1 Human Immunodeficiency Virus Diagnostic Testing: 30 years of Evolution

2 Running title- HIV Testing Evolution.

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Abstract

A concern during the early AIDS epidemic was the lack of a test to identify individuals who carried the virus. The first HIV antibody test, developed in 1985, was designed to screen blood products, not to diagnose AIDS. The first generation assays detected IgG antibody and became positive 6-12 weeks post infection. False positive results occurred, thus a two test algorithm was developed using a western blot or immunofluorescence test as a confirmatory procedure. The 2nd generation HIV test added recombinant antigens and the 3rd generation HIV tests included IgM detection, reducing the test negative window to approximately 3 weeks post infection. 4th and 5th generation HIV assays added p24 antigen detection to the screening assay reducing the test negative window to 11-14 days. A new algorithm addressed the 4th generation assay's ability to detect both antibody and antigen yet not differentiate between them. The 5th generation HIV assay provides separate antigen and antibody results and will require yet another algorithm. HIV infection may now be detected approximately two weeks post exposure, with a reduced number of false positive results.

Introduction

"I want to order the AIDS test on one of my patients". So began a phone call I received in late 1985 from an oncologist. I explained that the HTLV III (the term HIV had not been adopted at that time) antibody assay that had just been developed was not a test for AIDS, but was actually a test designed to prevent virus transmission via blood or blood products. The assay had not been FDA approved as a diagnostic test for AIDS (1). The oncologist went over my head to my department chairman in a futile attempt to have the "AIDS test" performed on his patient. Fast forward to 2016 and while we still don't have a specific AIDS test, diagnostic testing for HIV infection has evolved during the past 30 years. HIV infection now may be readily detected by

laboratory assays, but AIDS is the late stage of HIV infection and requires both clinical and laboratory parameters for diagnosis (2). In this article, I provide an historical background of HIV testing, concluding with a description of the current generation of HIV diagnostic assays and the current testing algorithm.

First Generation HIV antibody tests

Following the 1983 isolation and description of the virus associated with AIDS (3,4), diagnostic tests were developed using separate Human T cell Lymphotropic Virus (HTLV III) (Abbot and Electronucleonics) and Lymphadenopathy virus (LAV) (Genetic Systems) isolates. These ELISA and chemiluminescent methods used proteins isolated from virus infected tissue cultures as antigenic targets. The assays detected IgG antibody to HIV-1 only. The tests were empirically sensitive, but had an antibody negative window of up to 12 weeks or more post infection (5). The high sensitivity, while useful for protecting the blood supply, led to false positive results, especially when low risk individuals were tested. False positive results were associated with infections, autoimmune disease, pregnancy and unspecified conditions. Similar to syphilis testing, a second level of testing was added to improve specificity. Two procedures were FDA cleared as confirmatory tests for HIV-1 antibody only; the western blot (6) and an HTLV III immunofluorescent assay (7,8). Like the screening assays, each of these only detected IgG anti-HIV and had antibody negative windows of 6 weeks or greater. A testing algorithm was developed where reactive specimens were repeated in duplicate. If one or both of the duplicates were reactive, the confirming procedure was performed. Only specimens that were repeatedly reactive in the screening test and reactive on the confirmatory test had a final interpretation as positive. Positive predictive value of a reactive HIV screening test could be less than 50% in low risk populations (9). Clearly there was a need for better tests that could be used for the diagnosis of HIV infection.

