A PROPOSAL FOR THE VALIDATION OF P24 SPECIFIC ANTIBODIES USED IN RAPID HIV SCREENING TESTS

by

Raza Zaidi

B.S. (UMBC) 2009

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

January 2019

Accepted:

Craig Laufer, Ph.D. Committee Member	Ann Boyd, Ph.D. Director, Biomedical Science Program
Ann Boyd, Ph.D. Committee Member	
Jeffrey Rossio, Ph.D. Project Adviser	April Boulton, Ph.D. Dean, Graduate School

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DEDICATION

I would like to dedicate this work to my parents, Ali and Yasmeen Zaidi, and my sister Zehra Zaidi. I could not have accomplished all that I have so far without their unconditional love and support.

ACKNOWLEDGEMENTS

I would like to acknowledge the commitment and guidance provided to me by reading committee members: Dr. Ann Boyd, Dr. Craig Laufer, and my project advisor Dr. Jeffrey Rossio.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

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Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
A proposal for the validation of p24 specific antibodies used in rapid HIV screening tests.

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle)	3b. DEGREE(S)	3h. eRA Commons User Name
Zaidi, Raza	B.S.	N/A

3c. POSITION TITLE
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Department of Biology

3f. MAJOR SUBDIVISION
Biomedical Science Program

3g. TELEPHONE AND FAX *(Area code, number and extension)*
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4. HUMAN SUBJECTS RESEARCH	4a. Research Exempt	
☒ No ☐ Yes	N/A	
4b. Federal-Wide Assurance No.	4c. Clinical Trial	4d. NIH-defined Phase III Clinical Trial
N/A	☒ No ☐ Yes	☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes — 5a. Animal Welfare Assurance No. N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
05/01/2017	05/01/2018				

9. APPLICANT ORGANIZATION
Name: Hood College
Address: Department of Biology
Hood College
401 Rosemont Ave
Frederick, MD 21701

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☐ Private Nonprofit
For-profit: → ☐ General ☒ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. N/A Cong. District N/A

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
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14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*
N/A

DATE
07 Mar 16

PROJECT SUMMARY (See instructions):

HIV and AIDS have remained a consistent issue for many countries around the world, even though there has been a lot of progress made towards preventing and treating HIV over the decades (CDC). After years of research and multi-generation rapid diagnostic tests, scientists are still struggling to develop a robust efficient early rapid diagnostic test that can quickly with high confidence detect early HIV infection. This project will attempt to address this issue by a proposal to validate p24 specific antibodies utilized in rapid diagnostic tests for the detection of HIV during early infection.

RELEVANCE (See instructions):

According to the Centers for Disease Control (CDC), in 2015 there were an estimated 38,500 new HIV infections, and over 1.1 million people in the United States living with HIV, out of which 15% did not know they were infected (CDC). Current rapid diagnostic tests are unsuitable for early HIV detection due to their high variability in sensitivity for the p24 antigen component (Lewis JM). An estimated 5-20% of HIV infections are due to transmission from recently infected individuals. Diagnosing HIV in the weeks after an individual has acquired the virus is likely to be critical in preventing an epidemic as early detection and diagnosis can allow prompt entry into care and proper treatment, reducing the risk of further transmission (Lewis JM). A highly specific and robust antibody to p24 is critical for the early detection of HIV infection which will play a profound role in decreasing the incidence of disease and ensuring on time necessary patient treatments.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:		State:
Province:	Country:		Zip/Postal Code:
Project/Performance Site Congressional Districts:			

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

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eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

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Hood College, Frederick, MD	M.S.	2018 (anticipated)	Biomedical Science

A. Positions and Honors

2017 - Present Associate Scientist

2013 – 2017 Quality Control Analyst

B. Selected peer-reviewed publications (in chronological order)

N/A

C. Research Support

N/A

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

N/A

Clinical:

N/A

Animal:

N/A

Computer:

N/A

Office:

N/A

Other:

N/A

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

N/A

-
-
-
-
-
-
-
-

The following consumables will be purchased with grant funding:

N/A

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SPECIFIC AIMS

The goal of this proposal is to recommend a strategy for the validation of p24 specific antibodies utilized in early detection of HIV in rapid diagnostic tests (RDTs). P24 specific antibody validation is one way to ensure that the rapid diagnostic tools that are used frequently around the world show high specificity and robustness. The results produced from these tests, with validated p24 antibodies, can be relied upon with high degree of confidence with low likelihood of false positives and false negatives ensuring correct diagnoses and on-time appropriate patient care.

Following are the three main specific aims that will accomplish this goal:

1. Evaluate current status of p24 early HIV testing.

Rapid diagnostic tests (RDTs) are tests that produce results quickly, usually within 30 minutes and do not require a proper laboratory setting to be performed (Sands A, 2015). With proper training, these tests can be easily administered by health-care professionals and lay providers, making this test well suited for use in both community and facility based settings with limited infrastructure or resources (Sands A, 2015). Currently, an FDA approved, rapid point of care 4th generation test, the Alere Determine HIV-1/2 Ag/Ab Combo, detects both HIV-1/2 antibodies and free HIV-1 p24 antigen, and is able to identify HIV earlier than 2nd and 3rd generation antibody only tests (Alere, Green Oaks, IL). Whole blood, serum or plasma samples can be tested with results obtained in just 20 minutes (Alere). The sensitivity of the p24 antigen component of the test has been variable, from 2-92% in laboratory tests, which can translate into poor diagnostic accuracy in the field for the diagnosis of acute HIV-1 (Lewis JM, 2015).

2. Evaluate current proposed methods of antibody validation.

Realizing the need for the validation of binding agents, specially antibodies, Uhlen et. al in 2016 published a paper titled “A proposal for validation of antibodies”. In the publication, they discuss the need for validating antibodies and propose potential “pillars” for validating antibodies. These pillars are specific strategies that can be utilized to validate an antibody, which are: genetic strategies, orthogonal strategies, independent antibody strategies, expression of tagged proteins, and immunocapture followed by mass spectrometry.

3. Proposed validation for early HIV p24 antibodies.

It is now feasible to recommend a set path to validate early HIV p24 antibodies that would yield highly specific and robust antibodies. This would help eliminate false positives and false negatives and the need for retests and follow up tests.

BACKGROUND AND SIGNIFICANCE

HIV stands for human immunodeficiency virus and can lead to acquired immunodeficiency syndrome or AIDS if not treated (HIV). Unlike some other viruses, the human body is unable to get rid of HIV completely, even with treatment (CDC). HIV attacks the body's immune system, specifically the CD4 cells (T cells), which help the immune system fight off infections (CDC). Untreated, HIV reduces the number of CD4 cells (T cells) in the body, making the person more likely to get other infections or infection-related cancers, and over time, the body is unable fight off infections and disease (CDC). These opportunistic infections or cancers take advantage of a very weak immune system and signal that the person has AIDS, the last stage of HIV infection (CDC). There is no cure for HIV, but with proper medical care, HIV can be controlled (CDC). Antiretroviral therapy or ART is prescribed to people with HIV to decrease their viral load (amount of HIV in their blood) which can become undetectable, and if it stays undetectable, a long, healthy life and effectively no risk of transmitting HIV to an HIV-negative partner through sex is possible (CDC). Only certain body fluids such as blood, semen, pre-seminal fluid, rectal fluids, vaginal fluids, and breast milk from a person who has HIV can transmit HIV (CDC). These fluids must come in contact with a mucous membrane or damaged tissue or be directly injected into the bloodstream (from a needle or syringe) for transmission to occur (CDC).

