HIV Ag/Ac

ELISA 4ª Generación

Enzyme-linked immunosorbent assay (ELISA) for the simultaneous detection of p24 antigen to HIV-1 and antibodies anti HIV-1 and HIV-2

SUMMARY

The Human Immunodeficiency Viruses (HIV-1 and HIV-2) are the causative agents of the Acquired Immunodeficiency Syndrome (AIDS). These retroviruses are transmitted by exposure to certain infected body fluids, mainly genital secretions and blood or contaminated products derived from blood and through the placenta.

Serologic evidence of HIV-1 and HIV-2 infection may be obtained confirming the presence of antigens and antibodies in the serum of individuals suspected to have the infection. The antigens may generally be detected in the acute phase and during the symptomatic phase of the disease. The antibodies may be detected throughout the infection, beginning in the acute phase or immediately after it. Therefore, it is fundamental to use a high sensitivity determination able to detect antigens and antibodies.

The HIV Ag/Ac ELISA 4ª Generación test is designed to detect p24 antigen as well as HIV-1, HIV-1 group O and HIV-2 antibodies.

PRINCIPLE

The microtitration plate wells are coated with HIV-1 and HIV-2 recombinant proteins and synthetic peptides and anti-p24 monoclonal antibodies(*). The sample is incubated in the wells, if it contains p24 and/or HIV-1 (gp41) or HIV-2 (gp36) antibodies they will bind to the antigens and/or antibodies present in the wells. The unbound fraction is eliminated by washing. The next step is the addition of Conjugate 1 containing antibodies and antigens marked with biotin that will bind to antigens/antibodies is they are present in the sample. Then Conjugate 2 is added (peroxidase conjugated to streptavidin) that will bind to Conjugate 1. The unbound conjugate is removed by washing.

Then, a solution containing tetramethylbenzidine (TMB) and hydrogen peroxide is added. The reactive samples develop a blue color that changes to yellow when the reaction is stopped with sulfuric acid (Stopper).

PROVIDED REAGENTS

Coated microtitration plate: 96 wells microtitration plate coated with HIV-1 and HIV-2 recombinant proteins and synthetic peptides and human anti-p24 monoclonal antibodies.

Sample Diluent: 0.02 M Tris buffer containing bovine proteins, sodium chloride and surfactant agent, pH 7.2, blue color.

Conjugate 1: solution containing HIV-1 and HIV-2 recombinant proteins and synthetic peptides and human anti-p24 monoclonal antibodies biotinylated in 10 mM phosphate buffer with bovine proteins, sodium chloride and surfactant agent, pH 7.2.

Concentrated Conjugate 2: streptavidin conjugated to peroxidase, orange color.

Conjugate 2 diluent: 10 mM phosphate buffer with bovine proteins, sodium chloride and surfactant agent, pH 7.2.

TMB: 36 mM tetramethylbenzidine solution in 100% dimethylsulfoxide (DMSO), (100x).

TMB diluent: 40 mM citrate buffer and 1.27 mM hydrogen peroxide, pH 4.3.

Stopper: 1 M sulfuric acid.

Concentrated Wash Buffer: 250 mM phosphate buffer, 3.45 M sodium chloride and surfactant agent, (25x), pH 6.4.

Positive Control: inactivated human serum containing antibodies to HIV, red color.

p24 Positive Control: HIV-1 p24 antigen solution in 10 mM phosphate buffer with bovine proteins, sodium chloride and surfactant agent, pH 7.2.

Negative Control: inactivated, non-reactive normal human serum, yellow color.

NON-PROVIDED REAGENTS

Distilled or deionized water

REQUIRED MATERIAL (non-provided)

- Regulated or fixed automatic or semiautomatic micropipettes
- Disposable tips
- Volumetric material for dilutions
- Stopwatch
- Incubator at 37ºC
- Disposable gloves
- Absorbent paper
- Sodium hypochlorite
- (Automatic or manual) microtitration plate wash system
- Spectrophotometer for microtitration plate reading

WARNINGS

- The reagents are for “in vitro” diagnostic use.
- