for the catalytic subunit of PKA is the competitive binding assay. The assay can be designed to screen a compound library for compounds that bind to the catalytic subunit resulting in interfering with the binding of ATP or the substrate protein to PKA. The assay developer can exert a great level of controls on this simple assay system: The assay can be carried out in the presence or absence of ATP and the substrate; the assay can be performed at any concentrations of ATP or the substrate if they are present; and the assay can be done at any temperature, pH, and any salt concentration (before it denatures). With this much control, the assay is easy to perform. However, this is the least biologically relevant system, and the resulting inhibitors will be of less value compared to the other systems. Only competitive inhibitors that bind to the binding sites of either ATP or the protein substrate can be identified in this assay. Other types of PKA inhibitors will be missed in this assay (see Chapter 3). The functional assay for PKA's ability to phosphorylate a peptide substrate is a step closer to biological relevance. The transfer of the phosphate group from ATP to a peptide substrate is a reaction with a net loss of free energy. This means as long as there is sufficient ATP and peptide substrate, the active kinase will continue to turn the peptide substrate into a phosphorylated product. With one step closer to biological relevance, we lose some controls of our experimental conditions in this assay. To keep the kinase functional, the experiment has to be done in conditions with the following constraints: a small temperature range ($\sim$20–37°C); the presence of $Mg^{2+}$ or $Mn^{2+}$ in a small concentration range ($\sim$1–20 mM); the presence of ATP and peptide substrate; and a narrow pH range for PKA to be active. With this functional assay, all inhibitors that can affect the kinetics of PKA-catalyzed phosphorylation of the peptide substrate can be detected. Though the functional phosphorylation assay system is a step closer to native biological system than the binding assay, it is still far less biologically relevant because the peptide substrate is not the native substrate(s) for PKA in cells and the isolated catalytic subunits of PKA is not properly regulation. In addition, the ATP concentration in the artificial functional assay may be different from what it is in the native system (the cells).

The next step closer to physiological conditions is the cell-based assay in which the activities of PKA in live cells are measured by the phosphorylation of PKA's native substrates. In assays using cells as test systems, more experimental controls are lost. Cells can only grow in conditions with a very tight range of pH, temperature, and oxygen. In addition, the phosphorylation of the native protein substrates inside the cells can only occur with fixed ion species, at fixed ionic and ATP concentration maintained by the cells. Varying these conditions will destroy the assay system (the cells). Furthermore, test compounds have to be able to pass cell membrane to exert direct effect on PKA. After the test compounds pass the cell membrane, they may act on some other proteins that indirectly influence the PKA's activity. The interpretation of experimental data is more difficult in complicated uncontrolled biological systems. One of the major challenges in cell-based assays due to the loss of control is that many cell types require undefined media containing serum to survive. In this case, uncontrollable unknown matrix contain hundreds of thousands of substances (the serum), which must be present to maintain the survival of the test systems, can cause significant variations between assays at different times. However, the results obtained from these cell-based assays are more biologically relevant because the proteins are in a native state at native concentrations and are properly regulated by other interacting proteins.

The relationship among physiological relevance, assay complexity, controllability of the assay system, throughput, quantitation, and data quality for bioassays performed with different assay systems are summarized in Figure 1.2. Isolated proteins were the most used bioassay systems. Binding assays and enzymatic activity assays using proteins as the test systems are the simplest bioassay, and they were the

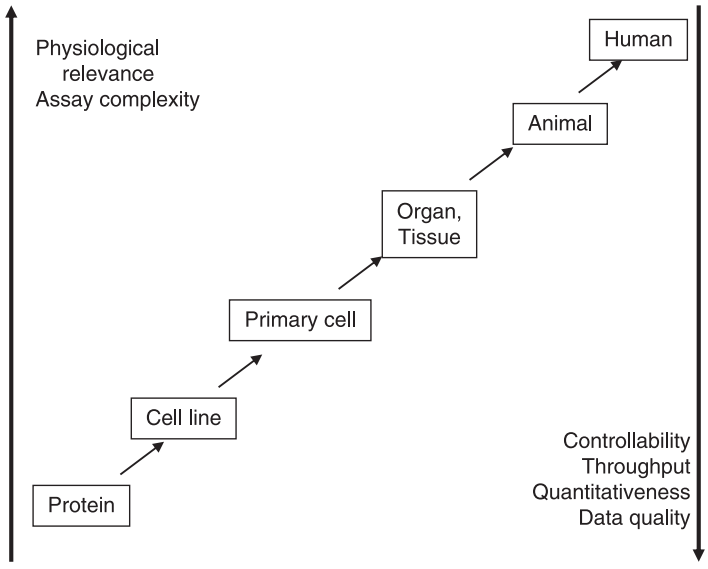


Figure 1.2 Relationship among physiological relevance, assay complexity, controllability, throughput, quantitation, and data quality for bioassays performed with different biological assay system. From Protein to Human, the physiological relevance and the assay complexity increase while the experimental control, assay throughput, ability to obtain quantitative data, and the quality of the acquired data decreases.

predominant assay format in the early day of bioassays. In modern drug discovery research, a drug target (a protein) is first identified and isolated. Molecules that can interact with the drug target are obtained by bioassays. Even with these simple protein assay systems, only a small portion of proteins (enzymes) is fully characterized before they are used as assay systems. The uncertainty in the test system will cause uncertainty in the assay outcome. Bioassays with transformed cell lines as assay systems are becoming more common recently because it is a step closer to biological relevance than isolated proteins. Transformed cell lines can be obtained in large quantities with homogeneous populations for large-scale screening, and they are easy to maintain. One advantage of using cell lines as assay systems is that many controls can be exerted on cell lines. Their genome can be readily manipulated to change the cells' characteristics to fit specific assay needs. With the advance of modern molecular biology, specific genes can be inserted or deleted from cells to make the cells gain or lose specific functions. However, the transformed cells may loss many properties of the primary cells from which they were derived. This makes the assay less biologically relevant compared with primary cells. Primary cells, especially human primary cells, are difficult to obtain in large quantity and cannot be easily manipulated. In addition, primary cells vary a lot among donors, which can cause large variations in assays. Because of these issues, primary cells are rarely used in primary screening. Cell-based assays are much more complicated than protein-based assays. Due to the complexity of the cell, it is safe to say that no cell is fully characterized. In addition, which response of the cell is relevant to the study and how the response is related to the study are not trivial to decide in a study. Further up the biological relevance ladder is to use tissue as a bioassay system in assays. Tissues preserve the interaction of cells with their native matrix environment, which is disrupted when cells are isolated. In some cases the isolated cells will not function well, and tissue or cells in artificial cell matrix should be used in bioassays. Beta cells in islet and chondrocytes in three-dimensional matrices are examples of such situations. Preserving these cells' native function is very challenging. Minimum control of the experimental condition can be exerted on these assay systems.