The HIV virus:

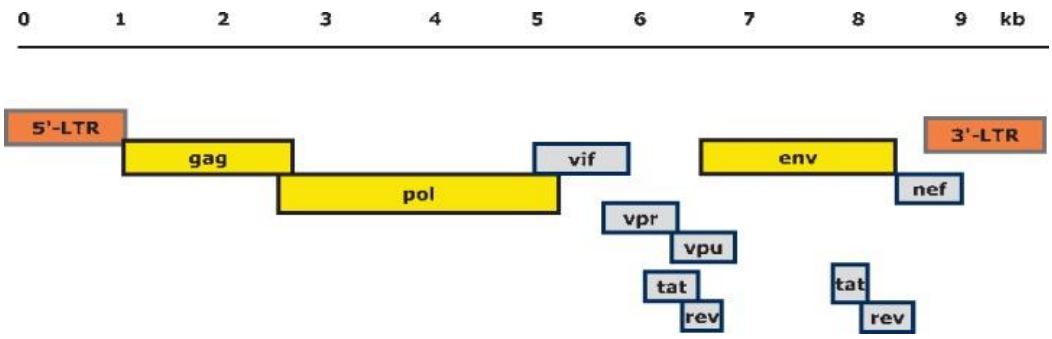
The human immunodeficiency virus (HIV) is grouped in the Lentivirus genus within the Retroviridae family (German, 2016). It is further classified into the types 1 and

2 (HIV-1, HIV-2) on the basis of genetic characteristics and differences in the viral antigens (German, 2016). Epidemiologic and phylogenetic analyses indicate that HIV was introduced into the human population around 1920 to 1940 (German, 2016).

Genome:

The HIV genome consists of two identical single-stranded RNA molecules that are enclosed within the core of the virus particle (German, 2016). The genome of the HIV provirus, also known as proviral DNA, is generated by the reverse transcription of the viral RNA genome into DNA, which then integrates into the human genome (German, 2016). See Figure 1 for the reading frames of the genes coding for structural and regulatory proteins in the HIV virus:

Figure 1: HIV genes coding for structural and regulatory proteins

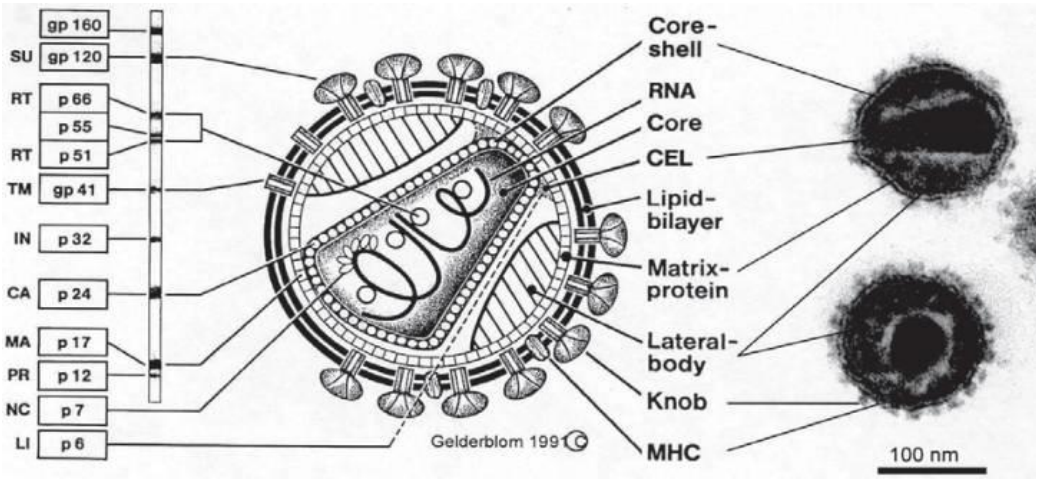


LTR = long terminal repeat; *gag* = group-specific antigen; *pol*= polymerase; *env* = envelope. In the case of the regulatory genes, the proteins of *tat* and *rev* are composed of two gene regions. In HIV-2, *vpx* corresponds to the *vpu* gene. The 5´ and 3´ LTR nucleic acid sequences are not translated into protein (German, 2016). The genome consists of 9,200-9,600 nucleotides in the case of HIV-1 and approximately 9,800 nucleotides in the case of HIV-2 (German, 2016).

Particle Structure:

The mature HIV particle is round and measures approximately 100 nm in diameter, with an outer lipid membrane as its envelope (German, 2016).See Figure 2 for the particle structure of the HIV virus. The envelope contains 72 knobs, composed of trimers of the Env proteins . The trimers of gp120 surface protein (SU) are anchored to the membrane by the trimers of the transmembrane protein gp41 (TM) (German). Conformation-

Figure 2: Structure of the HIV particle



dependent neutralising epitopes are found on the gp120 protein. These are present on the native protein but are only partially expressed on the unfolded denatured protein (German, 2016). The viral envelope is composed of a lipid bi-layer primarily derived during budding from the host cell. The envelope proteins SU and TM are present in mature virus particles (German, 2016). It covers the symmetrical outer capsid membrane which is formed by the matrix protein (German, 2016). The conical capsid is assembled from the inner capsid protein p24 (German, 2016). Two identical molecules of viral genomic RNA are located inside the capsid and several molecules of the viral enzymes RT/RNase H and IN bound to the nucleic acid (German, 2016). Virus particles also contain oligopeptides that are

generated after release from the cell during the maturation of virions by proteolytic processing of the precursor proteins (German, 2016).

Infection and disease state:

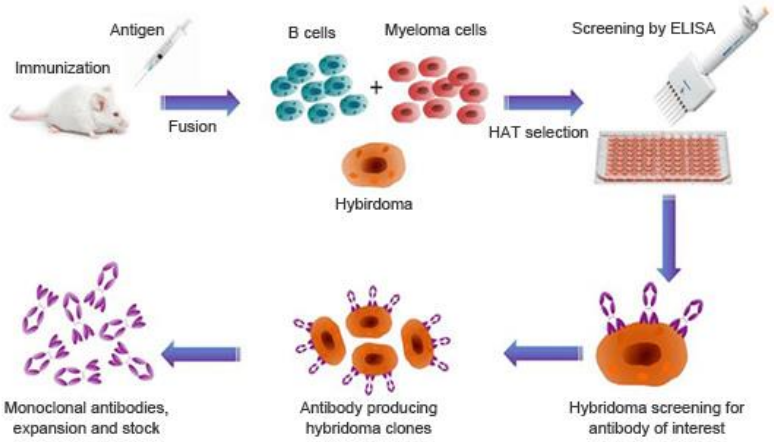
It is estimated that over a million people in the United States were living with HIV at the end of 2015 and of those people, about 15%, or 1 in 7, did not know they were infected (HIV/AIDS). 1987 was the first year HIV was listed as a cause of death on death certificates, and up through 2015, 507,351 people died from HIV disease (CDC). In 2015, 6,465 people died from HIV disease (CDC). HIV remains a significant cause of death for certain populations. In 2015, it was the 9th leading cause of death for those aged 25 to 34 and 9th for those aged 35 to 44 (CDC). World-wide there were 1.8 million new cases of HIV infection in 2016 (CDC). Globally 36.9 million people were living with HIV in 2017, and 21.7 million people were receiving antiretroviral treatment by the end of 2017 (Data). An estimated 1 million people died from AIDS related illnesses in 2016 (CDC). Sub-Saharan Africa accounts for about 64% of all new HIV infections (CDC). Other regions significantly affected by HIV and AIDS include Asia and the Pacific, Latin America and the Caribbean, and Eastern Europe and Central Asia (CDC).

