Analytical techniques used in the development of quantitative and qualitative assays for pharmaceutical and biological products in animal health

by

Donna L. Steve

B.S., University of Kansas, 1995

A REPORT

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2017

Approved by:

Major Professor
Dr. Alison Paige Adams

Copyright

Abstract

The animal health industry is a growing industry. Owners of pets and other animals want to ensure their animals are healthy. To do this, the animal health industry markets a variety of products from pharmaceutical products, such as antibiotics, to biological products, such as vaccines. These products are developed and marketed after the company provides regulators the necessary information as guided by a set of regulations. Pharmaceutical products follow *Title 21 of the Code of Federal Regulations,* while biological products follow *Title 9 of the Code of Federal Regulations*. During the product development process as well as after marketing, regardless of the regulations to follow, each product must go through testing for efficacy, safety, potency, and stability. The regulatory guidelines provide direction to companies on expectations of the testing requirements for each type of product. Different analytical techniques are used to provide the necessary data in support of product development. Discussed in this report, two analytical techniques are well known in the industry, and one is quickly becoming a technique of great value. Mass spectrometry, coupled with liquid chromatography, is an industry standard for testing product potency and purity as well as pharmacokinetics. The enzyme-linked immunosorbent assay (ELISA) is also used to measure potency of products as well as product stability. The newest technique is flow cytometry that characterizes cells within a suspension, most often with the use of cellular biomarkers as targets. By understanding the application of each technique as well as how it relates to regulatory requirements, the industry can provide assurances to regulators that their products are safe and efficacious for the treatment and/or prevention of animal diseases. This report outlines the history, theory, and use of three different analytical techniques currently used for pharmaceutical and biological products in animal health.

Table of Contents

List of Figures vi
List of Tables vii
Acknowledgements viii
Dedication ix
Chapter 1 - Introduction 1
Chapter 2 - Regulations 4
United States Code (U.S.C.) 4
History 4
Code of Federal Regulations (CFR) 4
History 4
Rulemaking Process 5
Animal Health Pharmaceutical Products 7
Animal Health Biological Products 10
Industry Testing of Products 13
Chapter 3 - Liquid Chromatography 15
History 15
Mass Spectrometry 16
Theory 16
Detection 18
Aspects of Mass Spectrometry 19
Coupling with Liquid Chromatography 21
Chapter 4 - Enzyme-Linked Immunosorbent Assay (ELISA) 26
History 26
Theory 26
Antibodies 27
Types of ELISA Methods 30
ELISA Methods 33
Chapter 5 - Flow Cytometry 36
History 36

Theory 37
Flow Cytometry Components 40
Fluidics system 40
Optics System 40
Electronics System 42
Reagents 43
Flow Cytometry Results 44
Chapter 6 - Practical Uses 46
GMP Analysis (*Title 21 of the CFR parts 210 and 211*) 46
GLP Analysis (*Title 21 of the CFR part 58*) 46
USDA Biological Analysis (*Title 9 of the CFR*) 47
Other Analyses 48
Chapter 7 - Practical Examples 50
Liquid Chromatography with Tandem Mass Spectrometry to measure Zilmax® (Merck Animal Health) in cattle urine and faeces 50
Enzyme-Linked Immunosorbant Assays to measure antibodies following vaccination with a new vaccine candidate in sheep 51
Flow Cytometry to measure IFNγ Expression after vaccination in cattle 52
Chapter 8 - Conclusion 54
References 56
Appendix A - Acronyms 58

List of Figures

Figure 2:1 Timeline for Animal Health Pharmaceutical Process 10
Figure 2:2 Timeline for Animal Health Biologic (Vaccine) Process 12
Figure 3:1 Chromatography Flow Diagram..... 18
Figure 3:2 LC Chromatogram..... 18
Figure 4:1 Antigen Y Shape 28
Figure 4:2 Schematic of ELISA Techniques 31
Figure 5:1 Diagram of Flow Cytometry Instrumentation..... 38
Figure 5:2 Mie Scattering Diagram 39
Figure 5:3 Forward and Side Light Scatter..... 43
Figure 5:4 Results Displayed by Flow Cytometry..... 45

List of Tables

Table 1:1 Animal Health Companies 2015 Reported Revenue 2
Table 2:1 Rulemaking Process..... 6
Table 2:2 Comment Period for Proposed Rules 6

Acknowledgements

This is for those who have seen me through this process. I want to thank my major professor, Dr. Paige Adams, for the support and guidance, as well as Dr. Justin Kastner and Dr. Brian Lubbers for their ideas and willingness to see this through. I want to thank my family, especially my husband Joe Steve, for the encouragement when I was ready to give up. This truly is a dream come true.

Dedication

To my Father, Richard Beaman.

He knew I was meant for more than just a typical career. He inspired me to go into applied science where I've learned and grown into who I am.

Chapter 1 - Introduction

Animals provide a few roles for humans. They are companions and a source of food. Because of these roles, the animal health industry is growing today. Taking care of the animals is up to the people who own them. Animal owners rely on pharmaceutical and biological companies to supply pharmaceutical products, such as antibiotics, and biological products, such as vaccines, to support the health and welfare of their animals. These products are developed and marketed as efficacious and safe products for the animals. This is accomplished by two United States (U.S.) regulatory entities. These regulatory bodies guide animal health companies through the product development process and are Center of Veterinary Medicine (CVM) of the Food and Drug Administration (FDA) and Center for Veterinary Biologics (CVB) of the United States Department of Agriculture (USDA). There are many more divisions within the FDA and USDA, but these two major divisions are important for animal health and the subsequent testing of the pharmaceutical and biological products used to treat animals.

The growing industry can be seen by the sale of their products. As of 2015, the top 10 animal health corporations are Zoetis, Merck Animal Health (Merck & Co.), Elanco (Eli Lilly), Merial (Sanofi), Bayer Animal Health (Bayer), Boehringer Ingelheim Vetmedica (Boehringer Ingelheim), IDEXX Laboratories, Ceva Sante Animale (Ceva), Virbac S.A., and Vetoquinol S.A. (Weintraub 2015). These companies produce products ranging from biological agents (like vaccines) to parasiticides (like flea and tick products). They also include such products as antibiotics, anthelmintics (de-wormers), and antifungals. Several of these companies are global companies, selling their products worldwide. In order to sell these products, each product must go through many stages of testing to show safety and efficacy for each animal species. Animal targets for these products range from bovine (cattle) and swine (pigs) to poultry (chickens and

turkeys), and also include dogs and cats among many others. Annual sales from animal health products for five of the top companies in 2015 are shown in Table 1:1.

Table 1:1 Animal Health Companies 2015 Reported Revenue

Company[1]	**2015 Year end Revenue (billions)**
Zoetis	$ 4.8
Merck Animal Health (Merck & Co.)	$ 3.324
Elanco (an Eli Lilly company)	$ 3.181
Bayer Animal Health (Bayer)	$ 1.60 (€ 1.49)
Merial (purchased by Boehringer Ingelheim)	$ 1.43 (€ 1.331)

[1]Dollars obtained from company annual reports available on their respective websites.

Animal health product development can take 5 to 15 years to get to marketing. The amount of money needed is several millions of dollars (Yarbrough 2016). This makes it important to know the content of each vial or capsule of product administered to the animal because the cost of a recall of product or re-development of a product could be substantial. The regulatory process includes testing at different stages of the process. Initial amounts of the organism (for biological products) or Active Pharmaceutical Ingredient (API) (for pharmaceutical products) are determined per regulations. Testing of the vial of vaccine or capsule of pharmaceutical product after manufacturing is also required to meet regulations. These regulatory requirements help ensure the products are administered with equivalent amounts of the active ingredient from batch to batch and provide the same efficacious quality each and every time. The testing is done through approved or validated procedures with quality control performed routinely. Because efficacy and safety are of concern for pharmaceutical and biological products, study designs and analytical techniques are needed to ensure that the final manufactured product is within acceptable limits. The purity and/or potency testing of the

different types of products uses different techniques during the development process of a pharmaceutical and biological product, and includes, but are not limited to, liquid chromatography, enzyme-linked immunosorbent assay (ELISA), and flow cytometry. Generally, liquid chromatography is used in the drug development process to test 1) the purity of the active pharmaceutical ingredient, 2) concentration of the analyte versus the administration time profile to determine the pharmacokinetics profile, and 3) as one of the stability assays for the reference material used in vaccine potency assays. ELISA is generally used to test 1) the potency of the vaccine product, 2) the reference material used in the potency assays, and 3) pharmacokinetic analysis. Flow cytometry assays are used for 1) biomarker analysis in which a sample is analyzed for the presence of disease, infection or environmental exposure, 2) the properties of cells, such as looking for antigen specific T cell response, and 3) other types of cellular analyses, such as cell viability.

The purpose of this report was to highlight three analytical techniques used to support regulatory requirements for marketing of pharmaceutical and biological products. This report is limited to these particular techniques because of their current (liquid chromatography, ELISA) and potential (flow cytometry) use within the industry. There are other analytical techniques available for testing; however, these particular assays are recognized standards, and governmental regulatory agencies accept the data from these assays in support of the marketed products. Some practical uses and examples of these techniques are discussed at the end of the report. To understand why the regulators accept the data from these analytical assays, this report will also review the evolution of the regulatory requirements for testing animal health products and explain how these testing techniques seek to fulfill these regulations.

Chapter 2 - Regulations

The United States (U.S.) governmental body puts laws in place to help keep the general public safe and ensure their rights as citizens of the U.S. are upheld. Some laws are intended to provide guidance to the industry on the expectations of the regulators for marketing of animal health products, such as pharmaceuticals or biologics. The U.S. government issues laws. These laws are codified; that is, laws are written based on their subject matter. The *United States Code* (U.S.C.) features the laws passed by the government. The *Code of Federal Regulations* (CFR) is developed in response to the *United States Code* (U.S.C.). The CFR is the regulation used to enforce the law. Each new law is recorded in the U.S.C. book, and subsequently, the new regulation issued is recorded in the CFR book. The law is not enforced without a regulation to govern it.

United States Code (U.S.C.)

History

The United States issued *Statutes*, a collection of laws, to provide some guidance to the public. In 1873, the revised *Statutes* were codified, gathered and written as collections of subject matter. By June 30, 1926, President Calvin Coolidge signed the U.S.C. as enforced. This date was when some of the laws had improved organization and became enforceable. The laws were considered positive laws, or enacted laws (McKinney 2004).

Code of Federal Regulations (CFR)

History

The *Code of Federal Regulations* is the rules used to enforce the U.S.C. The *Federal Register Act*, passed by Congress in 1935 gave the Archivist of the United States the ability to establish a division within the National Archives, which would publish a daily publication of the

Federal Register. This register made public all regulations as well as Presidential proclamations and executive orders. It was amended in 1937 to provide a codification of all regulations were made public every five years. By 1938, the first edition of the CFR was published which included all finalized regulations that were published since 1936 or before which were still in effect. Supplements were published yearly except during war times. In 1946, the *Administrative Procedures Act* was set to make any notice of proposed rules available and publicized. The second edition of the CFR was published in 1949. Starting in 1963, yearly publications are available. Yearly revisions to the CFR were determined were unmanageable. The CFR was divided into 4 groups, and the review process was staggered between the 4 groups. By the 1960's, each finalized rule was accompanied by brief explanations about the rule. In 1973, these explanations were part of the preamble to the rule. Since 1977, the requirement for the preambles included headings and related material. When a rule became effective, the comments to proposed rules, along with answers, were summarized (McKinney 2002).

Rulemaking Process

Regulations the pharmaceutical and biological companies follow go through a process to become effective. An "Advance Notice of Proposed Rulemaking" may be published in the *Federal Register* in the preliminary stages. The President of the U.S., as the head of the Executive Branch, can review a proposed rule with the assistance of the Office of Information and Regulatory Affairs (OIRA). Each rule goes through the steps outlined in Table 2:1. In step 1, the notice of proposed rulemaking (NPRM) is used to announce and explain the agency's plan for addressing issues or to reach a goal. Each proposed rule includes a preamble, which includes a summary of the problems or actions being considered. Dates for comment are listed. Table 2:2 summarizes the date period for comment. Step 2 of the process is to request comments from the

public. Step 3 gives advice on whether the rule is to continue in the comment process or proceed to finalization. Finally, step 4 is when the final rule is published under *Title 21 of the CFR*. Rules generally become effective within 30 days of publication in the *Federal Register*, but this timeline may be extended for complex or significant rules (Office of Federal Register 2017).