The next level of assay system based on biological relevance is the organ. There are a few examples of bioassays using organs as assay systems. For example, isolated animal heart has been used to perform bioassays. Whole animals are rarely used in initial bioassay, though they have been used extensively in preclinical research to investigate the toxicity and efficacy of drug candidates. Limited control can be imposed on these animals, though modern technology has allowed the creation of transgenic and knockout species. There are some attempts to use whole zebra fish as a bioassay system in initial compound screening. This approach still remains to be judged when more data is available. The advantage of using animals as test systems is that the results will be physiologically relevant. The disadvantage is that the results will be less quantitative and there is less control of the assay system. It is difficult to set up a predefined condition in an animal in order to measure the changes caused by an outside perturbation.

Because of less experimental control and the high cost associated with assay systems higher than tissues in terms of the biological relevance, these complicated bioassay systems are reserved for late-stage studies with a handful of compounds that have passed the hurdles in early-stage studies. The relatively simple biochemical

assays with isolated proteins and cell-based assays are the most common assays in early drug discovery research. Because many bioassays may exist for a given project, picking the right bioassay system is very important. For example, in the drug discovery phase, it is more important to find a chemical entity that is physiologically relevant than to be quantitatively accurate about its potency. Serious consideration may be given to more complicated bioassay systems depending on the stage to which the molecule has advanced. In the drug development phase, especially when the study is under regulated environments, quality of data is very important. A simple bioassay system is preferred whenever possible if it is appropriate for the study. Biochemical assays are more quantitative and are amenable to accessing the quality of a product and product-related variants. Since a majority of bioassays in pharmaceutical research and development are biochemical and cell-based assays, this book will focus on these two bioassay test systems. Furthermore, the terms assay and bioassay will be used interchangeably from here since most of the assays we will discuss are bioassays.

## 1.2 DRUG DISCOVERY PROCESS AND ROLE OF ASSAYS IN THE PROCESS

To understand the role assays play in the pharmaceutical industry, it is important to first understand the process to obtain approved drugs. This process can be divided into the drug discovery phase and the drug development phase.

### 1.2.1 Drug Discovery Phase

The first phase in the drug life cycle is the drug discovery phase. In this phase, one or more drug candidates for a particular disease are identified. In the early days, most drugs were discovered by chance or luck when scientists in academic research laboratories investigating particular diseases accidentally found the drugs. This model of drug discovery still exists, but it only accounts for a small fraction of drug candidates moving into the preclinical development stage. Most pharmaceutical and biotechnology companies follow more systematic methods to obtain drug candidates. The companies first identify the disease area they want to pursue based on a combination of factors, such as potential market size of the disease, expertise in the disease area, and tractable target for the disease. This is followed by thorough investigation of the disease by studying the literature and performing some key experiments to identify one or more disease targets. The disease targets can be an enzyme that is over- or less reactive, a receptor that is over- or less responsive to its ligand, a ligand for a cell receptor that is at too high or too low a concentration, a component molecule in a signal transduction pathway that can be modified to balance the abnormal signal coming from upstream diseased proteins, and the like. Some examples of drug targets are discussed below.

Gleevec (imatinib), the first approved drug for chronic myelogenous leukemia (CML) based on a kinase inhibitor, is a good example of selecting an overactive enzyme as the drug target. It was found that 95% of people with CML have a chromosomal abnormality called Philadelphia translocation. In this case, part of the *BCR*

(breakpoint cluster region) gene from chromosome 22 (region q11) is fused with part of the *ABL* gene on chromosome 9 (region q34). The fused *bcr-abl* gene codes for a protein that has tyrosine kinase activity. The *bcr-abl* transcript is constitutively active. It activates a number of cell cycle-controlling proteins and inhibits DNA (deoxyribonucleic acid) repairs. Thus, it is reasonable to hypothesize that inhibiting the kinase activity of BCR-ABL may offer a cure for CML. Thus, BCR-ABL was identified as the disease target for CML.

Tumor necrosis factor-alpha (TNFα) and its membrane-associated receptors offer a good example for drugs targeting ligand or its receptor. TNFα is a cytokine produced by monocytes and macrophages. TNF receptors are found on the surface of virtually all nucleated cells. TNFα mediates the immune response by increasing the transport of white blood cells to sites of inflammation, and through additional molecular mechanisms that initiate and amplify inflammation. Thus, it is reasonable to propose that interrupting the binding between TNFα and its membrane-bound receptor may offer a way to treat inflammatory disease, such as rheumatoid arthritis. Currently, there are three approved drugs for rheumatoid arthritis that block the activity of TNFα ligand: adalimumab (Humira), etanercept (Enbrel), and infliximab (Remicade). There is no drug targeting TNF receptor yet. This may be due to the fact that TNF receptors are expressed in too many cell types and its inhibition may lead to unwanted side effects.

In another scenario, if the disease target at the protein level cannot be identified, the cells involved in the diseases can serve as the disease target in the initial drug discovery effort. For example, scientists have tried to find drugs that stop B cells from causing inflammation. B cells cause joint inflammation in people with rheumatoid arthritis, though the detailed mechanism is not clear. Thus, it is reasonable to hypothesize that reducing the number of B cells in the body may reduce inflammation. A recently approved drug, rituximab, intercepts B cells and stops them from completing their tasks. Several other approaches to stopping B cells are under investigation. One investigational drug, belimumab, is a fully human monoclonal antibody that specifically recognizes and inhibits the biological activity of B-lymphocyte stimulator (BlyS). In this case, the protein target (BlyS) is known because BlyS is necessary for the maturation of B lymphocytes. In another example, isolated cancer cells can be used as the disease target for screening compounds that can selectively kill the cancer cells.