Antibody Production:

Generation of monoclonal antibodies

Monoclonal antibodies (mAbs) are created form a hybridoma cell line which are formed from the fusion of an antibody-producing lymphocyte and a histocompatible myeloma cell (Liddell, 2005). The hybridoma cell inherits the ability to survive in tissue culture from the parent myeloma cell and the capacity to secrete antibody products from the parent lymphocyte (Liddell, 2005). Each hybrid cell can develop into a clone of identical cells producing identical antibodies that bind to one specific epitope (Liddell, 2005). Figure 1 illustrates the process for obtaining monoclonal antibodies (Saeed, 2017).

Figure 3: Production of monoclonal antibodies



The production of mAbs begins by harvesting antibody producing lymphocyte cells found in the lymph nodes or the spleen (Genscript). The spleen cells typically have limited survival times in culture so they are fused with cancerous B-cells, or myelomas, to create an immortalized hybrid that can undergo many passages (Genscript). Usually polyethylene glycol or electric pulses are used to disrupt cell membrane and allow for the merging of two adjacent cells (Genscript). Selection of the myeloma-lymphocyte hybrid is carried out by utilizing hypoxanthine-aminopterin-thymidine medium (HAT) which inhibits DNA synthesis by aminopterin (Genscript). B-Cell and the fused hybrids contain

thymidine kinase which allows them to synthesize DNA polymerase precursors from the thymidine in the HAT medium, while myelomas that do not produce thymidine kinase are unable to and do not survive, and B-cells eventually die off in culture due to limited replication ability in culture (Genscript). Since the population is heterogenous producing different antibodies with different specificity, limiting dilutions are performed to dilute the culture to one hybridoma cell per well, which is then further expanded and screened using an ELISA test (Genscript). Once the monoclonal status is verified after successful performance in screening assays, the hybridomas are expanded and cryopreserved for long term storage (Genscript). The production of mAbs using hybridoma technology was first described in 1975 by Kohler and Milstein, and since then the technology has advanced with the antibody immunogenicity progressively decreasing from mouse to chimeric humanized to fully human mAbs (Jeskin, 2015).

Production of "humanized" antibodies by genetic engineering

The first monoclonal antibody therapy was approved for clinical human use in 1986 and was a complete, unmodified mouse antibody (Fusion, 2016). Antibodies derived from xenogenic sources, such as rodents, are highly immunogenic in humans and are rapidly removed form circulation and can cause inflammatory effects as well (Creative). Murine antibodies have a short half-life because they are recognized as foreign proteins by the patient immune system, resulting in a human anti-mouse response (Fusion, 2016). Three commonly used humanization techniques are employed to bypass the immunogenic responses elicited by the patient immune system: complementarity determining regision (CDR) grafting, phage display and transgenic mice (Fusion, 2016). In CDR grafting, hypervariable ends of an antibody are carefully selected from a parental antibody and

grafted onto a human framework (Fusion, 2016). Phage display utilizes antibody RNA from a given source which is then ligated to a phage display vector, resulting in expression of human IgG on bacteriophage hosts which can then be screened (Fusion, 2016). Transgenic mice are generated by suppression of their endogenous mouse antibody genes along with the introduction of human antibody heavy and light chain gene sequences, which results in the expression of fully human antibodies (Fusion, 2016).

Overview of the use of antibodies to detect HIV

There are three types of tests used to diagnose HIV infection: antibody tests, antigen/antibody tests, and nucleic acid tests (NIH). Current HIV antibody tests are often referred to as 2nd generation, detecting IgG antibodies; 3rd generation, detecting both IgM and IgG antibodies; and 4th generation, detecting both HIV antibody and the p24 antigen (HIV Insite, 2011). HIV antibody tests rely on ELISA or EIA technology to screen for HIV in blood, oral fluid or urine (HIV Insite, 2011). The antibody tests give a positive result based on antibodies to HIV, not the virus itself, and it can take up to 4 to 6 weeks for the body to produce these antibodies at levels that can be confidently detected (San Francisco, 2018). Antigen/antibody tests can detect both HIV antibodies and HIV antigens simultaneously (NIH). The p24 antigen can be detected by this type of test between 12 to 26 days (San Francisco, 2018). The nucleic acid tests detect the genetic material, RNA, of the HIV rather than the antibodies to the virus (HIV Insite, 2011). These tests can detect the presence of the virus 10 to 14 days after infection, however these tests are very expensive and not readily available in most places (San Francisco, 2018).

Antibody Validation:

General concepts and need for anti-HIV antibody validation

In order to reproduce results in research, and consistently provide sound test results in the clinic or a testing lab, it is crucial to use reagents that are reliable with very little to no variability. One of the most heavily relied upon reagents, for sound and consistent data, in the biomedical sciences are antibodies. In the lab they are most commonly used for western blots, immunoprecipitation, Enzyme Linked Immunosorbent assays, while on the clinical front they are used for diagnostic and therapeutic purposes (Bordeaux, 2010). Many of the antibodies that are available are not reliable and can cause issues with reproducibility of results (Bordeaux, 2010). Antibodies are among the most highly utilized tools in research and the biotechnology industry, however there are no universally accepted guidelines or standardized methods that can be utilized to determine the validity of these reagents (Bordeaux, 2010). Upwards of $2.5 billion a year is spent on research antibodies, but the waste as a result of poorly characterized antibodies has been estimated upwards of $800 million (Baker, 2015). An estimated 350 million dollars annually in the United States alone is attributed to the loss of time, effort and resources due to poorly characterized and ill-defined antibodies (Bradbury, 2015). As a result, it is paramount to have guidelines that allow for the validation of one of the most utilized tools in biomedical sciences.

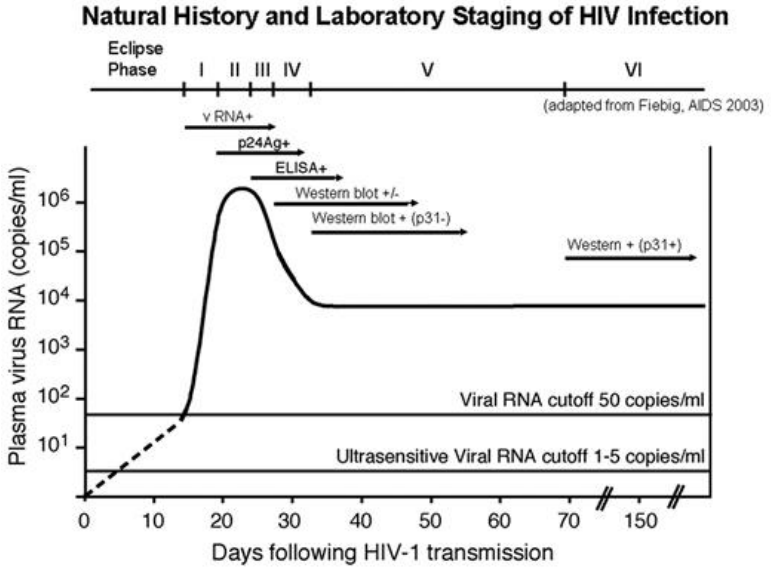
Documents such as FDA's Guidance for Industry Bioanalytical Method Validation and USP's Validation of Compendial Procedures provide a solid footing for scientists looking to validate laboratory procedures. In the biopharmaceutical industry, the analytical methods that are used for quantitative analysis of drugs and their metabolites need to be highly selective and sensitive for successful preclinical/clinical and biopharmaceutics testing (USFDA, 2001). Validation is a process where the performance characteristics of the procedure meet the requirements for the intended analytical application (US

Pharmacopeia). The validation process includes all of the procedures that demonstrate that a particular method used for quantitative measurement is reliable and reproducible for its intended use (USFDA, 2001). Typical characteristics that are used in method validation include accuracy, precision, selectivity/specificity, sensitivity, reproducibility, and stability (USFDA, 2001). Per the USP Validation of Compendial Methods, additional analytical parameters that are assessed which include detection limit, quantitation limit, linearity, range and robustness of the bioanalytical method (US Pharmacopeia).