Table 2:1 Rulemaking Process

Step #	Comment
1	Propose a new rule
2	Ask for Public comments
3	Regulators consider/synthesize/address public comments. a. If further action to the proposed rule is needed, issue a new proposed/revised rule b. If no further action to the proposed rule is needed, issue the final rule
4	Publish Final Rule in the *Federal Register* and include: a. Regulatory Requirements b. Impact of Requirements on industry and public c. Respond to comments on the proposed rule

Table 2:2 Comment Period for Proposed Rules

Period	Reason
Up to 30 to 60 days	General timeframe for taking comments
Up to 180 days	Timeframe for complex rules under consideration
Extension	If the public does not feel there is enough time to give proper comments

Animal Health Pharmaceutical Products

The Center for Veterinary Medicine (CVM) of the Food and Drug Administration (FDA) follows *Title 21 of Code of Federal Regulations* and associated guidance documents when inspecting any New Animal Drug Application and enforcing the U.S. laws. They are committed to "Protecting Human and Animal Health." (Center for Veterinary Medicine) *Title 21 of the CFR* is the regulation based on *Title 21 U.S.C.* chapter 9 sections 321 – 399f, of the *Federal Food, Drug, and Cosmetic Act*.

The *Food and Drugs Act* of 1906 was enacted to ban any movement of adulterated or misbranded food or drugs through interstate commerce. It also made any production of adulterated or misbranded foods or drugs unlawful within any territory of the U.S. and District of Columbia. The law defines drugs as all medicines or preparations recognized by the United States Pharmacopeia or National Formulary and are used internally or externally as well as any substance or mixture of substances intended for a cure, mitigation, or prevention of disease in both humans and animals. Adulteration is the process of selling of a product in which the official or recognized name is used without meeting the standards for strength, quality, or purity. If the label was clear with this information, the product was not considered adulterated so long as the company was producing a quality product according to the standards of the company. Misbranding is the falsification of the drugs identity on the label by using an imitation or false name. If the labels failed to show the quantities of the components or a lack of components, it was also considered misbranded (Congressional Research Service 1989).

In 1912, the *Sherley Amendment* was passed to prohibit false or fraudulent therapeutic claims on a label. Later, in 1938, the *Food and Drugs Act* was revised. This became the *Federal Food, Drug, and Cosmetic Act* of 1938. A deadly incident with "Elixir of Sulfanilamide" caused

death because the chemists manufacturing the drug failed to test for safety; they only tested for flavor, appearance, and fragrance. The sulfanilamide was dissolved in diethylene glycol, a deadly poison. Deemed the *Copeland Act*, the revision now encompasses cosmetics. The act required any new product have proof of testing. All new drugs then required submission of full reports of investigations to establish safety prior to marketing of the product, but the requirements for proving efficacy were not yet established (Congressional Research Service 1989).

By 1962, the *Drug Amendments* were added enhance regulatory authority and required products were manufactured in a licensed facility. This included batch certification where batches of drug product were required to show results of tests for efficacy and safety. The *Drug Amendments* were enacted in response to the "thalidomide disaster." Thalidomide was a drug product used as a sedative as well as to help pregnant women with morning or all day illness. Unfortunately, it caused birth defects in children when used while a woman was pregnant. This drug was not yet approved in the U.S. when the disaster happened; it was distributed to doctors for experimental use. The government now requires approval of a drug prior to marketing and distribution (Congressional Research Service 1989).

In 1968, provisions to the *Federal Food, Drug, and Cosmetic Act* were made to include all regulations of new animal drugs. This Amendment removed the requirement to regulate new animal drugs by the *Food, Drug, and Cosmetic Act* and as a food additive, thereby reducing the time to get to market (Congressional Research Service 1989).

Title 21 of the CFR is entitled "Food and Drugs." Chapter I under *Title 21 of the CFR* is entitled "Food and Drug Administration, Department of Health and Human Services." Subchapter E entitled "Animal Drugs, Feed, and Related Products." Parts 510, 511, and 514 of Subchapter E guide a pharmaceutical company on how to obtain a licensed product for sale.

Guidance for the Industry (GFI) as well as Veterinary International Conference on Harmonization (VICH) guidance documents provide additional guidance for licensing of products and are related to parts 510, 511, and 514 of subchapter E. These guidance documents are not enforceable by regulators, but provide the agency's current thinking on the topic. They are considered recommendations unless cited as a statutory requirement. Each GFI document lists the respective sections of the *21 CFR* under clarification in the document. Each GFI document also may relate back to the *21 U.S.C.* requirements. The GFI and VICH work in tandem with each other to help guide the company in their quest of marketing a new animal drug. Figure 2:1 illustrates the timeline a pharmaceutical company follows during the development process.

Analytical techniques to test the products are used throughout the discovery/development process. In the preclinical development phase, drug products go through feasibility studies in which the company determines the concentration of the active ingredient necessary to induce a therapeutic response. Analytical assays are developed to provide a way to measure the active ingredient for consistency during production. Also, analytical assays are developed to test for concentration of the active ingredient after administration. Over the next few years, research and development optimize and validate the assays to ensure reproducibility for subsequent uses. The data is compiled and submitted to the regulatory agency, CVM, for review and approval of the products intended therapeutic area. Testing continues to occur throughout the product's development period. Post-marketing testing for the stability of the product as it is stored in the warehouse, veterinary clinic, or owner's house provides assurance the product minimizes degradation when stored as per label requirements and used before product expiration.

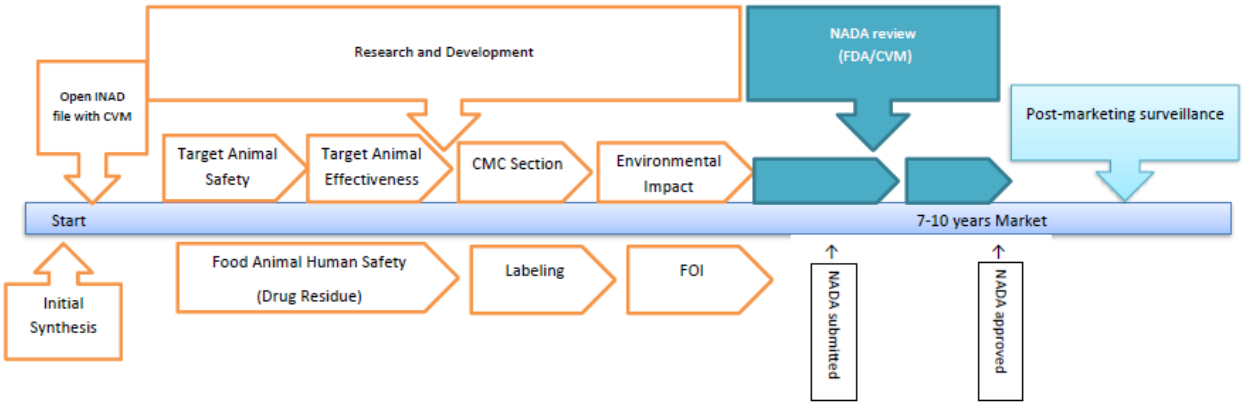

Figure 2:1 Timeline for Animal Health Pharmaceutical Process

Animal Health Biological Products

The Center for Veterinary Biologics (CVB) of the United States Department of Agriculture (USDA) and under the Animal and Plant Health Inspection Service (APHIS) follows *Title 9 of Code of Federal Regulations* and associated veterinary services memorandums (VSM) when inspecting a New Biological License Application and enforcing the laws. *Title 9 of the CFR* is the regulation based on *Title 21 U.S.C.* chapter 5 sections 151-159, the *Virus-Serum-Toxin Act.*

The *Virus-Serum-Toxin Act* of 1913 was enacted to provide the government the authority to issue licenses and regulate products and trade relating to biologics. This act was mainly in response to losses from the use of unregulated of anti-hog cholera serum. In 1985, the act was amended because of new challenges arising over the years. These challenges stemmed from modernization of the agricultural sector and more complicated regulations. The amendment was part of the *Food Security Act* which provided authority to the USDA to regulate intrastate (within states) and interstate (across states) movement of biological products. The amendment increased the Secretary of Agriculture's authority to issue regulations and enhance enforcement. The

amendment also recognized the need to prevent and eliminate issues in commerce as well as regulate commerce. In 2002, the *Homeland Security Act* transferred most of the major functions of border inspection to the Department of Homeland Security (DHS) without changing any provisions in the original act. DHS conducts all inspections while the USDA has jurisdiction, i.e. the ability to make legal decisions over the regulations and policies. The act specifies any activity related to the preparation, sale, barter, or exchange of any dangerous virus, serum, toxin, and equivalent product either shipped or delivered within the borders of the U.S. unlawful (Monke 2005).

Title 9 of the CFR is entitled "Animal and Animal Products." Chapter I of Volume 1 under *Title 9 of the CFR* is entitled "Animal and Plant Health Inspection Service, Department of Agriculture." Subchapter E entitled "Viruses, Serums, Toxins, and Analogous Products; Organisms and Vectors" Parts 101-124 guides a biological company on how to obtain a licensed product for sale. Veterinary Services Memorandums (VSM) numbers 800.50 to 800.301 provide additional guidance for licensing of products and are related to parts 101-124 of subchapter E. Each memorandum lists the respective sections of *9 CFR* that is clarified in the document. All these documents help a biological company in their quest to license and market a new biological product. Figure 2:2 illustrates the timeline a biological company follows during the development process.

During the biological development process, testing is initiated during the feasibility studies. The feasibility studies (pre-clinical studies) are where the company determines the concentration of the organism necessary to induce an immune response. Analytical assays are developed to provide a way to measure the viability of the organism for consistency during production. This testing begins with the development stage prior to submitting the development

plan. Over the next few years, the assays are optimized to maximize the assays ability to reproduce the results with each test of product. Testing occurs every time the product is produced during the development stage before the product is administered to the animals. One of the critical reagents for the product testing is the reference material. The reference material is used in the analytical testing technique to compare the newly produced product for determining potency. The data is compiled and submitted to the regulatory agency, CVB, for review and approval of the product's intended therapeutic area. Testing continues to occur throughout the product's development. Post-marketing testing for the stability of the product as it is stored in the warehouse, veterinary clinic, farmer's barn, or owner's house provides assurance the product does minimizes potency loss when the product is stored according to the label specifications.

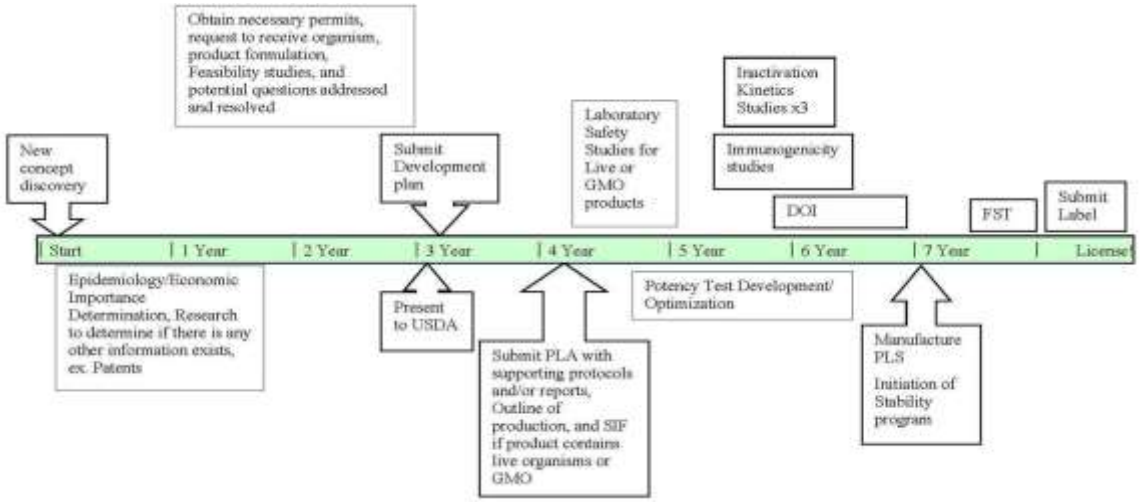

Figure 2:2 Timeline for Animal Health Biologic (Vaccine) Process

Abbreviations:

USDA	United States Department of Agriculture	GMO	Genetically Modified Organism
PLA	Product License Application	DOI	Duration of Immunity
SIF	Summary of Information Format	PLS	Pre-licensing Serials
FST	Field Safety Trial		

Industry Testing of Products

Figures 2:1 and 2:2 show the timeline a company would typically follow for development of a pharmaceutical or biological product, respectively. Each product goes through a series of testing to demonstrate safety and efficacy. Prior to and during the testing of the product for safety and efficacy, the pharmaceutical and biological companies must develop assays for testing of product concentration, testing for potency and purity of the product to show consistency between batches of manufactured product, and testing of components that are not the compound of interest, such as excipients or adjuvants, within each product administered to the animal to produce the desired medicinal result. Assays developed are standardized within each industry. The guidelines, either *Title 21 of the CFR* or *Title 9 of the CFR*, state what testing is done. The companies are responsible for developing and/or validating the assays. One type of assay companies validate is for the determination of sample concentration of pharmaceutical drug as a function of time to calculate toxicity, i.e. target animal safety, pharmacodynamics, and pharmacokinetics. Bioanalysis, a division of analytical testing to quantitatively measure drug products in a biological system, follows the guidelines of Good Laboratory Practices, GLPs, *Title 21 of the CFR part 58*. Assays used to test pharmaceutical drug potency, purity, and residual components must follow Good Manufacturing Practices, GMPs, *Title 21 of the CFR part 210, 211 or 226*. Assays used to test biological potency, purity, and other components of the product called excipients follow USDA *Title 9 of the CFR*. To accomplish these assays several analytical techniques are used and include liquid chromatography, enzyme-linked immunoassays (ELISA), and flow cytometry assays. There are other assay types available, such as titration techniques, polymerase chain reaction (PCR) for amplification, quantitative reverse transcription PCR (qPCR) for testing of RNA (ribonucleic acid) in starting material, thin layer chromatography,

nuclear magnetic resonance spectroscopy (NMR) for testing of magnetic properties of a material, and more; however, liquid chromatography and ELISA are considered the standard for use within the industry. Flow cytometry is relatively new to the industry and is rapidly becoming a state of the art technique. These three assays are the focus of this report.