After identification of the tractable disease target, the next task is to identify molecules that can interact with the disease target. There are two prevailing strategies in the current drug discovery paradigm. One strategy is to physically test a large library of compounds against the disease target with one or more predefined bioassay. This process is referred to as screening. The molecules that produce signals that meet the assay criteria are called "primary hit" or "hit." Since the late 1980s, most pharmaceutical companies have built a collection of molecules (or "library") that is tested against the disease targets with predefined assays. The compound library may range from a few hundred thousands to several million compounds. When the assay throughput is high enough, the process is called high-throughput screening (HTS), which will be discussed in Chapter 13. Molecules that interact with proteins or with diseased cells can be obtained through HTS. Many newly discovered drugs were the results of

significant contributions from HTS, such as Gleevec to treat CML. The other strategy is to use computational methods (or virtual screening) to identify molecules that interact with the disease target. This strategy requires the identification of the protein as the disease target and the knowledge of the target protein's structure. Many drugs were discovered successfully using this method, especially in the discovery of HIV (human immunodeficiency virus) protease inhibitors and reverse transcriptase inhibitors. The discovery of the HIV protease inhibitor darunavir (Prezista) is a good example of this approach. However, there are many limitations for computational methods. One is the availability of the crystal structure of the target protein. Further, a crystal structure does not always accurately depict how a molecule will behave in vivo. In addition, medicinal chemists often found it difficult to develop new structures for the "rational" approach. Computational methods have limited use in finding molecules that act on diseased cells. HTS remains the only way to find molecules that act on diseased cells. The advantage of the computation method is the large number of potential small molecules it can test and the resource and money needed to test the molecules. It is estimated that there are $>10^{30}$ conceivable compounds in the chemical space with molecular weight less than 500. It is not practical to physically screen every disease target with a library approaching 10 million compounds. Even 10 million is only a small number compared with the enormous chemical space. One strategy is to limit the potential pool of molecules going to HTS by first filtering out unlikely drug molecules through computation. Another approach is to build targeted screening libraries for specific drug targets.

After a collection of "hit" molecules is identified, the structure–activity relationship (SAR) is evaluated if the drug target is a known protein. This includes the determination of $IC_{50}$ values of a series of inhibitors and the characteristics of the inhibition (reversibility, binding kinetics, inhibition mechanism, etc.). In addition to studying the hit molecule's interaction with the disease target, other properties of the hit molecule are further evaluated in vitro to determine whether it meets initial criteria as a drug. This includes the molecule's solubility in aqueous solution, octanol–water partition (log *P*), permeability to biomembranes, and so forth. Since a majority of clinical drug candidates failed because of their cardiotoxicity and hepatotoxicity, initial in vitro toxicity evaluations are sometimes performed at this stage to avoid costly failure in late stage. For example, the interaction of a hit molecule with hERG potassium channels may hint at cardiotoxicity, and the interaction of the hit molecule with different forms of cytochrome C isozymes may indicate potential hepatotoxicity. Furthermore, the compounds may be tested with tissues or intact organs in drug discovery phase. Only a limited number of the compounds survived this phase. The survivors are further moved to the preclinical stage. The drug discovery phase is very dynamic and new ways of doing research are constantly evolving. It is expected that with the rapid advancement of understanding of human biology, new approaches to drug discovery will certainly emerge in the future.

### 1.2.2 Drug Development Phase and Regulations

The drug discovery phase only shows that a substance can interact with the disease target protein or diseased cells. There is no information about whether the substance

can be successfully delivered to the target protein in vivo, how long the substance remains in the body after administration to exert its effect, whether there is a disease modifying effect or not, and whether there are toxic side effects on the whole body. These questions are addressed in the drug development phase. To protect human safety, it is required by regulations that necessary safety information be collected from animal studies before any new substance is introduced to humans. Thus, drug development is divided into preclinical (animal study) and clinical (human) phases. The studies in the drug development phase must meet the regulatory requirements and they must be carried out by closely following regulatory rules and guidelines. The regulations have evolved slowly compared with the rapid advance of science and technology. This is understandable since the regulations' primary goal is to protect public health. However, sometimes it is difficult to obtain clear regulatory guidance when working with cutting edge technologies. In such cases, frequent communications with the regulatory authority is important.

Unlike in the drug discovery phase, there is less freedom to the approaches a researcher can adopt in the drug development phase. The drug development phase follows a process that is guided by government regulations and international standards. In the following discussion of preclinical and clinical development, related regulatory articles in the United States and international standards are cited so that the reader can further study the rules if in-depth knowledge is desired. In the United States, the Code of Federal Regulation (CFR) Title 21 deals with food and drug regulations. The Food and Drug Administration (FDA) in the United States publishes guidelines to further explain and clarify the regulations. Because drugs are marketed in different countries, there is a need for internationally recognized standard and harmonized regulations. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is a project that brings together the regulatory authorities of Europe, Japan, and the United States and experts from the pharmaceutical industry in the three regions to discuss scientific and technical aspects of product registration. The objectives of ICH are the more economical use of human, animal, and material resources, the elimination of unnecessary delay in the global development and availability of new medicines while maintaining safeguards on quality, safety, and efficacy, and regulatory obligations to protect public health. ICH publishes many guidelines that are divided into four major topics: Quality (Q), Safety (S), Efficacy (E), and Multidisciplinary (M).

Good laboratory practice (GLP), good clinical research practice (GCP), and good manufacturing practice (GMP) are managerial and regulation concepts dealing with the process and conditions under which laboratory studies, clinical research studies, and manufacturing are planned, performed, recorded, and reported. The basic philosophy for GxP (GLP, GCP, and GMP) is that the laboratory or the manufacturing facility should design and perform studies or manufacturing processes carefully. All activities should be performed according to predefined standard operating procedure (SOP) and documented in such a way that studies can be reconstructed in the future. At different drug development stages, different GxPs are applied as shown in Figure 1.3. GLP is applied in safety assessment of a drug's pharmacology, pharmacokinetics, and toxicity in animal studies. GCP is applied in all three phases of clinical studies to protect human subjects. GMP is applied to both the manufacturing

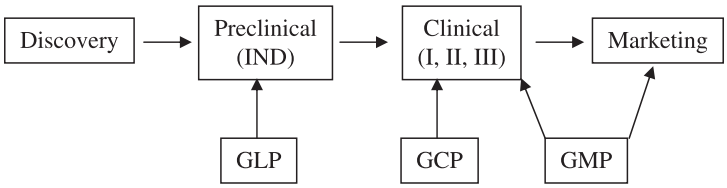


Figure 1.3 Required applications of GxP at different phases of drug development. There is no regulation in the drug discovery phase. GLP is applied in safety assessment of a drug's pharmacology, pharmacokinetics, and toxicity in animal studies. GCP is applied in all three phases of clinical studies to protect human subjects. GMP is applied to both the manufacturing of the reagents used in clinical testing and the manufacturing of the final marketed product after the approval of the drug.