Methods of antibody validation:

There are quite a few approaches that can be taken to validate an antibody. These approaches include the following: Genetic Strategies, Orthogonal Strategies, Independent Antibody Strategies, Expression of Tagged Proteins Strategies, and Immunocapture followed by Mass Spectrometry (Uhlen, 2016). Well-developed robust analytical assays within these strategies can be developed to validate the performance of p24 specific antibodies for early HIV detection. Acute human immunodeficiency virus infection (AHI) is defined as the time from HIV acquisition until seroconversion and during this time the subjects are maximally contagious because of high viral load

Figure 4:



and lack of any binding antibodies (Cohen, 2010). After transmission there is an initial "eclipse phase" of about 10 days (See Figure 4) in which infection is established at the exposure site, but dissemination at detectable levels in the systemic circulation has not yet occurred (Cohen, 2010). Once dissemination to lymphoid tissues and the systemic circulation occurs, HIV replication increases rapidly, with a doubling time of 20 hours (Cohen, 2010). During the ramp-up phase of viremia, a “window period” exists, during which HIV antibodies are still not yet detectable (Cohen, 2010). During the AHI period, a viral latent pool reservoir develops, as a result of which the immune system suffers irreparable damage, and the infected host may be most contagious (Cohen, 2010).. About 7 days later, it becomes possible to detect the p24 antigen, which transiently appears in the blood during the ramp-up phase once HIV-1 RNA levels rise above 10,000 copies/mL and before the development of detectable HIV antibodies (Cohen, 2010).. The emergence of p24 antigen occurs later than HIV RNA, but often disappears as antibodies form, because they complex with the antigen (Cohen, 2010). 5 days after positive p24 antigen test results, antibodies to HIV-1 reach levels that can be detected with sensitive enzyme immunoassays. (Cohen, 2010). It may be possible to intervene during AHI to limit HIV viral replication and integration into a latent pool that renders HIV incurable (Cohen, 2010). P24 antibody validation would provide sound data and concrete proof of the performance of the antibody before it is used in utilized for critical diagnostic testing.

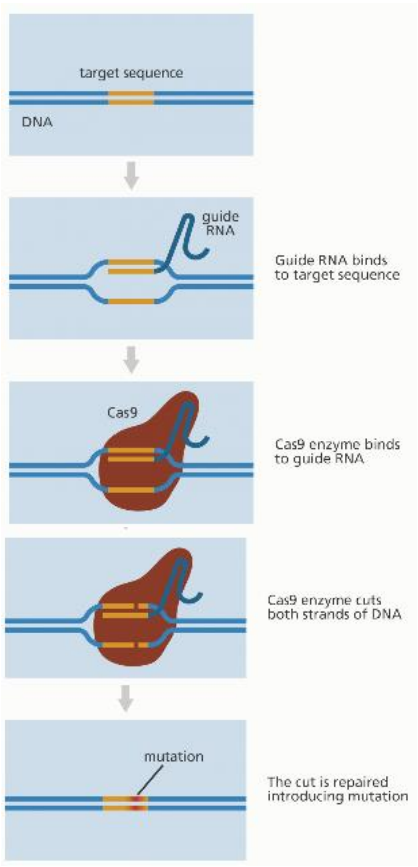
Genetic Strategies

One method of assessing antibody specificity is by using control cells or tissues with knocked out target genes or epitopes by using the CRISPR-Cas9 or RNAi technology (Uhlen, 2016). Expression of the target gene or protein is eliminated or reduced as a result

and any signal that is obtained can be concluded to be due to cross reactivity. This is particularly useful for blotting as it is easily visually observable (Uhlen, 2016).

The functions of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) genes are essential in adaptive immunity in select bacteria and archaea, enabling the organisms to respond to and eliminate invading genetic material (Biolabs). Cas9, can site-specifically cleave double-stranded DNA, resulting in the activation of the doublestrand break (DSB) repair machinery (Biolabs). DSBs can be repaired by the cellular Non-Homologous End Joining (NHEJ) pathway, resulting in insertions and/or deletions (indels) or, if a donor template with homology to the targeted locus is supplied, the DSB may be repaired by the homology-directed repair (HDR) pathway allowing for precise replacement mutations to be made (Biolabs). Refer to figure 5 for an illustration of the CRISPR-Cas 9 pathway.

Figure 5: CRISPR-Cas9 pathway

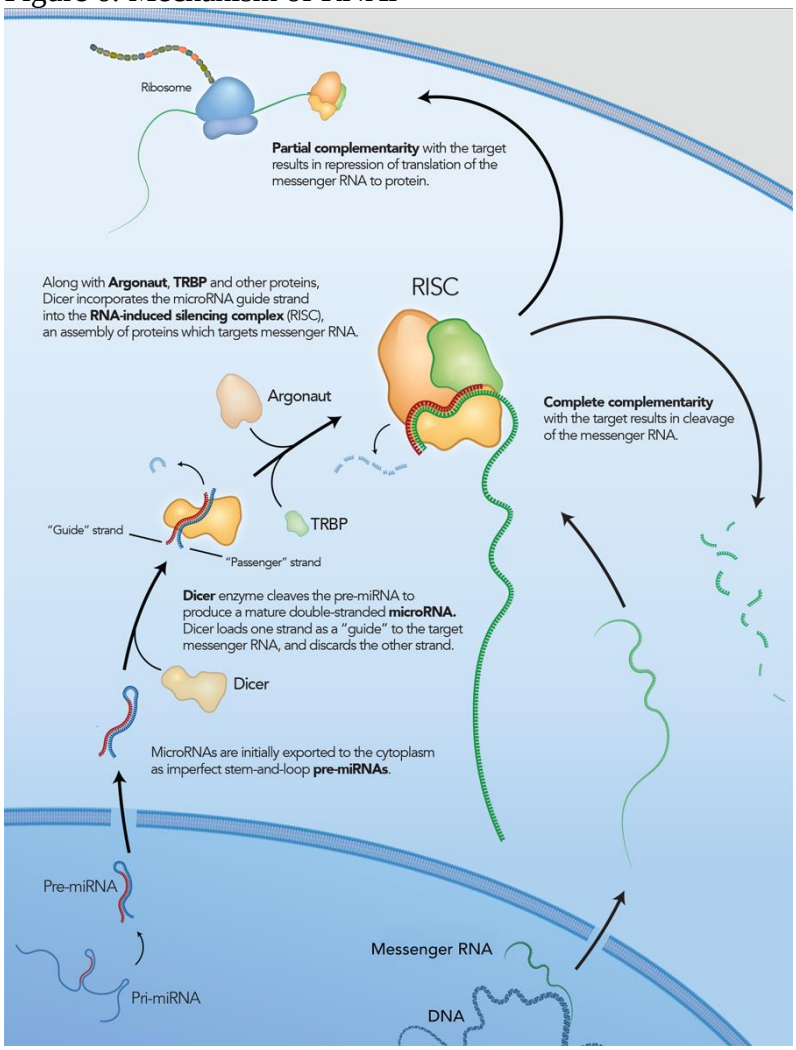


The CRISPR-Cas9 system consists of two key molecules, an enzyme called Cas9 and guide RNA (gRNA) (Genome.org, 2016). Cas9 acts as a pair of ‘molecular scissors’ that can cut the two strands of DNA at a specific location in the genome (Genome.org, 2016). The Guide RNA has RNA bases that are complementary to those of the target DNA

sequence in the genome, and also consists of a small piece of pre-designed RNA sequence (about 20 bases long) located within a longer RNA scaffold which 'guides' Cas9 to the right part of the genome, ensuring that the Cas9 enzyme cuts at the right point in the genome (Genome.org, 2016).