Chapter 3 - Liquid Chromatography

History

Liquid chromatography is an analytical technique used to separate molecules. A Russian botanist Mikhail Tswett was credited with the invention of chromatography in 1903. On March 8, 1903, Tswett gave a lecture entitled "On a New Category of Adsorption Phenomena and their Application to Biochemical Analysis." at the Biological Section of the Warsaw Society of Natural Sciences. The lecture described his wide-ranging investigations of leaf pigments, specifically a technique that used adsorption to separate the leaf pigments. Tswett published a book on the technique after refining the process entitled *Chlorophylls in the Plant and Animal World* (Ettre 2003). Tswett's technique utilized a glass column packed with adsorptive material, such as alumina or silica or even powdered sugar. Then, he added a solution of plant pigments to the top of the column and washed the column with an organic solvent. As the solvent flowed through the column, the plant pigments were washed down the column and separated into a series of discrete colored bands, with empty bands in between.

Another notable person in the development of chromatography was Archer J.P. Martin. Martin won a Nobel Prize along with his colleague Richard Synge for their development of partition chromatography in 1952. Where Tswett used a column of powdered chalk to separate the leaf pigments from the plants, Martin investigated the use of silica gel columns to separate amino monocarboxylic acids. This led Martin to develop partition chromatography. (Martin 1952). As time went on, Martin continued research using gas chromatography with a glass column measuring four feet long by one-fourth inch diameter. This was positioned vertically and filled with 200-mesh granules of thin layer of non-volatile hydrocarbon coated Celite (a diatomaceous earth or the remains of skeletal single cell algae). Next, Martin invented the gas

density balance for use as the detector. This simply made instrument used the imbalance of two flows of gas to measure the density difference between the gases. Both the column and detector were heated to 180°C. Martin utilized this for analysis of hydrolyzed fatty acids and lipids to methyl ethers. His success showed a reduction in time for analysis (Lovelock 2004).

Mass Spectrometry

Mass spectrometry is related to liquid chromatography. Where liquid chromatography is used as a separation technique, mass spectrometry is the detection/quantification tool and defined as an instrument used to separate a component into its associated ions according to their charge and mass. Mass spectrometry did not start out as a detector for the separation of components in tandem with liquid chromatography; instead, it was initially used to demonstrate the existence of isotopes. By the 1940's, the petroleum industry was using mass spectrometry to measure the amount of small hydrocarbons in their process streams. Then, in the 1960's, other chemists understood the range of applications a mass spectrometer was useful to support. Some examples are the determining of the earth's age, enriching compounds with carbon (^{13}C) for tracing studies, separating two isotopes of uranium, and more (Griffiths 2008).

Theory

Chromatography is a method of separation of a chemical mixture, which is carried through an apparatus by a liquid or gas. Figure 3:1 shows the flow diagram of liquid chromatography. The components are distributed between two phases, the stationary phase (the system in which the components are separated) and the mobile phase. As the mobile phase, liquid or gas, moves through the stationary phase, the chemicals are separated by repeated sorption/desorption during the movement. Gas mobile phase is an inert gas where as a liquid mobile phase is a liquid of low viscosity. The stationary phase is usually a porous, granular

powder packed into a tube, and referred to as the column. Each column can withstand a certain amount of pressure. Each small amount of sample, usually microliters in size, is carried through the column by the mobile phase. Liquid chromatography is a separation technique which requires an external instrument to detect the molecules as they leave the column. This is accomplished by the use of a detector and is subsequently by computer software. In the 1990's, electronic integrators and microcomputers were used to collect the data and translate electronic signals into what are called chromatograms (see Figure 3:2). These are a record of the concentration or profile of the sample components as a function of the movement of the mobile phase across the column. Information in the chromatograms used for quantitative or qualitative analysis is the retention time, peak height, peak area, and baseline noise (Poole and Poole 1991). Retention time is the time it takes to remove a specific analyte from the column by washing with a solvent. Peak height is the response height of the peak from the baseline to the center of the peak. Peak area is the area under the response curve. Baseline noise is the movement of the response that is detected from the mobile phase components and should be less height or area than the peak(s) of interest based on a signal to noise calculation between the peak of interest and the noise. Many validated assays set a criteria threshold for the baseline response in order to accurately identify the peak(s) of interest.

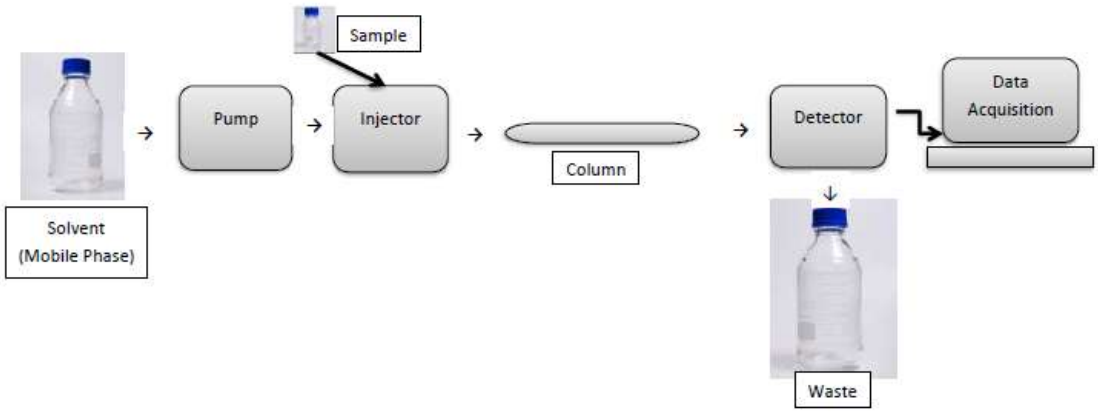

Figure 3:1 Chromatography Flow Diagram

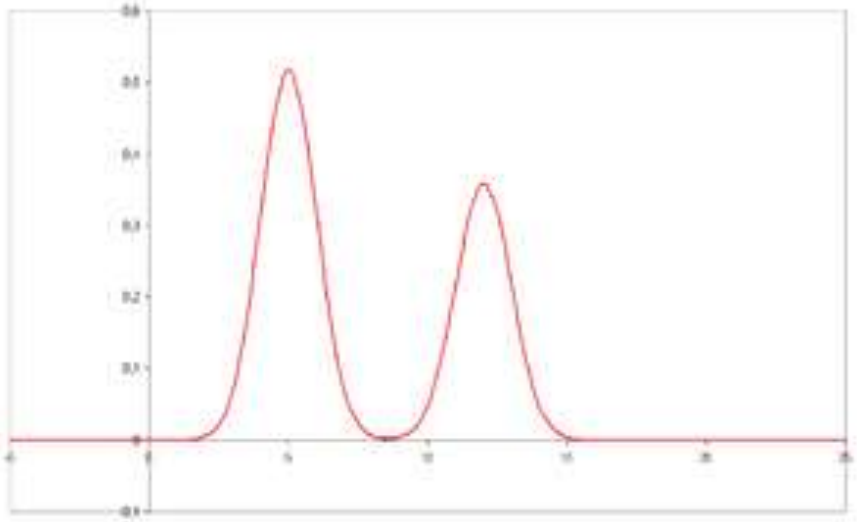

Figure 3:2 LC Chromatogram

Detection

Different instruments can accomplish detection of the molecule, each with their own strengths and weaknesses. Ultraviolet (UV)/visible absorption detector is in the ultraviolet-visible spectral region of light. It is the most widely used type of detector in liquid chromatography because many organic compounds are absorbed in the UV region of the electromagnetic spectrum. Sensitivity of the detector to detect the compound depends on the

ability to distinguish it from the liquid solvent, which is mainly in the range of 180 – 210 nanometers (nm) wavelength. Therefore, a detected compound by an UV/visible detector needs to absorb at a wavelength greater than 210 nm. For some compounds, the absorption of light can naturally fluoresce. Fluorescence detection is based on the ability to excite the molecule. The system is usually set to read the excitation and emission (Poole and Poole 1991). Mass spectrometers are used to measure the mass-to-charge ratio (*m/z*) of a molecule after ionization their ability to discriminate by molecular weight (Skoog and Leary 1992).

Aspects of Mass Spectrometry

Mass spectrometers use ion sources and mass analyzers to charge a molecule to an ionized or charged state by bombarding the molecule with electrons to produce fragmented ions and separate the ion of choice from others using the mass of the molecule. A mass spectrometer will sort the ions based on their mass-to-charge ratio. Three types of ion sources are electrospray ionization source (ESI), atmospheric pressure chemical ionization source (APCI), and atmospheric pressure photo-ionization (APPI) (Pitt 2009).

The ESI works with moderately polar molecules, such as metabolite, xenobiotics, and peptide analysis. The mass spectrometer detects positive or negative ions depending on the mode after the sample is pumped into a thin capillary where it is formed into a spray of charged droplets. These droplets are evaporated and an electrical charge is transferred to the analyte. Many small molecule analytes with a single functional group produce a singly charged ion to be detected. Larger molecules or molecules with several charge-carrying functional groups can have multiple charged ions (Pitt 2009).

The APCI functions similarly to the ESI by pumping the sample carried in a liquid through a capillary. The charge transfer happens near the tip of the capillary. This source is better

for small, thermally stable molecules, which are not ionized well by ESI. This technique works for lipids and fat-soluble vitamins (Pitt 2009).

The APPI is different from ESI and APCI, because it uses photons to excite and ionize molecules. The energy from the photons is used to minimize concurrent ionization of the solvents in the liquid phase and source gases. This technique is used for analysis of neutral compounds like steroids (Pitt 2009).

After the molecules are ionized, the mass spectrometer uses the ion charge and the molecule mass to detect the analyte of interest. Four types of mass analyzers are: 1) quadrupole analyzers, 2) time-of-flight analyzers, 3) ion trap analyzers, and 4) hybrid analyzers, each having their own advantages of use (Pitt 2009).

The quadrupole analyzer has a set of four parallel metal rods. The mass spectrometer varies the voltage along the rods to allow for scanning across a range of *m/z* up to 1000 *m/z* per second or more. When coupled with liquid chromatography, a triple quadrupole configuration is useful. This is when a collision cell is placed between two quadrupole mass analyzers. A type of collision cell is when a quadrupole maintains the low pressure of the collision gas used to disassociate or fragment the ions. This increases the specificity of analysis over a single stage mass analyzer. When using the triple quadrupole configuration, the first and third quadrupoles are stepped to different *m/z* values simultaneously, where the ion pairs are created to detect a large number of target analytes. This common process is called multiple reaction monitoring or MRM and is a very common liquid chromatography mass spectrometer (LC/MS) assay. Both the single and triple configurations are used in clinical biochemistry applications (Pitt 2009).