Second/Third Generation HIV Tests

Second generation HIV tests, developed in the late 1980s, improved the specificity, and thus the positive predictive value of the screening procedures by adding recombinant antigens, specifically, HIV-1 p24, to the antigen milieu. Often manufacturers added an HIV-2 protein and an HIV-1 group O protein to the antigen preparation in order to detect antibodies to those viruses. HIV-2 and HIV-1 Group O viruses are primarily found in West Africa but have been reported worldwide (10). These second generation assays reduced the antibody negative window period to 4-6 weeks post infection. Since these assays could detected antibody to HIV-2 in addition to HIV-1 antibody, HIV-2 confirmatory testing was added to the algorithm (fig 1). Adding IgM detection to the assay procedure resulted in the third generation HIV test. While specific IgM detection had not been clinically useful, the IgG/IgM combination reduced the antibody negative window to approximately 3 weeks post infection (5). A p24 antigen detection ELISA also could be performed which detected the virus as early as 2 weeks post infection. The overall testing algorithm remained the same, however, and repeatedly reactive screening results still were confirmed by a western blot assay or IFA. Including an HIV-2 protein in the antigen mixture added an additional level of testing to specimens repeatedly reactive in the screening test yet negative on the confirming procedure. Those specimens may have been positive for antibodies to HIV-2, which were not detected on the HIV-1 western blot, or they may have been false positives. Thus those specimens were then tested using an HIV-2 specific assay (fig 1). Quantitative and qualitative molecular assays could reduce the time from infection to detection even further; however, they were not cost effective to be used for generalized screening. HIV PCR assays are recommended for neonatal diagnosis, however, as antibody assays may be positive in the neonate due to maternal IgG crossing the placenta (http://www.who.int/hiv/paediatric/EarlydiagnostictestingforHIVVer_Final_May07.pdf) . In fact the testing and HIV staging requirements for individuals <18 years of age differ from those recommended for adults (2).

Fourth Generation HIV tests

In the late 1990s, manufacturers developed HIV assays that combined antibody and antigen detection. As before, these were ELISA and chemiluminescent based procedures. These tests reduced the test negative window to approximately 2 weeks. While both antibody and antigen were detected in these procedures, the test only gave a single result and did not differentiate whether a positive result was due to the presence of the HIV-1 p24 antigen or due to the presence of antibody to HIV-1 or 2. While these tests had been used outside of the United States for many years, the first 4th generation procedure cleared by the FDA was the Abbot Architect method that was approved in August of 2010. Chavez et al, found the Architect had a sensitivity of 99.94% and a repeat testing specificity of 99.5% in a cohort of 3386 HIV infected individuals, 7551 uninfected subjects and 58 patients with acute HIV infection (11). Bio-Rad's 4th generation GS ELISA was FDA cleared in 2011. The GS ELISA was evaluated on 9150 specimens and showed 100% sensitivity and 99.9-100% specificity (10). The Siemen's ADVIA 4th generation assay, to be used on the Centaur instrument, was approved in 2015. Siemen's package insert shows an antibody sensitivity of 100%, an antigen sensitivity of 97.87% and a specificity of 99.69% (http://www.fda.gov/downloads/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/PremarketApprovalsPMAs/UCM450406.pdf).

When the 4th generation HIV tests were adopted in the United States, a new testing algorithm was needed. Follow up testing required both antigen and antibody detection. The western blot which has a 4-6 week antibody negative window may yield false negatives in patients with early infection who could be identified with a 4th generation assay. Thus, the western blot would be replaced by an HIV antibody differentiation assay. Initially the CDC had proposed two separate 4th generation testing algorithms; one for areas at low risk for HIV infection and one for areas of high risk. In 2014, however, a single algorithm was finalized (fig 2). The new algorithm followed the 4th generation test with an HIV 1,2

differentiation procedure to determine whether the patient had antibodies to HIV-1 or HIV-2. Specimens that were repeatedly reactive on the 4th generation assay yet negative on the HIV 1,2 differentiation procedure were to be tested on an HIV-1 RNA qualitative PCR assay, to determine if HIV-1 was present, causing a positive antigen result on the 4th generation screening test. The molecular assay approved for this confirmation procedure is the Gen-Probe Aptima HIV-1 RNA assay. The current HIV viral load assays, while having improved sensitivity, have not been FDA approved for diagnostic use and are thus not recommended for use in this algorithm (S. Michele Owen, personal communication). Specimens that are negative on the molecular assay are considered to be false positive screening results. The fourth generation tests and algorithm have improved both sensitivity and specificity in detecting early HIV infection when compared to the previous algorithm using western blots as the confirmatory procedure (12,13, fig 3).