RNA interference (RNAi) or Post-Transcriptional Gene Silencing (PTGS) is a conserved biological response to double-stranded RNA that provides resistance against endogenous parasitic and exogenous pathogenic nucleic acids by regulating the expression of protein-coding genes (RNA). See Figure 6 for the mechanism of RNAi. RNAi pathway is based on two steps with each involving ribonuclease enzyme activity (RNA). In the first step, the trigger RNA (either dsRNA or miRNA primary transcript) is processed into short interfering RNA (siRNA) by

Figure 6: Mechanism of RNAi



the RNase II enzymes Dicer and Drosha (RNA). siRNAs are approximately 21 bases long with a central 19 bp duplex and 2-base 3′-overhangs (Dicer). In the second step, nascent siRNA associates with Dicer, TRBP, and Argonaut (Ago2) to form the RNA-induced silencing complex (RISC) which mediates gene silencing (Dicer). Once in RISC, one strand of the siRNA (the passenger strand) is degraded or discarded, while the other strand (the guide strand) remains to direct sequence specificity of the silencing complex (Dicer). The single-stranded guide RNA hybridizes with mRNA target sequence causing gene silencing, which is a result of nucleolytic degradation of the targeted mRNA by the RNase H enzyme Argonaute (Slicer) (RNA). If the siRNA/mRNA duplex contains mismatches the mRNA is not cleaved. Rather, gene silencing is a result of translational inhibition (RNA).

Bordeaux et al (2010), in their paper show that utilizing siRNA specific for the target of the antibody can down regulate the expression levels thereby proving antibody specificity to its epitope. Usually Western Blot techniques are used as the primary source for assessing antibody specificity for its target. Positive and negative cell line lysates are

Figure 7: Stathmin expression reduced by siRNA

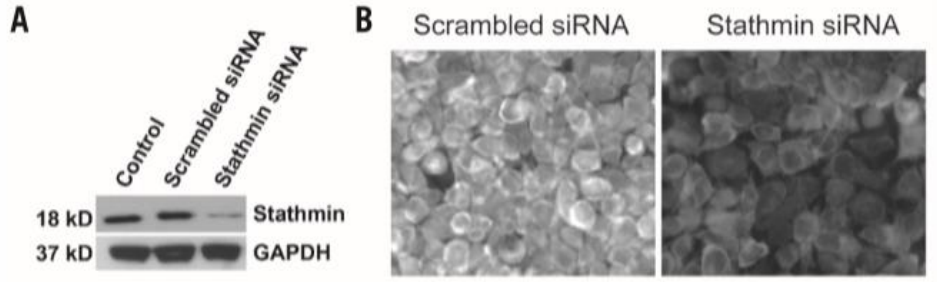


Figure 4. Stathmin expression is reduced by siRNA. (A) WB of BT-20 lysates 24 h post-mock; control scrambled siRNA or Stathmin siRNA transfection demonstrates specific reduction in Stathmin levels. GAPDH serves as a loading control. (B) BT-20 cells were fixed in 4% PFA 24 h post-transfection with scrambled control or Stathmin siRNA, and IF with Stathmin demonstrates a reduction in Stathmin expression.

assessed with known levels of target expression, however, when true negative lines are

unavailable, or when levels of target protein are highly dependent on specific growth conditions, lysates of cell lines where the target protein has been knocked down using RNAi are used (Bordeaux, 2010). They show this technique, using siRNA in validating an antibody, in an experiment where Stathmin, a microtubule-destabilizing protein is knocked down by the use of siRNA, see Figure 7. Lystates from BT-20 cells were transfected with scrambled siRNA control or siRNA specific for Stathmin. They confirmed using Western Blot analysis loss of Stathmin expression. The transfected BT-20 cells were also fixed and decreased Stathmin expression was visualized with immunofluoresence (Bordeaux, 2010). Stadler et al (2012), utilized the siRNA technology to build a platform for the validation of antibody binding through immunofluorescence. They analyzed and validated 65 human proteins, targeted by 75 antibodies and silenced by 130 siRNAs (Stadler. 2012). Their study showed a direct reduction of antibody signal as a result of siRNA silencing and confirmed it with Western Blot, indicating that this strategy is an objective approach for the validation of antibodies (Stadler. 2012).

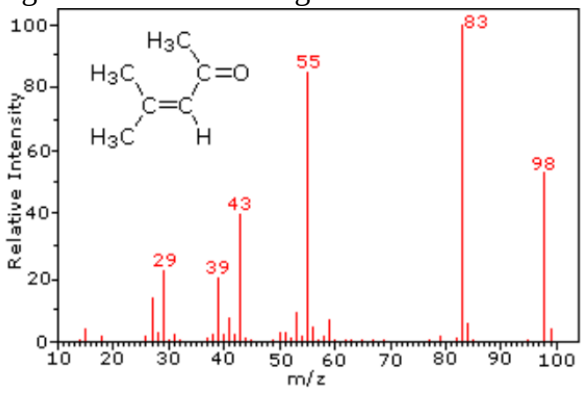
Generating knock out cell lines are a powerful tool in assessing antibody specificity. siRNA technology is not the only method in identifying non-specific antibody binding. CRISPR and other gene editing methods knock out a gene from DNA which then prevents the associated protein from being produced (Olds, 2017). Another method for creating negative control cell lines to determine non-specific binding of the antibody being validated is utilizing the CRISPR/Cas9 programable nuclease technology by creating targeted genetic changes to the gene of interest (Kanchiswamy, 2016).

Genetic approaches are powerful since they provide a direct link between gene and protein and its detection by the antibody, however it is not the best strategy to be used for human tissue sample and body fluids such as plasma and serum (Uhlen, 2016).