Time-of-flight analyzers use high voltage to accelerate the charged ions. The time it takes the ions to travel down the tube depends on the *m/z* value. This analyzer is highly sensitive and

quick with high mass accuracy to allow determination of molecular formulas for small molecules (Pitt 2009).

Ion trap analyzers, using static and radio frequency voltages, trap ions with the three hyperbolic electrodes. The ions are released from the trap based on their *m/z* value. If the molecule requires fragmentation, an inert gas is introduced into the trap, which will lead to isolation of ions in succession (Pitt 2009).

Hybrid analyzer is when the third quadrupole is replaced by a time-of-flight analyzer. These hybrid analyzers are used for proteomics but have limited scanning ability. Another hybrid analyzer is when the third quadrupole is operated in a different mode thereby trapping ions and then releasing them sequentially based on their *m/z* values. This hybrid analyzer is known as a linear ion trap referred to as the QTrap instrument. This configuration enhances the sensitivity of the ion scanning (Pitt 2009).

Coupling with Liquid Chromatography

Detectors are coupled with liquid chromatography to have an instrument that can generate sample throughput in an automated environment. This is accomplished by connecting separate systems, which include a pump, an injector, a column, the detector (previously described), and waste collection (see Figure 3:1). The pump delivers the solvent to the column and then into the detector. The injector delivers the sample with the help of the pumped liquid to the column and then to the detector. From the detector, the liquid waste is collected in a container and discarded appropriately at the end of the analysis when using an UV/visible or fluorescence detector or is pumped into the atmosphere outside the building when using a mass spectrometer. Each assay utilizes either an single pump, or two pumps for stepwise pumping using two reservoirs with different solvent concentrations (Poole and Poole 1991).

The flow rate of the pumping system is dependent on the amount of separation needed through the column. Typically, a flow rate of 0.1 to 10 milliliters per minute (mL/min) is used as long as the pressure generated by the system does not exceed instrument or column specifications. The flow rate of the pump is dependent on the column size and amount of separation needed. Three types of pumping systems are reciprocating pumps (90% of commercial industry uses this type), displacement pumps, and pneumatic pumps. The requirements of the pumping systems are pressure outputs, flow rates, pulse-free output, flow control, and flow reproducibility. It is also important that the systems have corrosion-resistant components, such as stainless steel and Teflon™. Most systems allow for isocratic flow (continuous flow from one reservoir) or gradient flow (step-wise flow of two or more reservoirs with a mixing chamber) (Skoog and Leary 1992).

Solvents are used as the carrier through the stationary phase. The solvents are usually termed the "mobile phase" of the system and are liquid or gas as previously discussed. Typical solvents used in a mobile phase are water, acetonitrile (ACN), methanol (MEOH), ethanol, and chloroform. These are most compatible with the ESI units. The grade of solvent, that is the purity of the composition of the solvent, can affect the ability to separate the components in a mixture. Buffers, inorganic ions of phosphate or sodium acetate, are common is mobile phases, but should be avoided in mass spectrometer applications as they can cause ion suppression. Ammonium acetate, formate, or bicarbonate is used instead, but these can also cause some ion suppression. Ion suppression is the competition of ions from more than one component, which reduces the mass spectrometer signals (Pitt 2009). Therefore, it is important to know and understand how each sample reacts in a mobile phase.

Baseline resolution, the ability to resolve a peak of interest from the baseline signal, is mainly required with using an UV/visible or fluorescence detector, due to the inability of this type of detector to separate signals (Pitt 2009). Typical columns range from 10 to 30 centimeters (cm) (3.9 to 11.8 inches). Inside diameter ranges from 4 to 10 millimeters (mm) (0.2 to 0.4 inches). Common particle size is 3, 5, and 10 micrometers (µm). For a typical separation, a 25 cm by 4.6 mm column packed with 5-µm particles may be used (Skoog and Leary 1992). Due to the ability to separation components based on their mass/charge ratio, mass spectrometers generally use smaller columns with particle size less than 2 µm. The shorter the column, the less the run time needed to achieve separation (Pitt 2009). Guard columns, a short column placed before the column to increase the life of the column and help with the separation, removes particulates and contaminants in the solvents. Stationary phase of the guard column is similar to the column of choice. Increasing the temperature of the column also helps with separation (Skoog and Leary 1992).

Quantitation of the response (height or area) is the purpose of chromatography. This can happen in multiple ways. Normalization method, external standard method, internal standard method, and the method of standard additions are four techniques for converting the response into relative composition data. Normalization method technique takes the responses gathered and sums the response, and then, each analyte is expressed as a percentage of the summed areas. Response factors are calculated when the detector response is not the same for all components. External standard method techniques use standards at a similar concentration of the anticipated sample. The precision of the method is dependent on maintaining constant chromatographic conditions throughout the analysis process. The sample responses are compared to the standard responses. When a compound is added to the sample at the earliest possible point in the method,

this type of technique is the internal standard method. The purpose of the internal standard is to compensate for sample loss during the extraction (preparation) to the final chromatographic analysis on the instrument. The internal standards for high performance liquid chromatography (HPLC) methods are chosen based on their ability to resemble the compound of interest (Poole and Poole 1991). For LC/MS applications, it is generally best practice to use isotopically-labeled compounds due to identical chemical properties to the analyte of interest and is distinguishable during the mass spectrometer analysis (Pitt 2009). The results are calculated based on the ratio of the analyte response (height or area) to the internal standard response. This technique helps standardize the results of each injection. Without the internal standard, variation is seen between injections. The technique of standard additions is not used widely. With this technique, samples are analyzed to estimate the concentration (amount) of the analyte in the sample. Then, the known amount is subsequently added to each sample, and the sample is analyzed thereby showing the approximate incremental increase in the detector response (Poole and Poole 1991). Each of these four techniques serves to provide a standardization of the response so that quantitation of the sample is determined. The most common practice in industry today is the use of the internal standards for analysis of concentration in a biological sample (bioanalysis) and external standard when calculating sample potency to determine how long a product can sit on a shelf (shelf-life).

The regulators review the data generated by liquid chromatography during the approval process. They rely on the companies to have validated software and instruments when producing the data. The industry is required to retain all data generated, either paper records or electronic records, for a period of time. The records are maintained appropriately so that the procedures are reconstructed when needed. The regulators will go to the company and inspect the records during

the approval process. All regulatory requirements are available to the pharmaceutical or biologic sponsor through the regulations and associated guidance documents. For biological companies, the regulators perform confirmatory testing of the products using the reagents and methods supplied by the companies to prove or disprove the results. Regulators inspect facilities and study records regularly to ensure compliance with the laws. Companies are expected to cooperate fully when the regulators are on site. When issues arise, the regulators issue their findings and expect the companies to answer and assure the regulators the issues were addressed. Therefore, it is important to follow the regulations and work with the regulators to ensure the products are fit for consumption.

Chapter 4 - Enzyme-Linked Immunosorbent Assay (ELISA)

History

Enzyme-Linked Immnosorbent Assay (ELISA) is an analytical technique that uses the paring of an antigen with an antibody for determining the concentration of either within a sample. Several scientists were involved in the development of the radioimmunoassay (RIA), a similar technique to ELISA. Rosalyn Yalow and Solomon Berson published a paper describing RIA in 1960; however, RIA was not economical and required a large amount of sample that was about 5 times as much sample as the ELISA methods require. It required special facilities that allowed investigators to work safely with the amounts of radioactivity required. In 1966, L. Wide and colleagues published a report highlighting their results from coupling antigens or antibodies with enzymes with the idea of using enzyme-labeled antigens and antibodies to detect antibodies and antigens by immunofluorescence. Peter Perlmann, a professor at Stockholm University with the help of his graduate student, Eva Engvall, were the first to publish a paper describing a method for enzyme-linked immunosorbent assay (ELISA) , in 1971. Perlmann and Engvall used the previous knowledge for RIA to create and develop the assay. Their paper described the results from quantitative measurement of IgG in rabbit serum with alkaline phosphatase as the reporter label (Lequin 2005).

Theory

ELISA is useful in providing measurements of antibody and antigen concentrations, as well as, for measuring hormones and other molecules. The assay is specific and sensitive enough to detect the presence of antigens that are recognized by an antibody and vice versa. The binding of an antibody to an antigen is the essential principle of an immunoassay. This complex interaction is exclusive to the antibody/antigen combination. In the body, the antigen will elicit a

specific antibody in response. Molecules with large molecular weights can function well as antigens, while small molecules, such as drugs, peptides or lipids, require a chemical coupled to it in order to elicit the immune response. The epitope is defined as site where the antigen binds to the antibody. For binding to occur, the epitope must be available and have the correct shape. Antibody affinity is the strength of the interaction at the antigen on a single site. This is usually represented as a constant and influenced by factors, such as temperature or pH. In the immunoassay, the antibody is detected for a target antigen or analyte. Plasma cells produce glycoproteins, antibodies, and function as a binder to foreign or non-self-molecules (Hnasko 2015).

Antibodies

Antibodies develop in the fetal liver and bone marrow and are produced by B lymphocytes (Wild 2013). Antibodies are the central part of an ELISA method. These glycoproteins are produced by the plasma cells and function to bind to foreign, non-self molecules (Hnasko 2015). The plasma cell is the cell in the immune system that is formed after a B cell interacts with foreign material. The B cell is genetically coded for a surface receptor to specific antigens. Once the B cell recognizes the antigen, they begin to multiply and produce the plasma cells. Plasma cells then produce antibodies (Lamont, Hajishengallis, and Jenkinson 2013). Antibodies have a basic Y-structure and are also known as immunoglobulins (Ig) (see Figure 4:1). Each immunoglobulin has two heavy chains and two light chains. Light chains consist of one variable and one constant region, while the heavy chains have one variable and 3 or 4 constant regions. There are five classes of immunoglobulins, IgG, IgA, IgD, IgE, and IgM, with IgG as the most abundant. IgG immunoglobulins are mainly used in immunoassays. This is because of the abundance in production, binding affinity to epitopes, and the ability to withstand the isolation and purification

process. IgM is also used in ELISA assays, but not to the extent of IgG. The remaining immunoglobulins are rarely used due to low binding affinity and abundance (Wild 2013). As previously stated, there are two types of antibodies, monoclonal and polyclonal, products which function similarly in the ELISA assays. Both provide binding of the antigen where the primary difference is the number of antigens that are bound (Hnasko 2015).

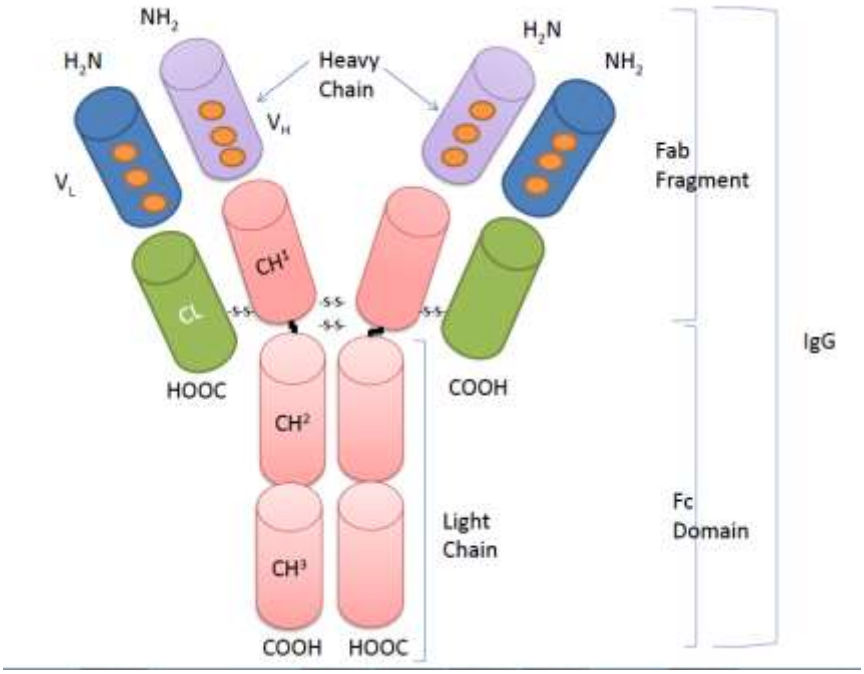

Figure 4:1 Antigen Y Shape

(Figure based on Figure 1 page 3 of 214 Hnasko, R. (2015). ELISA Methods and Protocols, Springer New York Heidelberg Dordrecht London.)