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During the *preclinical development* phase the drug candidate's initial safety and activity profile is investigated to support the investigational new drug (IND) application filing. The studies in this phase will determine the route of injection, duration, and total exposure in clinical patients based on pharmacological and toxicological evaluations. Biological activity of the substance may be evaluated using in vitro assays to determine which effects of the substance may be related to clinical activity. In vitro assays with cell lines derived from mammalian cells can also be used to predict specific aspects of in vivo activity and to assess quantitatively the relative sensitivity of various species. This study can assist in the selection of an appropriate animal species for further in vivo pharmacology and toxicology studies. The primary goals of preclinical safety evaluations are: (1) to identify an initial safe dose and subsequent dose escalation schemes in humans; (2) to identify potential target organs for toxicity and for the study of whether such toxicity is reversible; and (3) to identify safety parameters for clinical monitoring. Regulations require a set of tests being performed on animals to collect necessary information before the substance is introduced into the human body in clinical trials. At present, these studies have to be done with animals, though the industry and regulatory body have been working hard to find substitute methodology. In the United States, federal regulations on IND can be found in the Code of Federal Regulation Title 21 Part 312 (21 CFR 312). The IND submission for Phase I clinical study is required to contain the following sections:

**A.** Cover sheet
**B.** Table of contents
**C.** Introductory statement and general investigational plan
**D.** Investigator's brochure
**E.** Protocols
**F.** Chemistry, manufacturing, and control (CMC) information
**G.** Pharmacology and toxicology information
**H.** Previous human experience with the investigational drug

The most important section of the IND filing is the substance's pharmacology and toxicity profile. FDA guideline on pharmacology specifically indicates that "this section should contain, if known, (1) a description of the pharmacologic effects and mechanism of actions of the drug in animal, and (2) information on the absorption, distribution, metabolism, and excretions of the drug." The first part of pharmacology is a pharmacodynamic study that deals with how the drug interacts with its target cell or organ and how it exerts its effects or side effects. The second part of pharmacology involves pharmacokinetic studies that gather the data on the substance's absorption, distribution, metabolism, and excretion (ADME). The study of a substance's pharmacological effect (efficacy) and the mechanism of action in the preclinical stage may be important to address safety issues and may assist in the evaluation of toxicity data. However, the lack of efficacy information in IND filing will not cause a Phase I clinical hold. In vivo efficacy study of a substance requires the availability of the animal model for a particular disease. The lack of good animal model for a given disease is a major obstacle for new drug discovery and development. In contrast, in vivo pharmacokinetics and toxicity studies of a substance can be investigated with normal animals.

Toxicity studies gather information that is used to determine the safety range of dosing for phase I clinical studies in humans. ICH guideline M3 titled *Non-clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals* recommends the following basic required tests for preclinical development:

> The non-clinical safety study recommendations for the marketing approval of a pharmaceutical usually include single and repeated dose toxicity studies, reproduction toxicity studies, genotoxicity studies, local tolerance studies and for drugs that have special cause for concern or are intended for a long duration of use, an assessment of carcinogenic potential. Other non-clinical studies include pharmacology studies for safety assessment (safety pharmacology) and pharmacokinetic (ADME) studies.

Toxicity and pharmacokinetic studies are usually performed simultaneously with normal health animals, typically with at least two different species. Rodents are used for early studies and more expensive nonhuman primates are used in later studies when large amounts of data have been already collected from studying rodents. Acute and chronic toxicity studies are usually performed. With acute toxicity studies, animals are administrated with a single does of substance with different doses for different groups of animals. The animals are sacrificed a few days after administration and the organs are analyzed. In the same experiment, pharmacokinetic data can be collected too. In a chronic toxicity study, one or more administration of the substance to the animal per day is done, and the dosing lasts several weeks to several months with different doses for different dosing groups. At the end of the dosing schedule, the animals are sacrificed and the organs are analyzed. Again, pharmacokinetic data can be obtained in the same experiments. The IND-enabling toxicity studies should be performed in compliance with GLP. GLP is a quality system concerned with the organizational process and the conditions under which nonclinical health and environmental safety studies are planned, performed, monitored, recorded, archived, and reported. GLP regulations for the pharmaceutical industry is covered by Code

of Federal Regulation Title 21 Part 58 (21 CFR 58). Though an unaudited draft report is accepted for IND filing, fully quality-assured documents must be available to the FDA, upon request, within 120 days of the start of human studies.

Preclinical development of biotechnology-derived pharmaceuticals (biopharmaceuticals) follows the general guidelines discussed above. However, special properties of biopharmaceuticals require different treatment in some areas from their small-molecule counterpart. ICH guideline S6 titled *Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals* provides general principles for designing scientifically acceptable preclinical safety evaluation programs. The biological activity together with species and/or tissue specificity of many biopharmaceuticals often preclude standard toxicity testing designs in commonly used species (e.g., rats and dogs) for small-molecule drug development. It is important to select relevant animal species for toxicity testing. A relevant species is one in which the test material is pharmacologically active due to the expression of the receptor or an epitope of monoclonal antibodies. Biopharmaceuticals intended for humans are often derived from the source with human origins. Thus, they may be immunogenic in animals used in preclinical testing. The antibody detected in animals associated with the administration of biopharmaceuticals may complicate the toxicology studies. Some of the standard toxicity tests that are routinely performed with small-molecule substances are not applicable to biopharmaceuticals and are not needed, such as metabolic study of the substance's biotransformation, genotoxicity studies, and carcinogenicity studies. There is a significant difference in the physical properties between biopharmaceuticals and small molecules. The safety concerns may arise from the impurities and contaminants in the biopharmaceuticals because they are derived from host cells, such as bacteria, yeast, insect, plant, and mammalian cells. Thus, the product that is used in the IND-enabling pharmacology and toxicology studies should be comparable to the product proposed for the initial clinical studies. Though IND-enabling toxicity studies should be performed in compliance with GLP, some non-GLP compliant specialized tests needed for biopharmaceuticals are acceptable to regulatory agency. Areas of noncompliance should be identified and their significance evaluated relative to the overall safety assessment.