Summa Health System's 4th Generation HIV Testing Experience

Summa Akron City/St Thomas Hospitals, located in Akron, OH, implemented the 4th generation Abbott Architect HIV assay in December, 2010. Prior to implementing the 4th generation assay, Summa had been performing approximately 3500 HIV screening tests per year. Approximately 20 specimens per year were repeatedly reactive and 8-10 of those were confirmed as truly HIV positive resulting in a positive predictive value of 40-50% in a typical year during the time we performed the 3rd generation HIV ELISA. Through January 25, 2016, our volume for the 4th generation assay has remained approximately 3500 specimens per year. We had 79 repeatedly reactive specimens over the 5 years we have been performing that procedure. 56 specimens were confirmed by confirmatory testing as being positive for either antibody to HIV-1 (51 specimens) or viral nucleic acid positive (5 specimens). These values yield a positive predictive value of 70.8%. Thus while our annual total positivity rate has dropped since we instituted the 4th generation assay, our positive predictive value has increased, suggesting a

reduced number of specimens being reported as false positive . We also detected 5 acute infections that may have been missed using the third generation test and algorithm. Similar results for the Architect procedure were recently reported by Muthukumar, et al (14). Positive predictive values may be increased further by determining a different cutoff value appropriate for the population to be tested (15)

Fifth Generation HIV tests

In 2015, the FDA approved the Bio-Rad BioPlex 2200 HIV Ag/Ab fifth generation HIV screening test multiplex analysis method as a diagnostic assay. This test, like the 4th generation procedures, detects both HIV antibody and the HIV-1 p24 antigen, but provides separate results for each analyte. This assay will need a new algorithm, as there is no need for a supplemental HIV-1,2 differentiation assay for antibody positive specimens because the test also provides separate results for HIV-1 and HIV-2 antibody. Specimens reactive only for the p24 antigen do not need an antibody confirming procedure and specimens only reactive for antibody do not require an antigen confirmatory procedure. Salmona, et al, evaluated the BioPlex 5th generation assay and found 100% sensitivity and 99.5% specificity in a study of 1505 patients (16). As of this writing (Jan, 2016), the CDC has not published a 5th generation testing algorithm.

Rapid HIV Assays

The advent of HIV prophylactic treatments following an occupational blood or body fluid exposure, as well as a need to be able to provide HIV results to patients in a clinic, emergency room or labor and delivery, set the stage for manufacturers to develop rapid HIV assays. These card based assays have

gone through the same 1st-3rd generations as the main screening tests. Tests have been developed for whole blood, serum and oral fluid. The Orasure method is a third generation HIV antibody assay which is FDA waived and has also been approved for home testing (http://www.fda.gov/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/PremarketApprovalsPMAs/ucm311895.htm). Overall the rapid tests perform well.

The first HIV-1,2 antibody differentiation assay, the Bio-Rad Multispot procedure that is used in the 4th generation algorithm (fig 2), was initially developed as a 3rd generation rapid test. Bio-Rad will cease to market the Multispot assay during 2016 and is replacing it with another rapid procedure, the Geenius HIV 1-2 semi-automated HIV 1-2 differentiation assay. Although the Geenius is a "rapid" assay, it is approved to be used only as a supplemental assay in the 4th generation algorithm, not as a screening procedure. The Geenius has been reported to have a sensitivity of 100% and a specificity of 96% (17)

Currently there is a 4.5 generation rapid test, the Alere Determine HIV Ag-Ab Combo, which provides separate results for HIV antibody and antigen, but does not differentiate HIV-1 antibody from HIV-2 antibody. Like the 5th generation assay, this test would benefit from a different algorithm than has been developed for the 4th generation tests. Faraoni, et al, found this assay to have 100% specificity and positive predictive value with an overall sensitivity of 88.2% (18).