In an immunoassay, monoclonal antibodies or polyclonal antibodies are used for binding of the antigen to the antibody. Monoclonal antibodies are a single antibody directed at an antigen epitope. This type of antibody is very specific and usually not hard to obtain commercially. Polyclonal antibodies are produced to provide target detection and isolation where there are many antibodies able to interact with a single antigen. These antibodies are not in high quantity and are reactive with other antigens within a sample (Hnasko 2015).

Monoclonal antibodies are harder to produce yet they provide a higher affinity for an antigen, giving the assay better specificity. The disadvantage with using a monoclonal antibody is if there are subtle changes within an assay, the monoclonal antibody is more vulnerable, that is they could react differently in an assay and cause the assay to go through development again. It could cause problems with detection of the antigen (Hnasko 2015). Hybridoma technology is one way of producing monoclonal antibodies where a mouse is injected with the antigen, and the B cells that produce the antibodies are harvested and fused with myeloma or hybrid cell line. Monoclonal antibodies are also produced by chemical synthesis, purification, peptide synthesis, or recombinant protein expression. Monoclonal antibodies are continuous and renewable, limitless due to the ability to grow on cells, generally mouse cells, and can be frozen (Wild 2013). There is little variation between each production lot. Monoclonal antibodies are readily conjugated, linked to a molecule as a label for better detection, and easily characterized (Hnasko 2015).

Polyclonal antibodies are easier to manufacture. The antigen is injected multiple times in the non-target animal species, generally rabbits. The sera from the animal is harvested, drawn during peak antibody production, and then pooled (Hnasko 2015). The quality of the polyclonal antibodies depends on the purity of the immunogen as well as the purification process of the sera (Wild 2013). The ability of the polyclonal antibody to bind to multiple antigen epitopes increases the detection ability. This helps with the vulnerability of the assay. Assays are designed to detect certain characteristics of the antibody. For polyclonal antibodies, small changes in antigen presentation may not impede the ability to bind. One disadvantage of using polyclonal antibodies is that they may have poor binding ability. Another disadvantage is the inability to re-produce the polyclonal antibody exactly, thereby disabling the antigen's detection ability. The sera from the

non-target species may produce antibodies; however, the pool may be different or variable in some capacity. This may result in a change in the binding ability of the antibody (Hnasko 2015).

New technology has developed to allow production of recombinant antibodies where non-target animals were traditionally used to produce an immune response. These antibodies are isolated and cloned. The antibody heavy chains and light chains are fragmented, separated, then reproduced and amplified separately. The light chains are cloned first. Then, cloned heavy chains are inserted and linked to the light chains. This synthesized process is advantageous to use due to production speed, increase of diverse specificity, elimination of cross reactivity, and the optimization of binding affinity and specificity of the antigen. Some disadvantages of this technology include limitations of the semi-synthetic gene libraries and fusion proteins are describes but not fully available commercially (Wild 2013).

Types of ELISA Methods

Immunoassays function similarly by binding an antigen to an antibody. There are different techniques to obtain this binding function. The assays are designed to either detect the presence of antigen or test for antibodies that recognize an antigen. The four techniques are direct, indirect, sandwich, and competitive (see Figure 4:2).

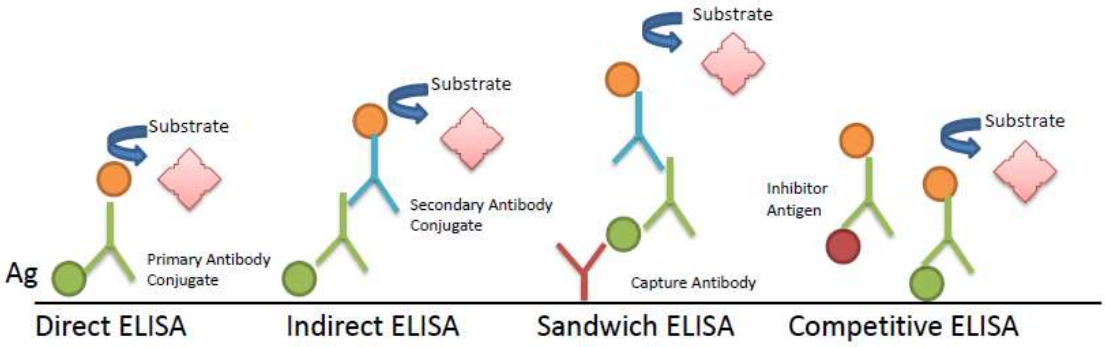

Figure 4:2 Schematic of ELISA Techniques

(Figure based on Figure 1 page 2 of 8 Heiat, Mohammad, Reza Ranjbar, and Seyed Moayed Alavian. "Classical and Modern Approaches Used for Viral Hepatitis Diagnosis." Hepatitis Monthly 14.4 (2014): e17632. PMC. Web. 9 Apr. 2017.) (Ag is antigen)

Direct ELISA methods use passive absorption of the antigen of interest to the microtiter plate. Direct methods are useful to qualitatively or quantitatively measure the antigen in the sample. It also is used for antibody screening and epitope mapping. One advantage to the direct ELISA method is the lack of cross reactivity in the microtiter plate. The antigen only binds to a single antibody. As a result, the assay is performed in less time. The assay is useful to analyze for epitope mapping and antibody screening (Hnasko 2015).

Indirect ELISA methods are similar to direct with the exception of the use of a secondary antibody. The secondary antibody is used to amplify the signal of the primary antibody bound to the antigen. The primary antibody is unlabeled, that is linking the antibody to an enzyme. An anti-species enzyme-conjugated secondary antibody is added to the microtiter plate and binds to the primary unlabeled antibody. The enhancement of the primary antibody signal makes the method more sensitive that direct ELISA methods. A disadvantage to the method is that a higher background signal is produced and can decrease the overall net signal of the assay (Hnasko 2015). It also may have more non-specific binding to interfere with the signal; therefore, the

purity and specificity of the antigen bound to the microtiter plate are critical components when evaluating specificity of the ELISA method (Wild 2013).

Sandwich ELISA methods use two antibodies to bind to different sites of the antigen. The primary antibody, or capture antibody, is bound to the microtiter plate. The microtiter plates are usually commercially available 96-well polystyrene plates. This primary antibody is highly specific to the antigen of interest. Samples containing the antigen, or analyte, are added to the plate. Conjugated, attached, detection antibody follows the sample addition. The detection antibody is conjugated to an enzyme. The result, the antigen is sandwiched between the primary antibody and the detection antibody (Wild 2013). This type of assay is very common due to the sensitive, precise, and robust format. However, the disadvantage is the antigen must have two different epitopes to bind with (Wild 2013). Sandwich assays are ideal animal safety samples (Hnasko 2015).

The final assay technique used for ELISA is competitive. This is based on direct, indirect, or sandwich ELISA methods. They are very complex methods and are called inhibition ELISA methods. Used mainly for the detection of small molecules with low molecular weights, the assays rely on enzyme-linked antigens to bind and compete with the antigens of interest in the microtiter plates. Signal detection is inversely related to the quantity of an antigen in the sample. The sensitivity of the assay is defined by equilibrium constant of the antibody, the precision of the signal measured, and the level of the non-specific binding. Therefore, it is not as sensitive of an assay as direct sandwich. An advantage for using competitive ELISA is the purification of the sample is not needed. One disadvantage to competitive ELISA methods is the timing of the incubation steps. These are critical to reproducibility of the assay. Small molecules, such as steroids, work well with this technique (Wild 2013).

ELISA Methods

Each assay is designed in a way to optimize the detection of the antigen or antibody. It starts with determining the reagents needed in the assay. The antigen-antibody binding affinity is maximized to create an environment where they are most adapted to each other. The antigen-antibody binding affinity is measured by the dissociation equilibrium between the two, the lower the dissociation between the antigen and the antibody the stronger the binding affinity. Determination of which antibodies work best for capture or detection is crucial to the performance of the method. These two roles are interchangeable. Each method should determine which role the antibody is best suited. If the method requires a capture antibody, the capture antibody is added to each well of the 96-well microtiter plate. It is bonded to the plastic of the plate by non-covalent bonds between the hydrophobic proteins and the plate surface. Blocking is the next step of the method and is essential to reducing non-specific binding of the primary or secondary antibodies to the solid surface of the plate. Washing buffers are added to the plate to remove any biological or chemical interfering components that may reduce assay signal. Wells of the plate should remain moist during most of the assay in order for the wash steps to be effective. The secondary antibody is added and is used to generate a signal. They are usually enzyme-labeled and are purchased commercially or produced by the company. These induce signal amplification as previously stated (Wild 2013).

The two most commonly used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (ALP). These enzymes are conjugated to either the primary or the secondary antibody, that is the labeling of the antibody, and are used in the detection of the antibody stage. Conjugating the primary antibody can be difficult because it lacks signal amplification and results in weak or no signal for detection which is why many assays conjugate the secondary

antibody. When this is the case, the conjugation of the secondary antibody allows for the primary antibody detection without direct conjugation. A substrate solution is added for color development. HRP and ALP require different substrates to produce light. Substrates tetra-methylbenzidine (TMB), 3, 3'-diaminobenzidine (DAB), and 2,2'-aziro-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) are recommended for HRP conjugate while chromogen *p*-nitrophenyl phosphate (PNPP) is recommended for ALP. After the substrate solution is added, the stopping solution is added to stop the chemical reaction of the substrate with the enzyme. At this point, the microplate is placed on a spectrophotometric plate reader, where a specific wavelength of light is transmitted through the microtiter plate and the absorption of light is read (Wild 2013).

Methods may include either a positive control or negative control sample. These samples are manufactured in the same manner as the product. Positive controls include all components of the product while negative controls remove the antigen or antibody of interest. These controls are used in methods for acceptance of the readings due to the known quantities of antigen in the positive controls or the lack thereof in the negative controls. The criteria of these are determined during development and validation of the assays. If either of these have a result that is not expected or outside validated criteria, the data is deemed not reliable then testing is repeated. If the results are within acceptable limits the second time, the data is usually accepted and reported. If the results show a similar unexpected trend, the data and assay are investigated to find the cause of the erroneous result(s). These controls help show assay reliability and reproducibility and ensure a rugged method by ensuring no false positive or false negative responses are reported.

Many plate readers are commercially available. Perkin Elmer VICTOR™ X3 Multilabel, Berthold LB962 CnetroPRO, and Molecular Devices Spectromax are some examples. For regulatory compliance, the instrument is validated, that it is tested several times and produce the desired result within a specified tolerance each time. The industry is moving towards instruments that are considered GXP where the X represents laboratory or manufacturing so that the software is valid for either Good Practice regulation. The data generated by a plate reader is in absorbance readings or optical density based on the wavelength used. The wavelength is determined during the development and validation of the assay. The absorbance readings or optical density are then quantifiable when transferred to a calculation software, usually excel, or some other system. A report is generated to show the method used and the outcome of the testing.

Chapter 5 - Flow Cytometry

History

Flow cytometry is the study of physical or chemical properties of cells. These properties or characteristics are then measured (Shapiro 2003). Since cell discovery in the 1600's, information about cells has expanded, such as structure and complexity as well as function within a cell and different cell types. In the 1800's, cell theory and germ theory were published. Cell theory is the idea that cells are the fundamental structure and functional unit in living organisms that propagate from other cells and is the foundation of modern biology. Germ theory is the development of disease by microorganism growth. Cells were observed and researched through the use of microscopes (Herzenberg et al. 2002). Around 1934, Moldovan developed a photoelectric method for counting individual cells while flowing through a capillary tube mounted on a microscope. These tubes unfortunately were problematic due to size. The tubes were difficult to manufacture to a standard size or flatten for ease of viewing under the microscope (Moldovan 1934). Later, Gucker et al. developed an instrument for the analysis of dust, which was also used for the study of microbiological specimens. This instrument was used by the U.S. Army to detect airborne bacterial spores during World War II (Gucker et al. 1947). In the mid 1950's, Wallace Coulter wanted to measure cell volume, and subsequently, developed a flow instrument. This instrument, deemed Coulter Counter, determined the number of cells in a suspension. The Coulter Counter measured the electrical conductivity difference between the cells and the suspension medium (Robinson 2013). The 1960's was the decade in which the first flow cytometer was made available with the ability to quantitatively measure the physical and chemical properties of cells. Cell sorters were also developed during this time period (Herzenberg et al. 2002).