Another important part in IND filing is the CMC section. The emphasis in Phase I CMC submission is placed on providing information to assure the proper identification, quality, purity, and strength of the investigational drug that will allow evaluation of the safety of subjects in the proposed study. For preclinical studies to be useful in assuring the safety of human studies, the drug product being proposed for use in a clinical study must be able to relate to the drug product used in the animal toxicity studies. The quantity of the drug candidates required for preclinical study is in several orders of magnitude more than what is required in the drug discovery phase. In the drug discovery phase, the quantity of a drug candidate used in studies, usually for physiochemical and biological characterization, is relatively small (on the order of a few grams). A bench chemist can make the neccessary quantity of small molecules in a flask and a biologist can make such quantities of proteins with several cell culture flasks. In preclinical studies, a gram or more of the drug may be administered to one animal in a single does. Multiply this by repeated doses, the hundreds of animals in one study, and the numbers of different studies, several hundred grams or

even several kilograms of the drug may be needed in the preclinical study. Thus, the substance used in preclinical testing may come from different batches of production. At this stage, the production group also constantly improves the process aimed at more efficient production processes and even larger production quantities for future clinical testing. Production process modification may result in large changes in biological activity between different batches of the substance, even though there is no detectable change in physiochemical properties. The problem is more prevalent for protein production than for small molecules. For protein production, many potential changes, such as degree of denaturization, different stable forms, posttranslational modification (phosphorylation, glycosylation, acylation, prenylation, methylation, etc.), can affect biological activity. For small molecules, the major activity changes may come from the different solid forms of the substance (polymorphs, amorphous, solvate, salt, and co-crystal). It is crucial to have good communication between material production groups and the preclinical testing groups so that any changes in the production process are taken into account in the interpretation of unexpected experimental results. The production process developed in preclinical phase should be quite stable or be similar to the substance production process for clinical testing in order to obtain consistent results and protect the safety of human subjects in clinical testing. Though different formulations of a drug may be used during drug development phases, links between formulations must be established by bioequivalence studies to allow interpretation of the preclinical and clinical study results. The substance used in the preclinical stage is not required by regulation to be manufactured by a GMP-approved facility. However, a GMP-produced substance is required at the clinical stage by FDA regulation. It will be of advantage if the substance is produced in a GMP facility in the late-stage preclinical testing to smooth the progress to clinical testing. The FDA guidelines suggest the following information being provided for review of the manufacturing procedures for drug products used in Phase I clinical studies.

1. Chemistry and manufacturing introduction
   a. Potential risk from the chemistry or the manufacturing of the drug substance or the drug product
   b. Chemistry and manufacturing difference between drug product for clinical use and the drug product used in the animal toxicity study
2. Drug substance
   a. Description of the drug substance (physical, chemical, biological)
   b. Manufacturer of the clinical drug substance
   c. Method of preparation of the drug substance
   d. Analytical methods to assure identity, strength, quality, and purity of the drug substance and acceptable limits
   e. Stability of the drug substance during the toxicology studies and proposed clinical studies
3. Drug product: Similar requirement as outlined in guideline 2 above
4. A brief general description of the composition, manufacture, and control of any placebo to be used in the proposed clinical trial

5. A copy of all labels and labeling to be provided to each investigator
6. A claim for categorical exclusion from or submission of an environmental assessment

The *drug substance* mentioned above means an active pharmaceutical ingredient (API) that is intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease or to affect the structure or any function of the human body, but does not include intermediates used in the synthesis of such ingredient. The *drug product* means a finished dosage form that contains a drug substance in association with one or more other ingredients (tablet, capsule, or solution).

The *clinical development* phase is aimed at determining the drug candidate's dosage range, safety profile, ADME, clinical end point, and efficacy. Clinical development is generally divided into three consecutive phases (Phase I, II, and III) to support the filing of a new drug to the regulatory agencies for marketing. In addition, the FDA Amendments Act of 2007, which went into effect on October 1, gives FDA the power to require drug makers to do postmarketing clinical trials (Phase IV). The phase concept is a description but not a set of requirement. The logic behind serial studies is that the emerging data from prior studies will guide the planning of later studies. This will also minimize the risk for trial subjects. Each phase of the clinical studies has a general objective: Phase I, human pharmacology; Phase II, exploratory therapeutics; and Phase III, confirmatory therapeutics. There are many individual studies in each phase of the clinical trials to answer different questions. Each individual study should contain objectives, design, conduct, analysis, and report. Dose–response information of the drug should be obtained at all stages of the development. In the United States, an NDA is filed with the Center for Drug Evaluation and Research (CDER) and a Biological License Application (BLA) is filed with Center for Biologics Evaluation and Research (CBER). Detailed format and contents requirements on NDA filing can be found in the Code of Federal Regulation Title 21 Part 314 (21 CFR 314) and BLA filing can be found in the Code of Federal Regulation Title 21 Part 601 (21 CFR 601). The drug used in the clinical phase is required by regulation to be manufactured by a GMP-approved facility. GMP is a part of quality assurance, which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use. Regulations for pharmaceutical GMP can be found in 21 CFR 210–226. ICH also published Q7: "Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients." In addition, the conduct of clinical research is highly regulated by federal regulations in the United States and throughout the world. ICH published guidelines for clinical trials in publication E8: "General Considerations for Clinical Trials," which describe the principle and practice in the conduct of both individual clinical trials and overall development strategy for an investigational drug. In clinical development, GCP must be followed. GCP is an international ethical and scientific quality standard for the design, conduct, performance, monitoring, auditing, recording, analyses, and reporting of clinical trials that provides assurance that the data and reported results are credible and accurate, and that the rights, integrity, and confidentiality of trial subjects are protected. GCP guideline is published in ICH publications E6: "Good Clinical Practice: Consolidated Guideline."

In the United States, the Phase I clinical trial is initiated 30 days after the submission of IND to FDA unless the FDA puts a clinical hold because of toxicology concerns or questions about the study design. The objectives of Phase I clinical development are typically nontherapeutic but are aimed at establishing the pharmacological and the toxicological properties of the drug candidate in humans and to determine the tolerability of the dose range to be used for later clinical studies. The emphasis here is to establish the drug candidate's safety characteristics. A small number of healthy volunteers or patients (20–100) are tested in this phase. The studies typically include both single and multiple dose administrations. Dose range and dose scheduling are commonly studied. For safety reasons, doses below the proposed treatment level are usually tested first and the dose is increased over time. Studies in this phase can be open, baseline controlled, or double blinded. The duration of clinical Phase I studies on average takes up to 1 year.

The primary objective of Phase II clinical study is the initial exploring of the therapeutic efficacy in patients. Usually 100–300 patients are enrolled in the study. The patients are selected by relatively narrow criteria with a relatively homogeneous population. Initial therapeutic exploratory studies may use a variety of study designs, including concurrent controls and comparisons with baseline status. Subsequent trials are usually randomized and concurrently controlled to evaluate the efficacy of the drug and its safety for a particular therapeutic indication. An important goal for this phase is to determine the dose and regimen for Phase III trials. Doses used in Phase II are usually less than the highest doses used in Phase I. Additional objectives of clinical trials conducted in Phase II may include evaluation of potential study end points, therapeutic regimens, and target populations. These objectives may be served by exploratory analyses, examining subsets of data and by including multiple end points in trials. The duration of clinical Phase II studies on average takes up to 2 years. The number of patients enrolled in Phase II trials is not large enough to obtain unambiguous statistical information to prove efficacy and safety.