Orthogonal Strategies

This strategy utilizes an antibody independent method (Uhlen, 2016). An antibody-independent strategy is used to quantify the target in many samples, which is then compared with an antibody-based method and then the correlation between the two methods is examined . In this type of strategy, antibody staining of the protein should be recognized by Western Blot, but also compared with measurement of the target protein's abundance from an MS-based targeted proteomics approach. The results then would be correlated, thus validating the antibody in question (Uhlen, 2016). In a large-scale inter-laboratory study, Abbatiello et al. (2015) show this type of analysis through the liquid chromatography multiple reaction monitoring mass spectrometry method. They use stable isotope labeled peptide standards for peptide-based protein quantification and as a result they have shown that this method produces limits of detection similar to that of ELISA based methods while being a very robust method overall. With LC-MS based method, solubilized compounds (the mobile phase) are passed through a column packed with a stationary (solid) phase, effectively separating the compounds based on their weight and affinity for the mobile and stationary phases of the column (ThermoFisher). This also

Figure 8: MS chromatogram

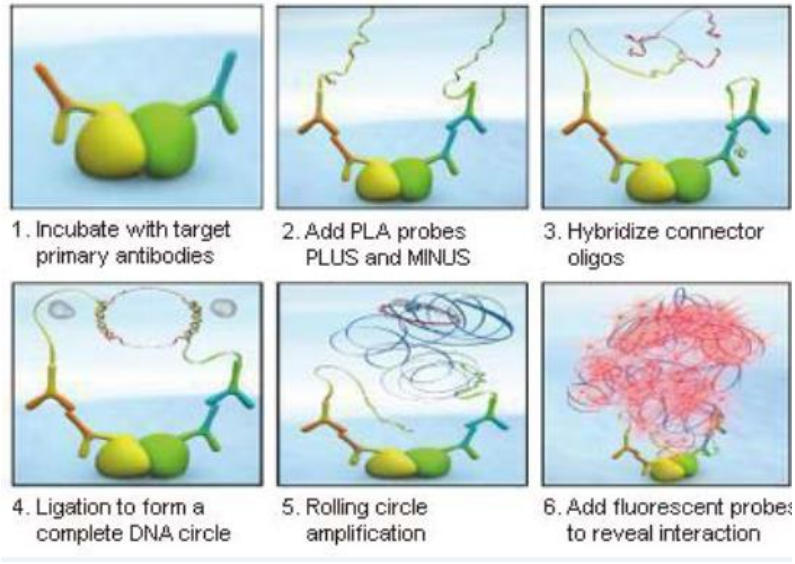


leads to fragmentation of the sample and its anionization through loss of H+ ions (ThermoFisher). After this step, the sample passes into the vacuum chamber of the mass spectrometer (ThermoFisher). Mass spectrometry (MS) is a technique that analyzes ions based on their mass-to-charge (*m/z*) ratio to determine their identity and quantity in simple and complex mixtures (ThermoFisher). Mass spectrometers typically consist of an ionization source, an analyzer and a detector (ThermoFisher). The introduced sample is ionized inside the ionization source, after which it is accelerated, under vacuum, through electric and magnetic fields (ThermoFisher). The Ions deflect and separate, and the radius of their path (and their eventual location on the detector) is a function of their masses (ThermoFisher). Spectral data are then generated through integrated computers and software platforms (ThermoFisher). See Figure 8 for typical data obtained from LCMS based method. For this type of approach, it is important to analyze a set of samples with variable expression of the target protein to be confident specificity and to allow for reliable statistical analysis (Uhlen, 2016).

Independent Antibody Strategies

This strategy relies on two or more antibodies recognizing the same target (Uhlen, 2016). This method requires that the expression pattern generated by the two antibodies match for a given environment. Independent means that the antibody recognize two different epitopes on the same protein. PLA (Proximity Ligation Assay), see Figure 9, is an example of this type of strategy where two antibodies conjugated to DNA probes bind to the same target within the sample, their proximity allows for ligation, amplification, and quantification via real-time PCR (Uhlen, 2016). The dual and proximal binding of the probes in the PLA and Proximity Extension Assay is the defining characteristic which significantly reduces cross-reactivity of the antibody (Lundberg, 2011). This makes these assays an attractive approach for antibody validation for targets contained in samples that are plasma and serum based.

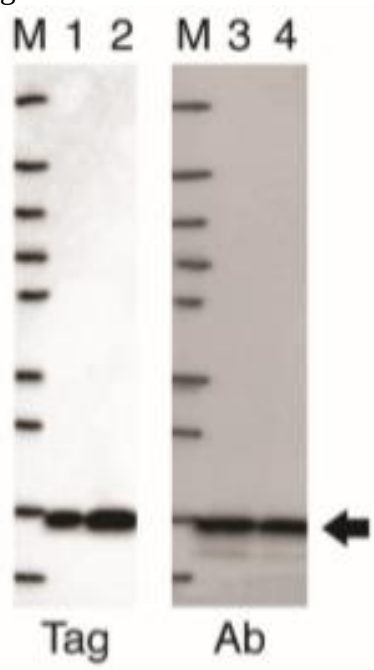
Figure 9: Proximity ligation assay diagram



Expression of Tagged Proteins

Antibodies are expressed with a protein containing an affinity tag which allows for parallel detection with additional well validated immunoreagents or direct observation (Uhlen, 2016). The detection pattern of the antibody being validated must match pattern demonstrated by the anti-affinity tag antibody or the fluorescent signal; a mismatch would be indicative of cross-reactivity. See Figure 10 for an example where human IL-8 is fused with tag peptide. In four cell line preparations, the target is detected with a tag specific antibody in samples 1 and 2, while the antibody to be validated is tested in samples 3 and 4. The detection pattern that is specific to the tag-specific antibody confirms the specificity of the antibody to be validated.

Figure 10:

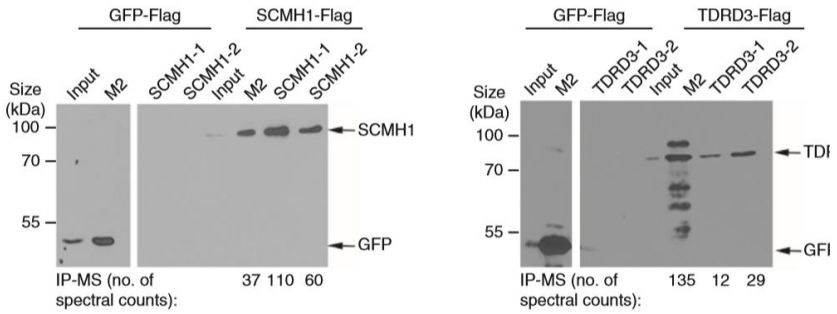


Immunocapture followed by Mass Spectrometry

Immunocapture isolates a protein from solution through binding with a target-specific antibody. With this technique coupled with mass spectrometry to identify protein that interact directly with the purified antibody as well as other proteins that interact with it. After immunocapture, proteins bound to the purified antibody may be directly digested off the bead followed by peptide analysis by mass spectrometry to identify target specific peptides (Uhlen, 2016). Marcon et al. (2015) developed a mass spectrometry-based procedure for scoring immunoprecipitation antibody quality. They expressed proteins as Flag-tagged derivatives in HEK293 cells and immunoprecipitated the epitope-tagged

antigens with an anti-Flag monoclonal antibody (M2) or with a set of respective recombinant antibodies (both Fabs and derivative IgGs) . The immunoprecipitated proteins from all three experiments were analyzed by WB to see whether the Flag-tagged antigen was present in the immunoprecipitate, and by MS to quantify the spectral counts corresponding to the antigen . See Figure 11 for results. The M2 anti-Flag antibody and the four recombinant Fabs were able to

Figure 11: IP-WB vs. IP-MS



immunoprecipitate Flag-tagged SCMH1 and TDRD3, whereas none of the recombinant antibodies immunoprecipitated the control antigen, GFP-Flag (Marcon). The intensities of the signals on the immunoblot varied depending on the antibody used, and were confirmed using the MS as the detection method, with the number of spectral counts correlating well with the intensity of the signal using WB . They were able to test 1,124 synthetic antibodies, both Fabs and IgGs. The antibodies were exposed to HEK293 cell lysate and immunoprecipitate their antigens, followed by mass spectrometry to identify bound proteins which were then compared to the intended antigen.