Mack Fulwyler at Los Alamos National Laboratories and Dr. Louis Kamentsky at IBM enhanced their flow cytometers to include a cell sorter. Kamentsky's instrument included a syringe pump, while Fulwyler's used an ink jet printer. Injected samples followed through the measurement system to be atomized into droplets. Each droplet with cells was electrically charged. The electrically charged particles within each droplet were collected in a vessel, while the remaining uncharged particles went to waste. Next, the use of fluorescence measurement to improve both the quantitative and qualitative analyses was implemented. Kamentsky left IBM to start Bio/Physics Systems, where the first fluorescence flow cytometer with an argon laser was built (Shapiro 2003). Fluorescence Activated Cell Sorter (FACS) was invented by Bonner, Sweet, Hulett, Herzenberg, and others. This instrument was and is used today for flow cytometry and cell sorting on viable cells, such as blood cells or organ cells. By the 1970's, Becton Dickinson Immunocytometry Systems introduced commercial machines with the help of the Stanford patent (Herzenberg et al. 2002). The optic improvements during this time period, along with the development of better fluorescent stains, lead to the use of the flow cytometer in the research of cells.

In the industry, flow cytometry is used as an analytical technique to provide support testing for products. Although, mainly used in the human health testing under medical device regulations, it is beginning to gain use in the animal health industry. It provides a way to test for biomarkers after administration of a biological product to test for immune response or pharmacodynamics responses after administration of a pharmaceutical product.

Theory

Flow cytometers use electrical and/or mechanical means to divert or collect cells of programmed characteristics. These instruments analyze the physical and chemical characteristics

of particles within a fluid stream as it passes by at least one laser. A single cell per second, up to thousands of cells per second, can be analyzed during the analysis of a sample. Each instrument is comprised of three main parts: the fluidics, the optics, and the electronics. See Figure 5:1 for flow cytometry diagram.

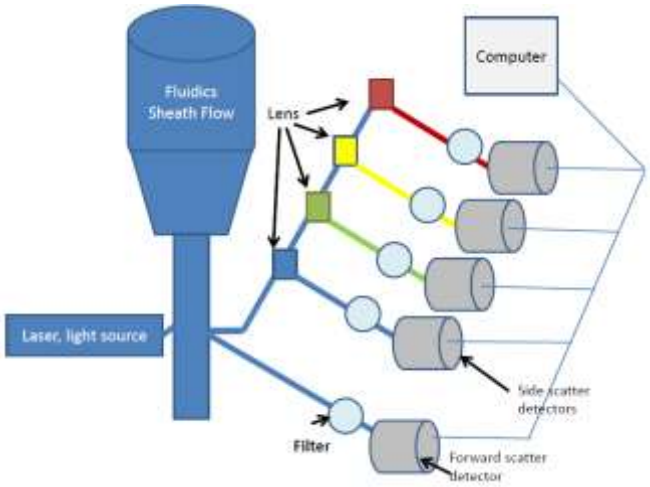

Figure 5:1 Diagram of Flow Cytometry Instrumentation

Flow cytometry measures the energy emitted by the photons after the laser lights up the cell. Photons are pure electromagnetic energy, where the absorption or emission of these by atoms or molecules is the only mechanism by which they are able to gain or lose energy. Electromagnetic radiation, or light, detects photons and behaves similar to a wave traveling at the speed of light. Light is emitted at wavelengths between 400 and 700 nanometers (nm). Using frequency or wavelength, the energy of a single photon is calculated. Photons were derived from incandescent sources, where temperature increases can cause the incandescent object to shift toward a high modal value and produce light (Shapiro 2003).

An alternative to incandescent objects is the use of a laser. A laser does not require incandescence to product light. It was previously determined that light interacts with matter in the electric field vector, and is the basis for all calculations (Shapiro 2003).

The flow cytometry principle is based on light scattering, more importantly, on Mie scattering seen in Figure 5:2. This principal, named for Gustav Mie, explains the process of light that is scattered in the forward direction. Flow cytometry measurements on cell size are based on this principal. Mie scattering involves interference, where light scatters by both reflection and refraction at the surface (Shapiro 2003).

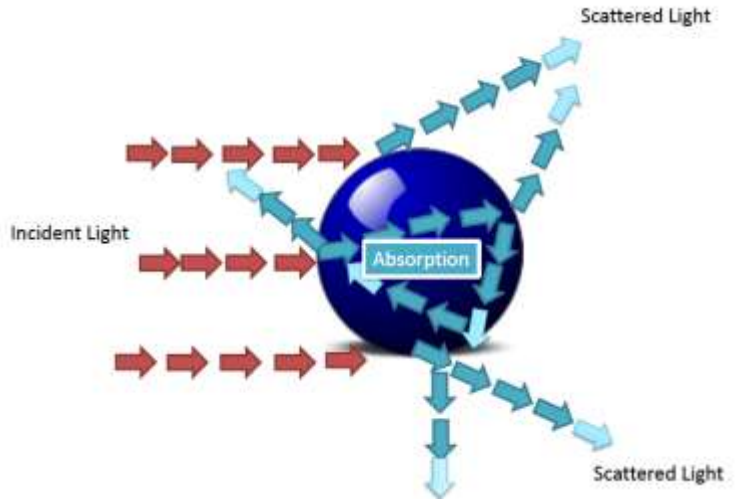

Figure 5:2 Mie Scattering Diagram
(Figure based on Figure 1 page 2 of 8 Malvern Instruments (2016). Mie theory: the first 100 years. United Kingdom, Malvern Instruments Limited.)

Absorption of the photons raises the molecule to a higher-energy excited state from its minimum-energy ground state. This change is called transition. Absorption of light by an atom or molecule produces electronic transition. Fluorescence of the atom or molecule causes the cell to emit all of some of the photons remaining for detection by the instruments as they approach their ground state. This is accomplished by fluorescent probes or dyes (Shapiro 2003).

Flow Cytometry Components

Fluidics system

The fluidics portion of the instrument is the stream of fluid used to transfer the sample containing the cells to the laser. Two components of the fluidics system are sheath flow and pressurized lines. The sheath flow, or hydrodynamic focusing, centralizes the sample, or core fluid, containing the cells to the central portion of the flowing stream. Flow requires stability through the whole process that is in a laminar flow pattern. Sheath fluid is pressure driven. Water is not ideal for the fluid of choice due to its high viscosity. However, phosphate-buffered saline or PBS is commonly used. Samples align in single file due to the pressure difference between the sheath fluid and the sample stream. Therefore, it is essential to keep the sheath flow debris free so the flow is not obstructed (Shapiro 2003). Flow rate of the sheath fluid can vary. The flow can be high for common applications, such as immunophenotyping of mammalian cells, or slow for quantitation of DNA content in fluorescent-stained nuclei. For the best uniformity and accuracy by the laser beam, slow flow rates are recommended (Wilkerson 2012).

Optics System

The optic system is where the laser illuminates the particles present in the fluidic stream as they pass through the instrument and scatter the light. This optics system uses lenses, glass, plastic or quartz, with a shape that makes rays of light reach a distant point. Lenses are used to decrease or increase the size of the image. A photon streaming along an optical axis is guided by the lens. The lens is convex in design where the material is of refractive index greater than that of air, and the center is the thickest (Shapiro 2003).

The laser is used as the light source because it is generally confined to the very small solid angle with the ability to focus all of the energy in the beam. The laser beam is directed at

the lens in the flow cytometer. Argon ion lasers are popular with flow cytometry for their fluorescence. Air-cooled argon-ion lasers are the most commonly used flow cytometry laser in the industry. Krypton ion lasers are another type that functions similarly to the argon-ion. Krypton lasers do not emit the same wattage of light as argon; 1 watt of light with krypton compared to 5 watts of light with argon. Other lasers include dye laser, helium-neon, diode lasers and more. These can pose some health risks where argon lasers do not. Argon-ion lasers usually operate at a wavelength of 488 nm. Plasma is the lasing medium, which is pulsed at high-voltage to ionize the gas to start the plasma. Argon ion lasers are suitable for measurements of weak fluorescence. Laser noise, background noise of the instrument readings, can come from a variety of sources. "Noise eaters" minimize the noise and are placed in between the laser and the rest of the mechanisms or by a noise compensation circuit which is more costly than the noise eaters (Shapiro 2003).

Optical filters are used to detect spectral responses in the flow cytometer. Filters are helpful in removing unwanted light wavelengths and direction of the wanted wavelengths to the detector. An enhancement to the flow cytometer was the use of monochromatic or polychromatic detectors. Monochromators divert different wavelengths of light into different directions then positioning a slit between the gating and the detector. Multiple polychromatic detectors are placed along several regions of the image. Despite this new technology, filters are the most cost effective option (Shapiro 2003).

The light is collected and is detected by detectors. On type of detector is a photodiode, which does not require an external power source and usually operate in the photovoltaic mode. Photodiodes converts light into current and are more efficient. Photomultiplier tubes are another type of detector where the tubes produce current at the anodes when photons reach the light-

sensitive photocathodes. They require shielding from stray light and magnetic fields. Photomultiplier tubes are used as detectors for weak signals, such as fluorescence scatter. Also available is the avalanche photodiode, which combines both the properties of photodiodes and photomultiplier tubes. Photomultiplier tubes, however, are the most desirable (Shapiro 2003).

Electronics System

The electronics system changes the detected light signals into electronic pulses for the computer to process. The instrument recognizes that the cell is present. When the cell is not present and not passing through the observation point, the response output is the background or baseline noise. Once the cell is acknowledged as present by the system, a pulse appears. The response remains active until the signal drops below the specific threshold. The output is either linear or logarithmic (Shapiro 2003).

To measure the cells, three physical parameters are measured. These are forward and side light scattering and fluorescence, see Figure 5:3. In forward angle scatter, the light is diffracted, or the process by which a beam of light is spread out, around a cell and proportional to the cell size. Sid angle scatter is at 90 degree angle and is proportional to the internal complexity of that cell. The light scatter properties help distinguish each cell based on their size and internal complexities (Wilkerson 2012). Fluorescence is the loss of some of the absorbed energy of the photons by light emission. This happens after the molecule first absorbs light at a wavelength shorter than or equal to the emitted light resulting in an excited electron state. The emission occurs at a wavelength that is longer than the excited wavelength (Shapiro 2003).

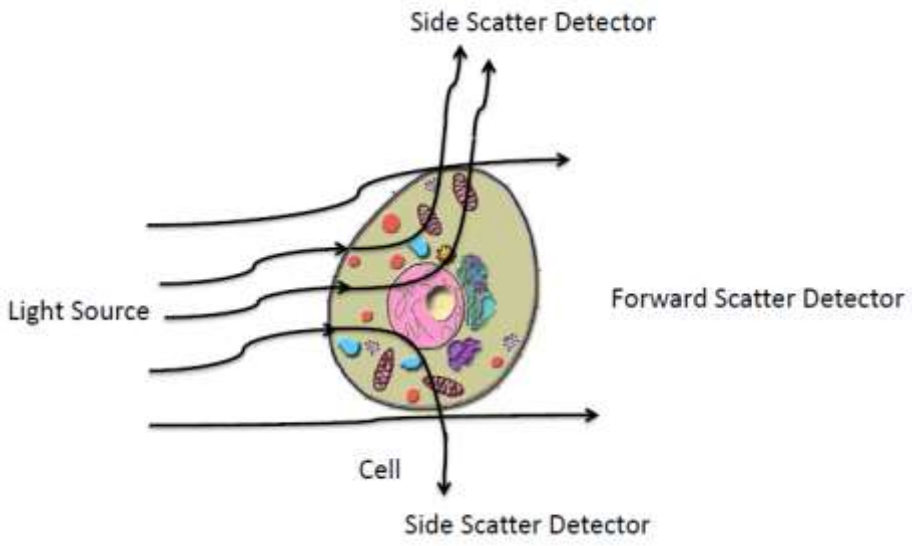

Figure 5:3 Forward and Side Light Scatter

(Figure based on Figure 3-1 page 10 of 52 Dickinson, B. (2002). Introduction to Flow Cytometry: A Learning Guide. Becton Dickinson and Company.)

Reagents

Not all samples analyzed by flow cytometry require the addition of reagents prior to analysis. However, some reagents are needed to help with analysis of the samples. For those samples that need to distinguish the different types of cell structures, the reagents, called probes, are added to the sample to bind to the structure of the cell of interest. They are essential in providing the means from which the detection of the cells happens. Probes, or fluorochromes, are added to the samples to help with the light absorption. Fluorochromes are used to label proteins, nucleic acids, and reporter molecules. Synthetic and natural dyes are available. Natural dyes include algae and phytoplankton. These are excited by an argon laser and results in a higher wavelength for emission. Synthetic dyes include fluorescein isothiocynate (FITC) and phycoerythrin (PE). FITC, excited by the argon laser, will emit in the green spectrum, 535 nm, while PE will emit in the orange spectrum, 585 nm (Wilkerson 2012).