Phase III clinical development is conducted with a large number of patients, usually in the range from 1000 to 3000. The objectives of Phase III studies are to gather enough statistically important evidence to confirm the efficacy and safety of the drug candidate for its intended indication in the targeted patient population. The data collected from Phase III clinical studies form the basis for market approval. Studies in Phase III may also further explore the dose–response relationship or explore the drug's use in wider populations, in different stages of disease, or in combination with another drug. Long-term effects of the drug candidate are studied for drugs that are intended for long-term use. The duration of clinical Phase III studies on average takes up to 3 years.

### 1.2.3 Role of Assays in Drug Discovery and Development

From the drug discovery and development processes described above, it is clear that assays play very important roles from the initial drug discovery phase all the way to the clinical phases. In the drug discovery phase, assays are performed for testing hypothesis of unknown biological processes, for detecting specific biological pathways, and for testing the effect of a substance on proteins, cells, tissues, or organs.

Bioassay is a major part of high-throughput screening. Bioassays also play a vital role in the computational drug discovery approach because the lead molecules obtained from computational methods must be confirmed experimentally. In drug development phases, assays play important roles in pharmacological and toxicity testing, substance characterization, manufacturing process development and validation, formulation development, manufacturing quality control, evaluating long-term and short-term (accelerated) stability, establishing comparability between batches of manufactured drug substance and drug product. As discussed before, physiochemical and biological characterization of a drug substance is required in the specification part of the document for regulatory submission to access the substance's potency. The assay here is not to identify an unknown species but to make sure a known molecule is detected over and over with the same accuracy. The biological characterization is a measurement of the substance's ability to elicit a biological response or potency. While physiochemical characterization can be performed by physical/chemical techniques, the biological characterization can only be accessed by bioassay.

When the drug is a small molecule, physical/chemical measurement alone usually can establish the equivalency between different batches and between different manufacturing processes because small molecules usually can be precisely characterized with a set of well-established techniques, such as gas chromatography (GC), HPLC, NMR, mass spectrum, ultraviolet (UV), infrared (IR), and the like. There is a direct correlation between a substance's structure and activity for small molecules. Thus, assay for biological activity may not be required for each batch of small molecules. For protein-derived drugs, the identity, purity, and quantity can be estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), HPLC, amino acid sequencing, sugar analysis, and the like. However, these physical/chemical methods may not be able to fully characterize the drug; or the physical/chemical properties do not correlated with the biological activity due to post-translational modification, protein folding, media effect on protein, and so forth. Bioassay is especially important in protein-based drug development. One specific example is to detect neutralizing antibodies for a given protein drug that can only be measured with bioassays.

## 1.3 BIOASSAY DEVELOPMENT

Bioassay development is the process to obtain a final assay system that is appropriate for its intended use and can be reliably performed repeatedly. The final bioassay protocol is developed through careful evaluation of all potential parameters that may affect the assay. The bioassay development process can be divided into following parts:

**A.** Carefully studying the biological target to determine what biological parameters should be determined to answer specific questions

**B.** Setting up a bioassay system in which some of its components, having relationships with the predefined biological parameter, can be directly measured with well-established methods

**C.** Understanding the selected measurement's application range (boundary conditions) and making sure the intended uses are within the boundary

**D.** Obtaining a control substance known to generate the intended responses in the test system

**E.** Building algorithms that can mathematically relate the final detected signal to the intrinsic biological function

Part B can be divided into two stages: (1) a series of manipulation of the test system to generate a detectable signal and (2) the measurement of the signal. When developing an assay, the first step is to develop the detection system before putting much effort to manipulate the test system. Bioassay usually involves several steps of manipulation of the test system to reach the final stage that a detectable signal is generated. The intermediate stages usually cannot be detected. When an assay does not give an expected signal, it is very difficult to tell which step among a series of steps leads to the wrong signal. In some cases, the signal is not detected not because of malfunction of the test system but because of the wrong detection system. The first step in developing an assay is to artificially generate the detectable signal and then test whether the signal can be properly measured. This can be achieved by obtaining the substance that gives off the detectable signal or by establishing an artificial test system mimicking the final stage of the assay to generate the detectable signal. When unexpected results happen, a diagnostic procedure should be performed from the last step closest to the signal generation backward stepwise to the beginning of the assay. The reason for going from back to front is that the signal can only be detected at the last step.

Here is a real case example to demonstrate that the proper sequence of experiments can save a lot time and effort: An inexperienced postdoc was working on a project to generate bacteria strings that secreted the most surfactant. He found in the literature a method using measurement of surface tension as the final assay reading because the more surfactant secreted the more changes in the surface tension. After he generated many strings of bacteria and tested them with his detection system, he could not see changes in surface tension among all the bacteria tested. He suspected that he might not generate a good string of bacteria and continued generating more bacteria. After all these efforts, he learned that he should first separate the bacteria from detection. He artificially applied different concentrations of the same surfactant secreted by the bacteria to a test solution to establish an artificial system mimicking the final stage of the assay. He then tried to detect the change using the detection method described in the literature. To his surprise, there was no change at all for any concentration of the surfactant he tested. Now he realized that he could not detect any change in surface tension with the method described in the literature even if there is a change. He then went back to the beginning of the assay development: to obtain a reliable detection system.