RESEARCH DESIGN / METHODS

The validation process includes all of the procedures that demonstrate that a particular method used for quantitative measurement is reliable and reproducible for its intended use (USFDA, 2001). Antibodies should be validated in an application and a context specific manner (Uhlen, 2016). Just because the antibody works with one cell or tissue extract does not mean that it would work similarly in another cellular context.

Validation strategy:

The validation strategy for the validation of p24 specific antibodies would follow the standard set forth by the guidance for industry by the United States Food and Drug Administration's (USFDA, 2001) Guidance for Industry Bioanalytical Method Validation and the United States Pharmacopeia's (USP) Validation of Compendial Procedures with regards to the analytical method validation, and will utilize one of the strategies proposed earlier. All validation activities must be performed under Good Laboratory Practices (GLP) requirements.

Highly conservative approach for the analytical method validation ensures that all aspects of antibody performance are highly controlled. Analytical performance characteristics for antibody validation performance should provide data to show accuracy, precision, selectivity/specificity, sensitivity and reproducibility for the particular antibody being validated.

Accuracy: The accuracy of the of an analytical procedure is the closeness of the acquired mean test results of that procedure when compared to the true value (concentration) of the analyte being measured (US Pharmacopeia). This is determined by

replicate analysis of samples containing known amounts of the analyte (USFDA, 2001) For a drug substance its accuracy may be determined by utilizing the analytical procedure to an analyte of known purity, like that of a reference standard, or by comparing the result of the procedure with those of a second, well-characterized procedure, whose accuracy has been defined (US Pharmacopeia). Accuracy is measured using a minimum of five determinations per concentration. At least three are recommended to be within the expected range. The mean value should be within 15 percent of the actual value except at the lower limit of quantitation, where it should not be more than 20 percent (USFDA, 2001).

Precision: For an analytical procedure, precision is described as the agreement or closeness of the test results when the procedure is performed repeatedly to multiple preparations of a single homogenous sample. This parameter is further subdivided into within-run, intra-batch precision, in which the precision is analyzed during a single performance of the method, and between-run, inter-batch precision where precision is measured over time and can involve different analysts, equipment, reagents and different laboratories (USFDA, 2001).Precision is usually expressed as the standard deviation or percent coefficient of variance, of a series of measurements which are obtained through assaying a sufficient number of aliquots of a homogenous sample .

Selectivity/specificity is defined as the ability of a method to differentiate and quantify an analyte in the presence of other components in the sample. Usually in case of analytical methods, specificity requires that the method show that the procedure is unaffected by the presence of impurities or excipients. Usually this is performed by spiking the sample with appropriate concentrations of impurities or excipients. The result should

show that the assay result is unaffected by the presence of extraneous material in the sample.

Reproducibility: Reproducibility is defined as the precision between two laboratories and represents precision over a short period of time. Technically, precision runs can be a measure of either the degree of reproducibility or repeatability as well when the procedure is performed in two different labs. If the test is performed within the same laboratory over different days, that is referred to as intermediate precision. Intermediate precision is also known as ruggedness and expresses within-laboratory variation when the analytical procedure is performed in the same lab over different days, or with different analysts or different laboratory equipment. Like precision, reproducibility is also evaluated by looking at the percent coefficient of variance.

Linearity: The linearity of an analytical procedure is its ability to produce data that is directly proportional to the concentration of analyte in sample within a given range. In order to establish linearity, sometimes the data requires transformation. If linearity is unattainable, a non-linear model can be used so as long as it is able to show a concentration-response relationship.

The functionality of the antibody is dependent on both application and context (Uhlen, 2016). Once the context of use for the antibody is determined the next step is to determine which strategy would be best to validate the antibody. From among the five conceptual pillars of antibody validation (genetic strategies, orthogonal strategies, independent antibody strategies, expression of tagged proteins, immunocapture followed by mass spectrometry), at least two of unrelated strategies will be used to perform the

validation of the antibody. Utilizing a multidimensional strategy to perform validation for its intended use would produce high quality antibodies.

The Method:

The p24 specific antibodies in RDTs are adsorbed onto a solid phase surface with their specificity to the p24 antigen playing a critical role in the early detection of acute HIV infection. A well-developed immunoassay in conjunction with two of the antibody validation strategies discussed above will be utilized for the validation of p24 specific antibodies.

Among the 5 validation strategies (genetic strategies, orthogonal strategies, independent antibody strategies, expression of tagged proteins, immunocapture followed by mass spectrometry), the two that are recommended to be utilized for the validation of p24 specific antibodies are expression of tagged protein and immunocapture followed by mass spectrometry. Genetic strategies would be difficult to employ for validating a p24 specific antibody since p24 protein expression in a controlled cell line would be difficult to control with basal level of endogenous expression. Independent antibody strategies are good for polyclonal antibodies. Immunocapture followed by mass spectrometry and expression of tagged protein strategy is a good way to check specificity of the antibody for its antigen, in particular monoclonal anti p24 antibodies.

Immunoassay:

A well-developed p24 immunoassay will be conducted to assess the performance of the p24 specific antibody to be validated, against well-established gold standard reference antibodies. Use Abcam's anti-HIV1 p24 antibody (catalog#: ab9071) and/or ThermoFisher's HIV1 p24 monoclonal antibody (D45F) to prepare an immunoassay to test

the performance of the antibody to be validated. Test 5 different vials of the stock antibody to be validated, prepared independently and tested on three plates in triplicate. See plate layout below in figure 12:

Figure 12: Plate layout with staggered locations of individually prepared samples

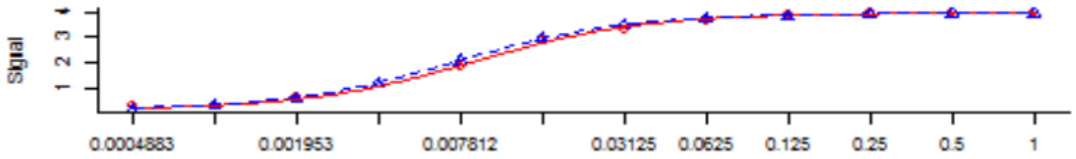
Plate 1 replicate 1												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Reference Antibody											
B	75% Control											
C	125% Control											
D	Antibody to be validated Prep 1											
E	Antibody to be validated Prep 2											
F	Antibody to be validated Prep 3											
G	Antibody to be validated Prep 4											
H	Antibody to be validated Prep 5											

Plate 2 replicate 2												
	1	2	3	4	5	6	7	8	9	10	11	12
A	125% Control											
B	Antibody to be validated Prep 1											
C	Antibody to be validated Prep 2											
D	Antibody to be validated Prep 3											
E	Antibody to be validated Prep 4											
F	Antibody to be validated Prep 5											
G	Reference Antibody											
H	75% Control											

Plate 3 replicate 3												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Antibody to be validated Prep 2											
B	Antibody to be validated Prep 3											
C	Antibody to be validated Prep 4											
D	Antibody to be validated Prep 5											
E	Reference Antibody											
F	75% Control											
G	125% Control											
H	Antibody to be validated Prep 1											

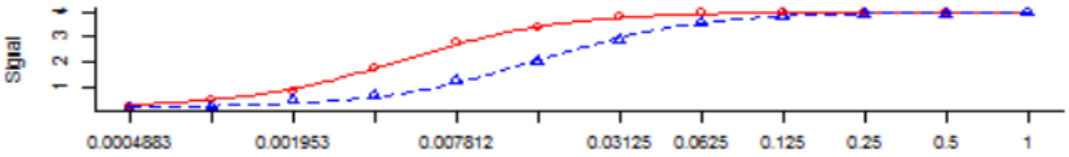
After completion of the assay, generate binding curves of the antibody being validated using a computer software. Curves should follow a 4-parameter logistic fit and should look similar to the curves in figure 13.