When the sample is a whole blood sample, the blood needs is collected so that there is no cell activation (that is the activation of the immune response and cell division), death, or aggregation. Using K_2EDTA (Dipotassium ethylenediaminetetraacetic acid) anticoagulant in the collection tube will keep the cells from changing before analysis. After collection but prior to analysis on the instrument, the addition of the fluorescent-labeled antibody is added. The red blood cells are removed by the addition of a lysis buffer. Each type of sample requires a method specialized to it. For analysis that requires a labeled intracellular antigen, cells are made permeable by the addition of a permeable agent that will allow the antibody to enter the cell. In animal health, secondary conjugated antibodies are mainly required because primary antibodies are not as readily available (Wilkerson 2012).

Flow Cytometry Results

In order to gain understanding of the data, results are usually graphically displayed. The electronic system of a flow cytometer sends the pulse data to a computer with software capable of producing the numbers as well as graphs. Frequency distributions, or histograms, are used to examine the measured values of cell parameters. Single parameter analysis was widely used due to restrictions of the instrument. Recently, two parameter or multicolor analyses are available. For multicolor and two parameter data, dot plots or scatter plots are used to show relationship between parameters/colors. Generally, the pulse data is collected into bins. The binned data is then used to produce the distribution of the measured values. The dyes or stains are added to help identify the different phases of the cell cycle as well as the cell behavior and chemical composition (Shapiro 2003). See Figure 5:4 for flow cytometry results display. Here the scatter plot shows on the x-axis the forward scatter and the y-axis is the side scatter of the cells. When the cells are dense, these are considered small cells because they are compact, the lower part of

the scatter plot. Larger cells are not as densely displayed, the middle of the scatter plot. After the cells are initially read by the instrument, the computer software is used to segregate a population of cells by refining the gating. With each refinement, the scatter plots reveal more about the cells of interest.

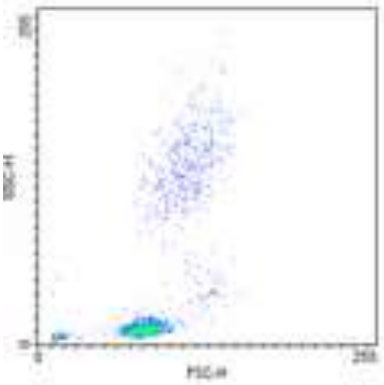

Figure 5:4 Results Displayed by Flow Cytometry

Chapter 6 - Practical Uses

In the animal health industry, analytical techniques are used for a variety of applications. Figures 2:1 and 2:2 showed the timelines for the product development. Each company is responsible for developing and validating the methods used when submitting reports for market approval. Guidelines provide direction on governmental expectations of the methods.

Each method is to describe the chemicals and reagents, instrumentation, concentrations, and more in order for the analysis by the method. Acceptance criteria of the run is either included in the method or in Standard Operating Procedures provided by the company and are in line with regulations.

GMP Analysis (*Title 21 of the CFR parts 210 and 211*)

For Active Pharmaceutical Ingredient and veterinary product, analytical procedures are used. These procedures test for purity of the API and product or potency of the vaccine, as well as stability of the product during storage at the warehouse or in a home, which is considered shelf-life. The results of the testing are recorded in a Certificate of Analysis for each lot or batch of product. This document describes the tests conducted, and the results of the tests to show the quality of the product and leads to shelf-life of the product. For an API or drug product, the testing is evaluated using LC/MS techniques. Stability testing for shelf life gives each product an expiration date as it awaits distribution.

GLP Analysis (*Title 21 of the CFR part 58*)

When determining safety of a pharmaceutical product, the testing is conducted as bioanalysis. These methods are developed to show the concentration of the compound through dosing time. The test protocols are designed to determine the length of time for the drug to move through the animals system and be eliminated. This is to make sure the drug is not given at too

high of level to cause an adverse reaction, such as death. These bioanalytical methods are described and include the chemicals and reagents, instruments, standard concentrations necessary to determine sample concentration. LC/MS is a typical technique used for small molecule bioanalysis. Small molecules are drugs with low molecule weights, such as Aspirin with a molecular weight of 180 g/mol. Molecules with high molecular weights are considered large molecules. These are molecules based on proteins with a molecular weight as high as 150,000 g/mol. These can be analyzed by LC/MS or HPLC, as well. ELISA is generally used for these types of compounds. Each method is specific to each compound.

USDA Biological Analysis (*Title 9 of the CFR*)

Sometimes the analysis is not limited to the analyte or organism, such as bacteria or virus of interest. In some cases the technique is used to analyze the reagents of the test method. When using an ELISA to test for the antigen of interest in a vaccine, the reference material, conjugate, monoclonal or polyclonal antibodies, and more critical reagents are analyzed and compared to the previous batch to ensure the method is producing results similar to the originally validated method. These analyses help to keep the method uniform. Reference material for use in vaccine testing is usually stored for an extended period of time. To ensure the reference material has not degraded over time, the material is analyzed and compared to itself after several analyses and trending is reviewed. If the trend does not show an increase or decrease over time, the reference material is considered stable. The reference material is used during analysis of the vaccine sample to ensure the newly produced vaccine is within the potency guidelines. The target of the vaccine production is to have a response equal to or greater than the reference material.

Reference material for vaccine production has several requirements for stability. One determination of stability is the back comparison to the analyzed data collected over the life span

of the storage. Another way to support the stability of the reference material is by development other analytical techniques. One technique for stability evaluation of the reference material is the use of LC/MS. The reference material is analyzed on the instrument and compared to the historical data with trending. Trending is the study of the material over time. The reference material is evaluated every 2.5 years to see if it behaved similar between each use. If the data trends up or down from the average, the methods and the reference material are investigated to determine the problem. If the method is found to have little changes, the reference material is changed to a new lot and compared with the historical data of the old lot for assay results. VSM 200.211 guides the process and expectations to follow.

Stability of the vaccine is also determined by assay techniques. The product stability is tested with ELISA using current reference materials and reagents or by other assay techniques; however, the other techniques are not addressed in this report. When the results of the stability test are within the guidelines, the test is accepted.

Other Analyses

In the industry, flow cytometer use is for analysis of cells of a sample. Typical analysis for samples is to count the viable cells of interest. The instrument sorts the cells, and then, it evaluates the number of cells based on approved protocols. Protocols, such as determination of DNA, immunological studies, and stages of cell death, are developed. Flow cytometry is quickly gaining acceptability within the animal health community. Immunological studies use flow cytometry for determination of T lymphocytes and the roles they play in autoimmune diseases.

Biomarker analysis is another type of analysis a flow cytometer can produce. This type of analysis is used to characterize an animal sample by objectively measuring and evaluating to indicate the normal biological process, pathogenic process, or pharmacologic response to a

therapeutic intervention, such as pharmaceutical product or vaccine administered for the health and wellbeing of the animal. A vaccine would illicit a normal biological response because it encourages antibody production in the presence of antigen. Disease is the outcome of a pathogenic process after infection with a bacteria or virus when these replicate within the body. The biochemical or physiological effects of a treatment by a pharmaceutical product is a pharmacological response. Biomarkers are associated with each type of assay and are evaluated. This analysis is used in support of the drug or vaccine getting to market.

Modern flow cytometry is a useful tool in diagnosing of cancer or other health problems at the cellular level. The instrument can detect changes in the cells when compared to a normal cell and show what stage of cancer a small animal, such as a dog, might have if diagnosed with lymphoid neoplasia or leukemia.

Each of these assay protocols would be used to provide qualitative data in support of the approval process. In the next chapter, some practical examples are provided to describe how each technique is applied in the animal health industry.

Chapter 7 - Practical Examples

Liquid Chromatography with Tandem Mass Spectrometry to measure Zilmax® (Merck Animal Health) in cattle urine and faeces

Beta-agonists are useful in the health and welfare of animals to help fatten the cattle prior to processing. Beta-agonists belong to the chemical class of catecholamine and bind to beta-2-receptors to stimulate the receptors to relax the muscle. Also used as bronchodilators to treat pulmonary diseases such as asthma in humans, these drugs improve carcass composition as the animal decreases their fat content in favor of muscle. The drug product is given as a low dose to cattle to help fatten the animal by allowing the animals body to focus on turning feed into muscle The European Union (EU) has banned the use of beta-agonist products. A product produced by Merck Animal Health called Zilmax, or zipaterol, is a beta-adrenergic agonist compound that is different from the well-known *N*-alkyl-beta-agonists and di-aromatic beta-agonists due to its chemical structure. Zilmax is given to cattle in their feed to help promote better muscle gains. A LC/MS method was developed for concentration of zipaterol in cattle urine and feces to help determine the elimination withdrawal time (Hoof et al. 2004).

Chromatographic separation of zilpaterol in urine was achieved by LC/MS using the following components. The column was Inertsil ODS C_{18} (3 micrometer particle size, 3.0 mm diameter by 100 mm in length). A gradient pumping scheme was deployed with a mixture of water and MeOH in the presence of ammonium acetate for 25 minutes total run time. The flow rate was set at 0.6 mL/min. The mass spectrometer was set to MS^3-mode, which is triple quadrupole mode. Zipaterol in feces was achieved by LC/MS using an Alltima C18 (5 micrometer particle size, 3.5 mm diameter by 250 mm in length) column with isocratic pumping at 0.5 mL/min for 4 minutes total run time. The mobile phase was a mixture of

pentafluorpropionic acid and acetonitrile. The mass spectrometer was set to MS^2-mode, which is also a triple quadrupole mode. Both methods gave results that were reproducible for testing of urine and feces after the oral administration of Zilmax to male veal calves (Hoof et al. 2004).

Enzyme-Linked Immunosorbant Assays to measure antibodies following vaccination with a new vaccine candidate in sheep

Bluetongue disease is monitored by the World Organization for Animal Health (OIE) due to the increase in transmission rate due to the environmental changes and economic loss due to the loss of trade for those animals that are infected. This disease is caused by a virus (bluetongue virus) and transmitted by *Culicoides* biting midges. Midges are a group of insects that includes small flies. Bluetongue disease causes significant economic loss to cattle and other ruminant farmers. Bluetongue virus (BTV) is usually a temperament climate organism; however, it was recently discovered in areas of milder climate. Vaccination for BTV is the most effective way of controlling outbreaks of the disease. Vaccines are currently available as both live attenuated or killed inactivated products. A new novel product for vaccination was developed. This uses the Disabled Infectious Single Animal (DISA) concept, in which this platform has removed the NS3/NS3a expression. NS3/NS3a is a non-essential protein which is synthesized in the BTV replication. This platform has the advantages of the live attenuated vaccines without the risks of viremia, or the presence of virus in the blood (Tacken et al. 2015).

To determine the presence or absence of BTV after vaccination and challenge, a competitive ELISA method was developed. The NS3 was produced, then tagged and purified. The purified NS2 tagged antigen, monoclonal antibody 33H7, and rabbit alpha-mouse serum conjugated to horseradish peroxidase were used to coat the maxisorp plates and incubated overnight at 4°C. Prior to incubation, a solution of phosphate buffered saline (PBS) with 0.01%

Tween 20 and 5% fetal bovine serum (FBS) was used to coat the wells to reduce non-specific binding. The collect sera from the animals was diluted with the PBS/Tween/FBS solution and added to the wells then incubated again. The plate was washed to remove unbound sera antibodies. Monoclonal antibodies were added and the plate was incubated. The plate was washed and conjugated rabbit alpha-mouse serum was added. After incubation, TMB substrate was added and color development was stopped with addition of a stopping solution. Optical density was read at 450 nm. The results of the study showed the ELISA assay detected BTV infections after vaccination and challenge for BTV serotypes 1-24. The ELISA test is not 100% sensitive or specific for antibodies directed against BTV NS3; however, it is a good tool to monitor DISA vaccinated populations for BTV circulation (Tacken et al. 2015).

Flow Cytometry to measure IFNγ Expression after vaccination in cattle

Bovine tuberculosis has caused significant economic loss; for British taxpayers it costs £100 million (~$107 million U.S. dollars) each year. In 2016, bovine tuberculosis caused the slaughter of 28,000 cattle for control of the disease (Leadsom 2016). This global disease problem for animals is considered an increasing economic issue for the future; therefore, vaccination is needed to effectively fight against outbreaks. A better understanding of the protective immune response to the disease will help with development of a vaccine. Memory CD4 T-cells were identified and defined in cattle. Four distinct CD4 T-cell populations were purified by cytometric cell sorting and purified (Blunt et al. 2015).