Social Aspects of HIV Testing

A complete discussion of the societal aspects of HIV testing is beyond the scope of this article, however, no discussion of the evolution of HIV testing can be complete without noting the social stigma originally associated with HIV infection and testing, the issue of mandatory vs voluntary testing, the legal restrictions concerning release of HIV testing related information and the eventual acceptance of HIV testing as part of routine medical practice. Most cases of AIDS were initially described in male homosexuals and iv drug users. Although heterosexual and blood product transmission were also

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documented, just having an HIV test was often interpreted as an indication that the patient was a member of a high risk group. Many states, including Ohio where I practice, passed laws requiring specific informed consent for HIV testing, counseling prior to and after the test was performed, and limiting the disclosure of HIV test results. HIV test results were often not put into laboratory or hospital computer systems. Summa Health System, where I practice, did not put HIV results in the laboratory and hospital computer system until 2005. The CDC recommended, in 2006, that HIV testing become a routine procedure and that all adults be screened for the presence of HIV or antibody to HIV (19). This required amendments to many state laws. As of January 2016, 41 states have at least some law concerning HIV testing and/or counseling (http://www.cdc.gov/hiv/policies/law/states/index.html).

Conclusions

HIV testing has evolved from being used as a method to safeguard the blood supply to being offered as a routine diagnostic test. The major deficiencies of early HIV tests have largely been overcome with the advent of the 4th and 5th generation assays. The test negative window from infection to detection has been reduced, positive predictive values have improved and tests are available in a variety of formats. Testing algorithms need to be continually updated to be used appropriately with newer assays. HIV testing has progressed to where infection can be detected approximately two weeks post exposure with a reduced number of false positive results when compared to the early HIV assays (fig 3). Despite the improvements in HIV testing, the oncologist I mentioned in the first paragraph would not be satisfied. There remains no specific diagnostic test for AIDS.

Acknowledgement

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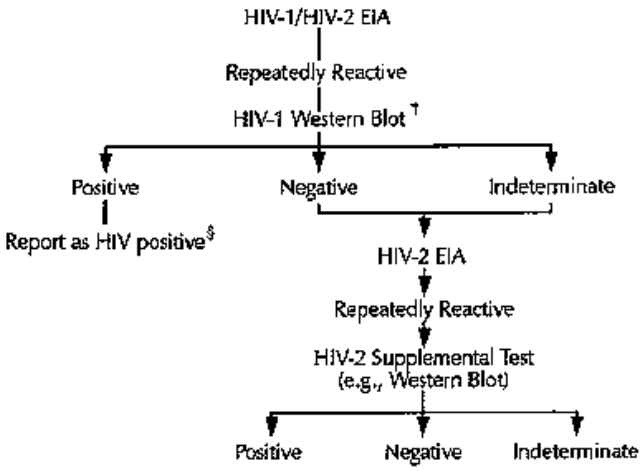
FIGURE 1. Centers for Disease Control/Food and Drug Administration testing algorithm for use with combination HIV-1/HIV-2* enzyme immunoassays (EIAs)



*HIV = Human immunodeficiency virus.
†An immunofluorescence assay (IFA) for HIV-1 antibodies has recently been licensed by the Food and Drug Administration and can be used instead of Western blot. Positive and negative IFA results should be interpreted in the same manner as similar results from Western blot tests. An indeterminate IFA should first be tested by HIV-1 Western blot and then as indicated by the Western blot results.
§Perform HIV-2 EIA only if there is an identified risk factor for HIV-2 infection.