An example of bioassay development is shown to illustrate each step in the process of assay development (see Fig. 1.4). The background of this study is based on a real case scenario. A growth factor (L) is a potential therapeutics found in drug discovery phase and it is currently under development. It is known that L may denature in

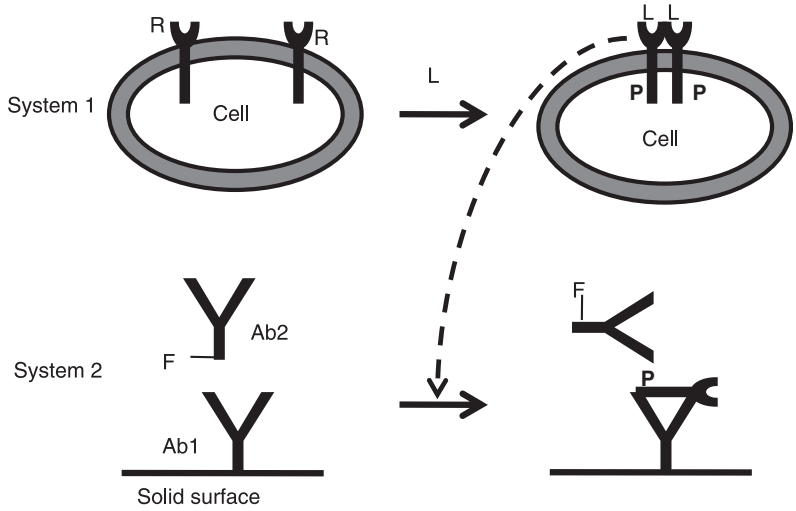


Figure 1.4 Illustration of bioassay for active ligand (L) of a growth factor receptor (R). The first biological test system (System 1) is a cell line that expresses the receptor on its surface. The R receptors dimerize and phosphorylate each other upon binding to ligand L. The second biological test system (System 2) is an ELISA system with the first antibody (Abl) attached to a solid surface that captures solubilized receptor R. The fluorescence-labeled second antibody (Ab2) binds only to phosphorylated R. Upon removal of the unbound Ab2, the fluorescent signal can be measured with a fluorimeter. The intensity of the fluorescence is proportional to the quantity of the phosphorylated receptor R that in turn is proportional to the quantity of the active ligand L.

some formulation resulting in lose of activity. An assay for L is needed to support the preclinical testing in mice.

**A.** *Define the Goal of the Assay* The goal here is to determine the active substance in each batch of L to guide the administration of correct amount of L into mouse. In this case, physiochemical characterization of each batches of L in formulation is not good enough to determine the quantity of the active molecules administrated into mouse. A functional bioassay is required.

**B.** *Design an Assay* It is known that L binds to a receptor (R) to elicit the downstream biological effects that form the basis for the intended therapeutic use of L. Thus, a biological test system that contains receptor R can be constructed. The response from the test system can be directly measured when L binds to receptor R. It is also known that L's biological activity started with its binding to the cell surface receptor R. The binding of L initiated the dimerization of receptor R on the cell surface. This is followed by autophosphorylation of receptor R that is followed by many other biological responses. From this information, a test system based on cells that express receptor R at their surface can be established. With this test system, the biological activity of L can be measured by the binding between L and R, the dimerization of R, the

phosphorylation of R, or other downstream biological responses. After detailed analysis with these different readouts, the phosphorylation of R is chosen as the final assay readout. After a literature search, a stable cell line that expresses receptor R on its surface is found readily available, and it will be used as the biological assay system.

**C.** *Understanding the Measurement* There are many bioassays to measure the phosphorylation state of membrane receptors. Here we choose to measure the phosphorylation of receptor R with a sandwiched enzyme-linked immunosorbent assay (ELISA) system. In this assay, one antibody attached to the bottom surface of a microplate captures solubilized receptor R. Another fluorescently labeled antibody recognizing the phosphate group is used for detection. It is important to establish the boundary conditions for the ELISA system and to find the most sensitive part in the detection window (linear range). Many assay conditions should be tested, such as pH, buffer, temperature, and duration.

**D.** *Obtaining a Control Substance Known to Elicit the Desired Response from the Test Systems* The proposed assay contains two consecutive assays with two test systems. The first test system is the cell line that expresses receptor R and is responsive to active L. The second test system is the ELISA, which is responsive to the phosphorylated R. A preferred control to qualify the ELISA bioassay system is phosphorylated receptor R with a known amount of phosphorylation. In the absence of this control, a ligand known to induce phosphorylation of receptor R in the cell line can be used as a control to test the two assay systems together. The biological activity of this control ligand must be known. The same commercially available growth factor L with known activity is used as a control here.

**E.** *Relating Measurement to Intrinsic Biological Function* The raw data obtained from the above bioassay is the relative fluorescence unit (RFU). Unlike absorbance measurement that is an absolute measure, RFU changes between fluorimeters and within a given fluorimeter when instrument parameters (such as gain, slit width, voltage on photomultiplier) change. Thus, the measurement of RFU at one experimental condition should be converted to an absolute measurement. In this case, the RFU is converted to units of control ligand L (either activity unit or concentration because there is a correlation between the two in the control) based on the response generated. The first attempted experiment generated the initial standard dose–response curve (Figure 1.5). These data indicate that the assay is sensitive in the region between 1500 and 9500 RFU, which corresponds to the biological response of the test system to 0.05 and 1 nM of the control ligand L. Further experiments should be performed to obtain more data points in this region to plot a higher quality dose–response curve. These data also help setting the boundary condition as to how much test sample containing phosphorylated R from test system 1 can be applied in test system 2 (ELISA system).

A well-developed assay is a finished product that has many intrinsic properties and performance characters. It is important to bear in mind the performance

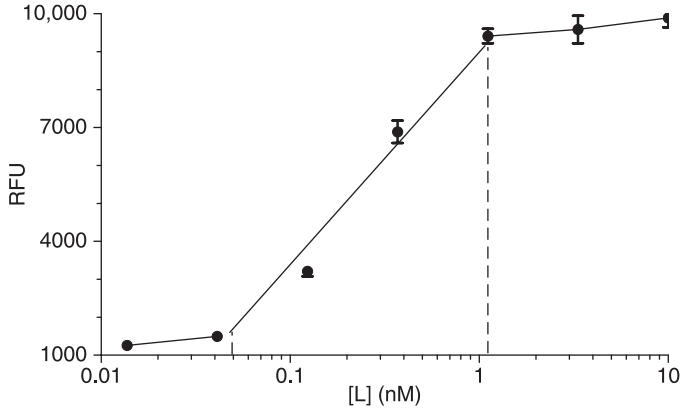


Figure 1.5 Standard dose–response curve of control ligand L known to cause receptor dimerization and autophosphorylation. The sensitive region for this assay is between 1500 to 9500 fluorescence count that corresponds to a dynamic range between 0.05 and 1 nM of the known ligand L.

characters when developing bioassays. Documentation of the performance characters is important for the transfer of the assay and for satisfying the regulatory requirement if the assay is intended for IND, NDA, or BLA filing. Following are important assay performance characteristics that should be investigated in the assay development process.