Figure 13: 4-Parameter logistic curve:



If the curves are different and shifted as in the case of the following graph, then it means that the antibody vs. the reference is different or lower in potency and not performing as intended relative to the reference standard. See figure 14 for an example in discrepancy between the reference antibody and antibody to be validate.

Figure 14: Variable performance of reference antibody versus antibody to be validated:



With an immunoassay system, the following parameters (see Figure 15) can be assessed quite easily and provide a good indication of how the antibody to be validated is performing relative to the gold standard antibodies.

Figure 15: validation parameters:

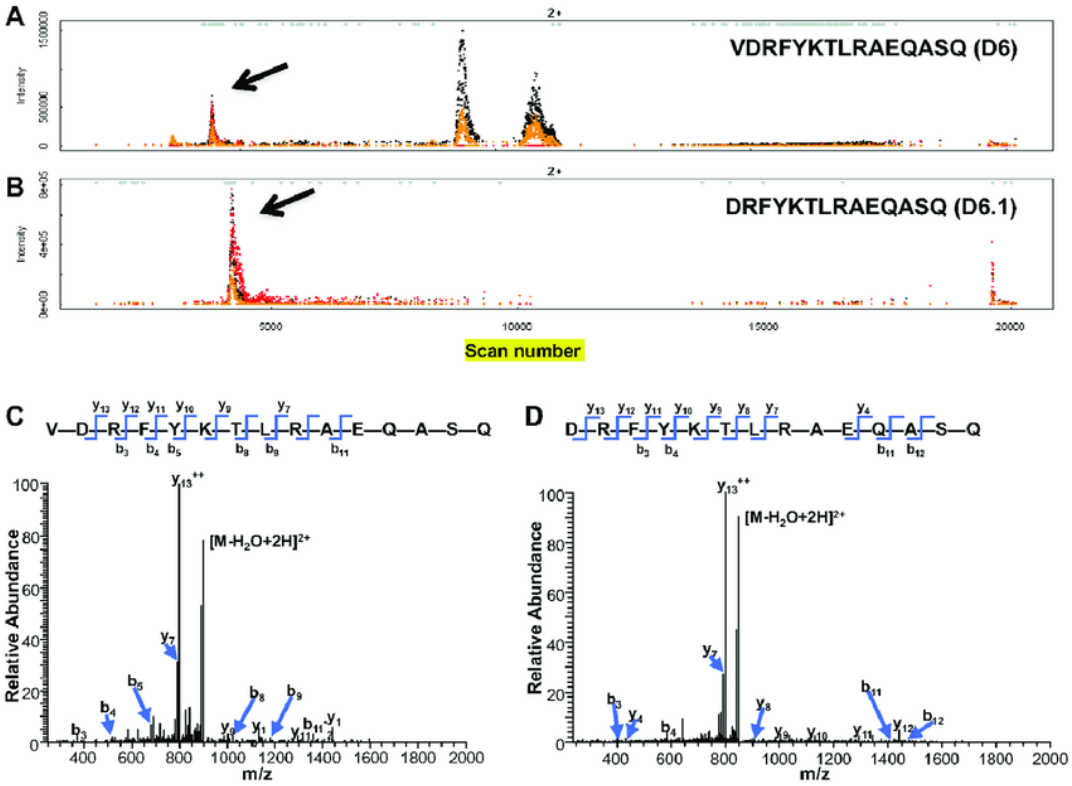
Parameters	Results
Accuracy	% Recovery = 80 to 120
Range	Report the range which meets the following criteria. At least 5 consecutive points of the linearity fall within the range. % Recovery = 70 to
Limit of Quantitation	Report the lowest point within the range.
Repeatability	%CV ≤ 20
Intermediate Precision	%CV ≤ 20
Robustness	%CV ≤ 20
Sample Matrix Verification	% Recovery = 80 to 120

In conjunction with a well-developed immunoassay showing performance of the antibody to be validated in reference to the gold standard antibodies, an Immunocapture followed by mass spectrometry and/or expression of tagged protein strategy should be employed to fully validate the antibody for its intended use.

Immunocapture followed by mass spectrometry will utilize human serum samples spiked with recombinant p24 proteins. Antibody to be validated will be subjected to Immunocapture protocol followed by a purification step before being analyzed for MS. At least three different samples will be tested in three different independent triplicate preparations. The mass spectrometry result analysis should indicate that the antibody to be validated is specific if top three peptides derived from the expected target protein, p24.

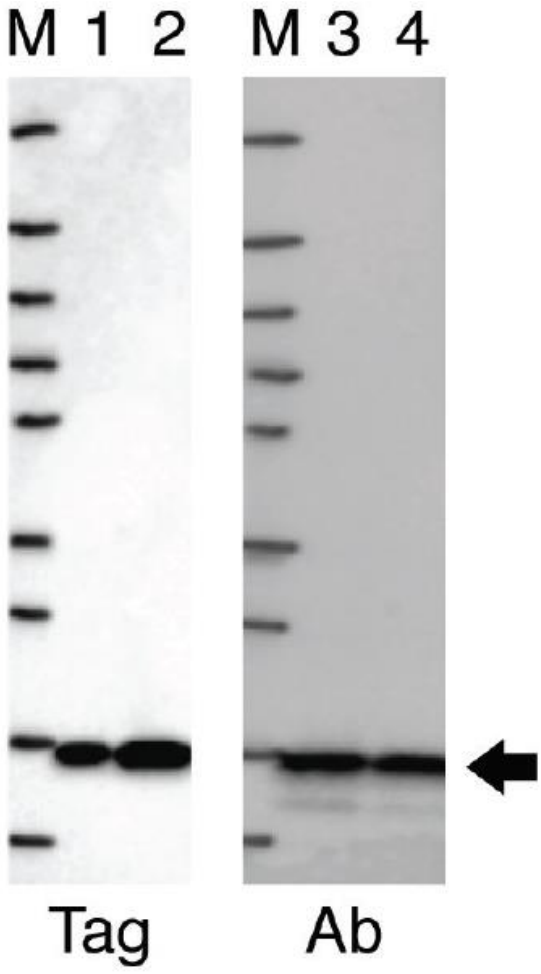
Typical data for mass spectrometry of the p24 HIV antigen is shown in Figure 16 (Poorinmohammed, 2014):

Figure 16: p24 HIV anigen mass spectrometry data:



The expression of tagged protein strategy will include the comparison of the detection pattern of the antibody to be validated with tag specific antibodies. The antibodies will detect p24 protein fused with a tag peptide. The expression profiles of the two antibodies must match to show that the antibody to be validated is specific and thus validated for its intended use. A typical expression profile will look like the expression profile in figure 17, with the expression of the tag specific antibody matching that of the antibody to be validated. If expression profiles are different of bands appear where they should not be, then the antibody is not specific and will fail this part of the validation.

Figure 17: Expression of Tag specific antibody versus antibody to be validated



A multidimensional approach to antibody validation, a well-developed immunoassay in conjunction with the Immunocapture followed by mass spectrometry and/or expression of tagged protein strategy will lead to well characterized validated antibody.

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