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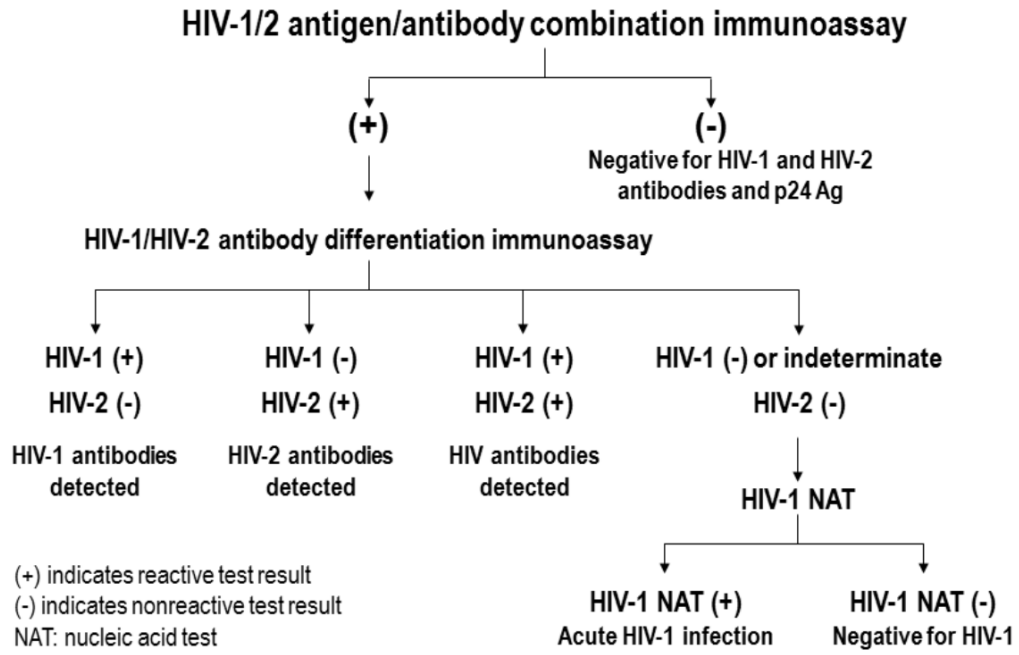
207 Fig 2. CDC Algorithm for use with a 4th Generation HIV AB/Ag screening test



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209 From MMWR, June 27, 2014. Downloaded from the CDC.gov website 1/26/2016

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211 Fig 3. HIV assay diagnostic testing evolution (4).

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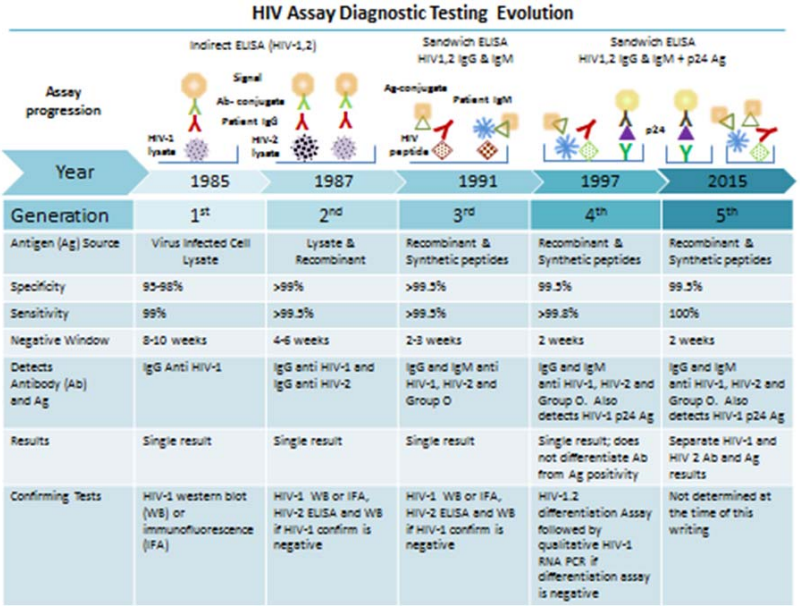