1. *Accuracy* The closeness of the *mean* test results obtained by the assay to the true value or the accepted reference value of the analyte. For many bioassays, it is very hard to obtain the true value. Accepted reference value is commonly used as a standard for accuracy measurement.
2. *Precision* The closeness of *individual* measurements of an analyte when the same assay procedure is applied repeatedly to multiple aliquots of a single homogenous sample. Precision measurement is obtained in an assay with a particular concentration of the analyte without referencing to a standard sample. Precision of an assay at a particular analyte concentration is commonly expressed as the coefficient of variation (CV). Precision can be further divided into: within-run precision (also called repeatability) and between-run precision (also called intermediate precision).
3. *Sensitivity* The ability of the assay to discriminate between small differences in analyte concentration (detailed discussion can be found in Chapter 2).
4. *Specificity* The ability of the assay to differentiate and quantify the intended analyte in the presence of other components expected to be present in the sample. Specificity is especially important in multiplex assay in which the assay is designed to analyze more than one analyte simultaneously. Specificity is also a very important factor in cell-based assays in the presence of potentially interfering serum.

5. *Detection Limits* The lowest concentration of the analyte in the sample that produces an assay signal that can be distinguished from the assay background (the assay signal in the absence of the analyte).
6. *Lower Quantitation Limits* The lowest concentration of the analyte in a sample that can be quantitatively determined with predefined precision and accuracy. Quantitation limits are always higher than the detection limits.
7. *Upper Quantitation Limits* The concentration of the analyte at which and beyond that cause the assay to fail to obtain quantitative results [e.g., deviate from known biology, oversaturating the detection instruments, oversaturating one of the assay components, unreasonable phenomenon such as "hook effect" (a phenomenon in an assay in which the response initially going up with higher concentration of analyte and then turns lower with even higher concentration producing a hooklike dose–response curve)].
8. *Linearity* The phenomenon in an assay by which the measured test results are directly proportional to the concentration of the analyte in the sample within a range of the analyte concentration.
9. *Dynamic Range* The concentration of the analyte between lower quantitation limits and the upper quantitation limits.
10. *Robustness* The measurement of an assay's tolerance to small perturbations in one or more components in the assay system.
11. *Boundary Conditions* The range of the assay components beyond which the assay is not valid (e.g., pH, temperature, buffer components and their concentration, enzyme concentration, substrate concentration, cell number).
12. *Reproducibility* The ability to carry out the assay and obtain specified results with a combination of any of the following: a different scientist, a different time, a different location, a different batch of assay components, and different instruments, etc.
13. *Scalability* The ability of the assay to perform in different formats (test tubes, different microtiter plates: 12-well, 24-well, 48-well, 96-well, 384-well, 1536-well) and in different scales (screen a few sample per run vs. screen a few hundred thousand sample per run). The scalability is especially important for high-throughput screening operations and for large-scale clinical lab testing.

## 1.4 BIOASSAY CLASSIFICATIONS

There is no unified systematic classification of bioassays. Some commonly used bioassay classification and associated nomenclatures are listed below.

1. *Classification According to Test System Used in Bioassay* Isolated protein-based assay (or biochemical assay), cell-based assay, tissue-based assay, organ-based assay, and animal-based assay. This book is organized using this classification method by first discussing isolated protein-based assays

(Chapters 3 to 7) that is followed by discussing cell-based assays (Chapters 8 to 12).

2. *Classification According to Assay Target Class* Protein-binding, protease, kinase, GPCR, ion channel, metabolite transporter, and so forth.
3. *Classification According to Whether Employing Separation Methods in the Assay* With this method, all the assays are divided into homogeneous assay and heterogeneous assay. Homogenous assays sometimes are also referred to as "mix and read" assays. There is no separation step to remove the interfering species in the assay systems from the analyte in homogenous assays. Higher background as the result of the signal from the interfering species in the assay system is the major issue affecting homogeneous assay. Opposite to homogenous assay is heterogeneous assay, which involves separation steps, such as washing, filtration, and centrifugation, to physically remove the interfering components from the analyte (the techniques are discussed in Chapter 4). Separation steps usually are tedious to perform and can result in higher variations in the assay. In addition, heterogeneous assays are more difficult to implement in HTS operations compared with homogeneous assays. Evolving technologies have made this classification system based on separation difficult to apply. For example, the "off-chip" kinase assay marketed by Caliper Technologies is a homogenous assay from operation point of view. However, physical separation is incorporated into the detection. It is a homogeneous assay but with high signal-to-background ratio comparable to heterogeneous assay (see Chapter 14).
4. *Classification According to Whether a Label is Introduced in the Assay System* Most traditional bioassays employ a foreign tag (or label) attached to one or more components in the assay system. The foreign label can be a small molecule (fluorescent molecules, biotin, etc.), a small peptide (epitope peptide, peptide substrate for biotin attachment, etc.), or a large protein (GST, streptavidin, etc.). This scheme allows the assay detection to focus only on the label (intensity or distribution), while other changes in the test system are invisible unless they indirectly cause changes in the label intensity or distribution. The downside of this scheme is that the foreign tag may interfere with the native biological system and make the assay less biological relevant. Label-free technologies have emerged in recent years that do not use a label in the test systems. The systemwide changes in the whole test system are monitored instead of just monitoring the label. By introducing specific detection techniques, the technologies may be able to detect a specific signal in the background of all the other signals. Marketed label-free detection technologies can detect the mass changes after binding events occurred (surface plasmon resonance technology from GE Health Life Sciences, formerly Biacore Life Sciences; bio-layer interferometry technology from Fortebio; EPIC from Corning), the impedance change between cells and the electrode that they stick to (RT-CES from ACEA Bioscience and Cellkey from MDS-Sciex), and extracellular microenvironment changes (XF96 Extracellular Flux Analyzer from Seahorse Bioscience).

5. *Classification According to the Format or Specialized Technology* ELISA, SPA (scintillation proximity assay, from GE Health Life Sciences, formerly Amersham Biosciences), AlphaScreen (amplified luminescent proximity homogeneous assay, from PerkinElmer), HTRF (homogeneous time-resolved fluorescence, from Cisbio International), microfluidic (Caliper Life Sciences & others), EFC (enzyme fragment complementation assay from DiscoverX), Branched DNA (QuantiGene, from Panomics), ECL (electrochemiluminescence assay from Meso Scale Discovery), and the like.

## Useful Websites

http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm

http://www.fda.gov/oc/gcp/

http://www.fda.gov/cdrh/comp/gmp.html

http://www.ich.org/cache/compo/276-254-1.html

http://www.oecd.org/document/63/0,2340,en_2649_34381_2346175_1_1_1_1,00.html

http://www.who.int/medicines/areas/quality_safety/quality_assurance/production/en/

http://www.emea.europa.eu/Inspections/GMPhome.html

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