Six Holstein-Friesian cattle were used in the study. Three were given the vaccine, and three were considered naturally infected. The study was designed to determine if there is a difference between responses of vaccinated or naturally infected cattle.

Cattle blood was collected and the peripheral blood mononuclear cells were prepared from peripheral blood by gradient density centrifugation, and the $CD4^+$ T-cells were enriched by paramagnetic bead isolation using monoclonal antibody CC8 (IgG_{2a}). The $CD4^+$ T-cells were then stained with monoclonal antibodies CC32 (IgG_1) and IL-A116 (IgG_3). A second staining was done with rat monoclonal antibody to mouse IgG_1 (brilliant violet 421), goat anti-mouse IgG_{2a} (FITC), and goat anti-mouse IgG_3 (R-phycoerythrin). The cells were incubated, washed, and re-suspended in RPMI 1640 medium used to culture cells in a suspension. Next, the cells were passed through a 30 micrometer filter for sorting. The flow cytometer was set at 60 psi with a 70 micrometer nozzle. After sorting, the cells were greater than 98% pure for each of the four populations (Blunt et al. 2015).

The sorted CD4 T-cells were evaluated for cytokine response. The flow cytometry results prior to sorting showed the majority of cells expressed cell surface CD45RO. The sorted cell populations were used in the experiments to show the different cell populations in vaccinated and infected animals. The cells were stimulated *in vitro* to show the frequency of IFNγ secreting cells. Additional flow cytometry analysis was performed to test for CD4 T-cell plasticity during culture. Plasticity is defined as the switch phenotype that is up- or down- regulated within stimulation (Blunt et al. 2015).

The study used the flow cytometry data to support the testing *ex vivo* ELISPOT. The *in vitro* testing of the cells surface markers showed plasticity, which would make it difficult to directly correlate the observed *ex vivo* results (Blunt et al. 2015).

Chapter 8 - Conclusion

The animal health industry continues to grow as the demand of the importance for the welfare of the animals continues. There are many animal health products available for purchase to help in multiple therapeutic areas. These products go through rigorous testing to prove their viability for use before marketing approval. The top animal health companies spend billions of dollars and many years developing products following the governmental regulations and guidelines. The regulations enacted to enforce each law by the U.S. government depend on which type of product is marketed.

Pharmaceutical products for animal care are governed by the Center of Veterinary Medicine (CVM) of the Food and Drug Administration (FDA). The CVM follows *Title 21 of the CFR*, the regulation based on *Title 21 U.S.C.*, the *Federal Food, Drug, and Cosmetic Act.* The *Federal Food and Drugs Act* of 1906 was enacted to ban interstate transportation of adulterated or misbranded food or drugs. *Title 21 of the CFR* gives guidance and expectations the FDA regulators inspect against when determining if a product is ready for marketing. They will inspect for compliance with *Title 21 of the CFR* Part 58 for GLP analysis and Parts 210, 211 or 226 for GMP analysis and all associated guidance for the industry documents.

Biologic products, such as vaccines, bacterins, and antisera, for animal welfare are governed by the Center for Veterinary Biologics (CVB) of United States Department of Agriculture (USDA). The CVB follows *Title 9 of the CFR*, the regulation based on *Title 21 U.S.C.*, the *Virus-Serum-Toxin Act.* The *Virus-Serum-Toxin Act* of 1913 was enacted to provide the government the ability to give license for biological products as well as regulate trade of those products biologics. The regulators inspect each product license application for safety and efficacy of the product for the target animal. The regulators will inspect each application against

the *Title 9 to the CFR* and associated veterinary service memoranda to determine if the product met the requirements to show its purity, safety, potency, and effectiveness.

In order to fulfill the testing requirements, companies are asked to validate testing protocols or instrumentation methods. Analytical techniques for testing include high performance liquid chromatography (HPLC), liquid chromatography with tandem mass spectrometer (LC/MS), enzyme-linked immunosorbent assay (ELISA), and flow cytometry. Each technique has several components to make the assays work. Depending on what the need is, the technique is used for practical applications. Analytical analysis employs the use of HPLC or LC/MS for determining stability of the active pharmaceutical ingredient or drug product shelf life. Bioanalysis for determination of concentration versus time in sampling employs HPLC, LC/MS, and ELISA. Vaccines use ELISA and LC methods when determining reference material stability and critical reagents as well as potency and shelf life. When a study protocol requires more information, flow cytometry could be used to detect cellular biomarkers. Animal health is dependent on the accuracy of these analytical methods to show the regulators the usefulness of the product and ensure the regulators the products are safe and efficacious to treat animal health and welfare.

References

Blunt, Laura, Philip j. Hogarth, Daryan A. Kaveh, Paul Webb, Bernardo Villarreal-Ramos, and Hans Martin Vordermeier. 2015. 'Phenotypic Characterization of bovine memory cells responding to mycobacteria in IFN gamma enzyme lined immunospot assays', *Vaccine*, 33: 7276-82.

Center for Veterinary Medicine. 'About the Center for Veterinary Medicine (CVM)'. http://www.fda.gov/aboutfda/centersoffices/officeoffoods/cvm/default.htm.

Congressional Research Service. 1989. *Food, Drug, and Cosmetic Act* (Library of Congress).

Ettre, Leslie S. 2003. 'M.S. Tswett and the Invention of Chromatography', *LCGC North America*, 21.

Griffiths, Jennifer. 2008. 'A Brief History of Mass Spectometry', *Analytical Chemistry*, 80: 5678-83.

Gucker, Frank T, Chester T O'konski, Hugh B Pickard, and James N Pitts. 1947. 'A photoelectric counter for colloidal particles.', *Journal of the American Chemical Society*, 69: 2422–31.

Herzenberg, Leonard A, David Parks, Bita Sahaf, Omar Perex, Mario Roederer, and Leonore A Herzenberg. 2002. 'The History and Future of the Flourescence Activated Cell Sorter and Flow Cytometry: A View from Standard', *Clinical Chemistry*, 48: 1819-27.

Hnasko, Robert. 2015. *ELISA Methods and Protocols* (Springer New York Heidelberg Dordrecht London).

Hoof, N Van, R Schilt, E van der Vlis, P Boshuis, M Van Baak, A Draaijer, K De Wasch, M Van de Wiele, J Van Hende, . Courtheyn, and H De Brabander. 2004. 'Detection of zilpaterol (Zilmax) in calf urine and faeces with liquid chromatography-tandem mass spectrometry', *Analytical Chimica Acta*, 529: 189-97.

Lamont, Richard J, George N Hajishengallis, and Howard F Jenkinson. 2013. *Oral Microbiology and Immunology* (ASM Press: Washington, UNITED STATES).

Leadsom, Andrea. 2016. 'Bovine TB:Written statement - HCWS376', Department for Environment, Food and Rural Affairs, Accessed March 22, 2017. https://www.parliament.uk/business/publications/written-questions-answers-statements/written-statement/Commons/2016-12-16/HCWS376/.

Lequin, Rudolf M. 2005. 'Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA)', *Clinical Chemistry*, 51: 2415-18.

Lovelock, James. 2004. 'Archer John Porter Martin CBE. 1 March 1910 - 28 July 2002: Elected F.R.S. 1950', *Biographical Memoirs*, 50: 157-70.

Martin, Archer J P. 1952. *The development of partition chromatography*.

McKinney, Richard J. 2002. 'A Research Guide to the Federal Register and the Code of Federal Regulations', *Law Library Lights*, 46: 10-15.

———. 2004. *United States Statutes and the United States Code: Historical Outlines, Notes, Lists, Tables, and Sources* (Law Librarians Society of Washington, D.C.).

Moldovan, Andrew. 1934. 'Photo-electric technique for the counting of microspoical cells', *Science*, 80: 188-89.

Monke, Jim. 2005. *The Virus-Serum-Toxin Act: A Brief History and Analysis* (Library of Congress. Congressional Research Service.).

Office of Federal Register. 2017. *A Guide to the Rulemaking Process* (Washington, D.C.).

Pitt, James J. 2009. 'Principles and Applications of Liquid Chromatography-Mass Spectrometry in Clinical Biochemistry', *Clin Biochem Rev.*, 30: 19-34.

Poole, Colin F, and Salwa K Poole. 1991. *Chromatography today* (Elsevier Science Publishers .V.: The Netherlands).

Robinson, J Paul. 2013. 'Wallace H. Coulter: Decades of Invention and Discovery', *International Society for Advancement of Cytometry*, 83A: 424-38.

Shapiro, Howard M. 2003. *Practical Flow Cytometery* (Wiley-Liss).

Skoog, Douglas A, and James J Leary. 1992. *Principles of Instrumental Analysis* (Saunders College Publishing: United States of America).

Tacken, Mirriam GJ, Franz J Daus, Femke Feenstra, Rene GP van Gennip, and Piet A van Rijn. 2015. 'Development of a competative ELISA for NS3 antibodies as DIVA test accompanying the novel Disabled Infectious Single Animal (DISA) vaccine for Bluetongue', *Vaccine*, 33: 5539-45.

Weintraub, Arlene. 2015. 'Top 10 animal health companies of 2015', FiercePharma, Accessed 1/9/2017. http://www.fiercepharma.com/special-report/top-animal-health-companies-2015.

Wild, David. 2013. *The Immunoassay Handbook: Theory and applications of ligand binding, ELISA and related techniques* (Elsevier Science).

Wilkerson, Melinda J. 2012. 'Principles and Applications of Flow Cytometry and Cell Sorting in Companion Animal Medicine', *Veterinary Clinics of North America: Small Animal Practice*, 42: 53-71.

Yarbrough, Cathy. 2016. "Why Animal Health Is The Next Big Growth Area." In *Life Science Leader*.

Appendix A - Acronyms

Acronym	Full Description
U.S.	United States
CVM	Center for Veterinary Medicine
CVB	Center for Veterinary Biologics
FDA	Food and Drug Administration
USDA	United States Department of Agriculture
APHIS	Animal and Plant Health Inspection Service
API	Active Pharmaceutical Ingredient
ELISA	Enzyme-Linked Immnosorbent Assay
LC	Liquid Chromatography
CFR	*Code of Federal Regulations*
U.S.C.	*United States Code*
OIRA	Office of Information and Regulatory Affairs
NPRM	notice of proposed rulemaking
GFI	Guidance for the Industry
VICH	Veterinary International Conference on Harmonization
CMC	Chemical Manufacturing and Controls
INAD	Investigational New Animal Drug
FOI	Freedom of Information
NADA	New Animal Drug Application
VSM	Veterinary Services Memorandums
DHS	Department of Homeland Security
PLA	Product License Application
SIF	Summary of Information
GMO	Genetically Modified Organism
DOI	Duration of Immunity
PLS	Prelicensing Serial
FST	Field Safety Trial

Acronym	Full Description
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
PCR	polymerase chain reaction
qPCR	quantitative reverse transcription PCR
RNA	Ribonucleic acid
NMR	nuclear magnetic resonance spectroscopy
°C	degrees Celcius
^{13}C	Carbon-**13**
UV	Ultraviolet
nm	nanometers
m/z	mass-to-charge
ESI	electrospray ionization source
APCI	atmospheric pressure chemical ionization source
APPI	atmospheric pressure photo-ionization
MRM	multiple reaction monitoring
LC/MS	Liquid Chromatography Mass Spectrometry
mL/min	milliliters per minute
ACN	Acetonitrile
MEOH	Methanol
cm	centimeters
mm	millimeters
µm	micrometers
HPLC	High Performance Liquid Chromatography
pH	potential of hydrogen
Ig	immunoglobulins
HRP	horseradish peroxidase
ALP	alkaline phosphatase
TMB	tetra-methylbenzidine
DAB	3, 3'-diaminobenzidine

Acronym	Full Description
ABTS	2,2’-aziro-bis (3-ethylbenzthiazoline-6-sulfonic acid)
PNPP	*p*-nitrophenyl phosphate
GXP	Good Practice where X can be Laboratory or Manufacturing
FITC	fluorescein isothiocynate
PE	phycoerythrin
K_2EDTA	Dipotassium ethylenediaminetetraacetic acid
g/mol	grams per mole
EU	European Union
ODS	Oxide dispersion strengthened alloys
DISA	Disabled Infectious Single Animal
OIE	World Organization for Animal Health
BTV	Bluetongue Virus
PBS	phosphate buffered saline
FBS	Fetal Bovine Serum
CD4	cluster of differentiation 4