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217 **References**

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219 1. Pear R. 1985. AIDS blood test to be available in 2-6 weeks.
220 http://www.nytimes.com/1985/03/03/us/aids-blood-test-to-be-available-in-2-to-6-weeks.html.
221 Accessed Jan. 26, 2016
222 2. Selik, RM, Mokotoff, ED, Branson, B, Owen, SM, Whitmore, S, Hall, HI. 2014. Revised Syrveillance
223 Case Definition for HIV INFection. MMWR **63**(RR03): 1-10.
224 3. Gallo RC, Sarin PS, Gelmann EP, Robert-Guroff M, Richardson E, Kalyanaraman VS, Mann D,
225 Sidhu GD, Stahl RE, Zolla-Pazner S, Leibowitch J, Popovic M. 1983. Isolation of human T-cell
226 leukemia virus in acquired immune deficiency syndrome (AIDS). Science **220**(4599):865-7.
227 4. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin
228 C, Vezinet-Brun F, Rouzioux C, Rozenbaum W, Montagnie L. 1983. Isolation of a T-
229 lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).
230 Science 220(4599):868-71.
231 5. Chappel RJ, Wilson KM, Dax EM. 2009. Immunoassays for the Diagnosis of HIV: Meeting Future
232 Needs by Enhancing the Quality of Testing. *Future Microbiol*. **4**(8):963-982.
233 6. Centers for Disease Control. 1989. Interpretation and Use of the Western Blot Assay for
234 Serodiagnosis of Human Immunodeficiency Virus Type 1 Infections. MMWR **38**(S-7):1-7
235 7. Hedenskog M, Dewhurst S, Ludvigsen C, Sinangil F, Rodriguez L, Wu YT, Volsky DJ. 1986. Testing
236 for antibodies to AIDS associated retrovirus (HTLV-III/LAV) by indirect fixed cell
237 immunofluorescence: specificity, sensitivity and applications. J Med Virol **19**(4):325-34.

8. Imrie AA, Kehrer S, Smith GW, Penny R, Cooper DA. 1986. 757. Seroimmunology of AIDS retrovirus infection. I. Use of immunofluorescence assay to confirm sera with ELISA reactivity. Pathology **18**(4):438-443.
9. Alexander TS, Lee J, Yen-Lieberman B. 1999. Incidence of human immunodeficiency virus antibody in a prenatal population at a community hospital. Clin Diag Lab Immunol **6**(1):140-141.
10. Bentsen C, McLaughlin L, Mitchell E, Ferrara C, Liska S, Myers R, Peel S, Swenson P, Gadelle S, Shriver MK. 2011. Performance evaluation of the Bio-Rad laboratories GS HIV combo Ag/Ab EIA, a 4th generation HIV assay for the simultaneous detection of HIV p24 antigen and antibodies to HIV-1 (groups M and O) and HIV-2 in human serum or plasma. J Clin Virol **52S**:S57-S61.
11. Chavez P, Wesolowski L, Patel P, Delaney K, Owen SM. 2011 Evaluation of the performance of the Abbott Architect HIV Ag/Ab combo assay. J Clin Virol **52S**:S51-S55.
12. Geren K, Moore E, Tomlinson C, Hobohm D, Gardner A, Reardon-Maynard D, Gay C, Hightow-Weidman LB, Pandori MB, Moss N, Westheimer E, Tsoi B, Branson BM, Peters PJ. 2013. Detection of Acute HIV Infection in Two Evaluations of a New HIV Diagnostic Testing Algorithm — United States, 2011–2013. MMWR, **62**(24) 489-494.
13. Cardenas AM, Baughan E, Hodinka, R. 2013. Evaluation of the Bio-Rad Multispot HIV-1/HIV-2 rapid test as an alternative to western blot for confirmation of HIV infection. J. Clin Virol **58S**:e97-e103.
14. Muthukumar A, Alatoom A, Burns S, Ashmore J, Kim A, Emerson B, Bannister E, Ansari, MQ. 2015. Comparison of 4th generation HIV antigen/antibody combination assay with 3rd generation HIV antibody assays for the occurrence of false-positive and false-negative results. Lab Med **46**(2): 84-89.

15. Kim S, Lee JH, Choi, JY, Kim JM, Kim HS. 2010. False-positive rate of a "fourth-generation" HIV antigen/antibody combination assay in an area of low HIV prevalence. Clin Vaccine Immunol. 17(10):1642-1644.

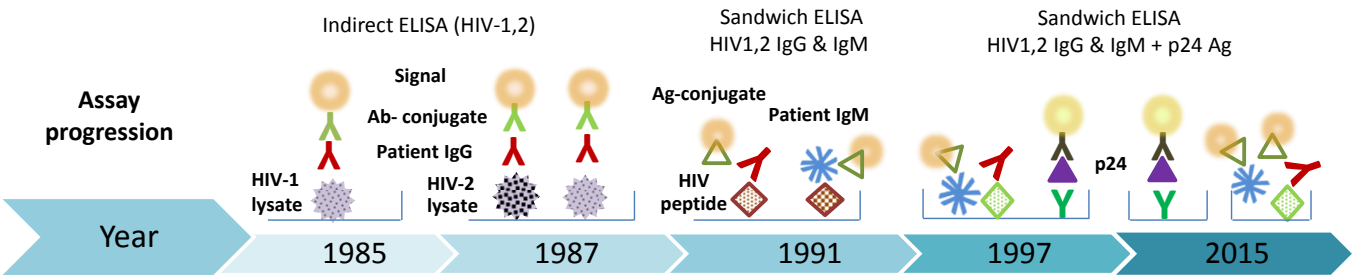
16. Salmona M, Delarue S, Delaugerre C, Simon F, Maylin S. 2014. Clinical Evaluation of BioPlex 2200 HIV Ag-Ab, an Automated Screening Method Providing Discrete Detection of HIV-1 p24 Antigen, HIV-1 Antibody, and HIV-2 Antibody. J Clin Micro **52**(1):103-107.

17. Mallocha L, Kadivara K, Putzb J, Levett PN, Tangc J, Hatchetted TF, Kadkhodae K, Nga D, Hoa J, Kima J. 2013. Comparative evaluation of the Bio-Rad Geenius HIV-1/2 Confirmatory Assay and the Bio-Rad Multispot HIV-1/2 Rapid Test as an alternative differentiation assay for CLSI M53 algorithm-I.J Clin Virol **58**(S-1):e85-e91.

18. Faraoni S, Rochetti A, Gotta F, Ruggiero T, Orofino G, Bonoar S, Chisetti V. 2013. Evaluation of a rapid antigen and antibody combination test in acute HIV infection. J Clin Virol **57**:84-87.

19. Branson BM, Handsfield HH, Lampe MA, Janssen RA, Taylor AW, Lyss SB, Clark JE.2006. Revised Recommendations for HIV Testing of Adults, Adolescents, and Pregnant Women in Health-Care Settings. MMWR Recommendations and Reports **55**(RR17):1-17.

HIV Assay Diagnostic Testing Evolution



Generation	1st	2nd	3rd	4th	5th
Antigen (Ag) Source	Virus Infected Cell Lysate	Lysate & Recombinant	Recombinant & Synthetic peptides	Recombinant & Synthetic peptides	Recombinant & Synthetic peptides
Specificity	95-98%	>99%	>99.5%	99.5%	99.5%
Sensitivity	99%	>99.5%	>99.5%	>99.8%	100%
Negative Window	8-10 weeks	4-6 weeks	2-3 weeks	2 weeks	2 weeks
Detects Antibody (Ab) and Ag	IgG Anti HIV-1	IgG anti HIV-1 and IgG anti HIV-2	IgG and IgM anti HIV-1, HIV-2 and Group O	IgG and IgM anti HIV-1, HIV-2 and Group O. Also detects HIV-1 p24 Ag	IgG and IgM anti HIV-1, HIV-2 and Group O. Also detects HIV-1 p24 Ag
Results	Single result	Single result	Single result	Single result; does not differentiate Ab from Ag positivity	Separate HIV-1 and HIV 2 Ab and Ag results
Confirming Tests	HIV-1 western blot (WB) or immunofluorescence (IFA)	HIV-1 WB or IFA, HIV-2 ELISA and WB if HIV-1 confirm is negative	HIV-1 WB or IFA, HIV-2 ELISA and WB if HIV-1 confirm is negative	HIV-1.2 differentiation Assay followed by qualitative HIV-1 RNA PCR if differentiation assay is negative	Not determined at the time of this writing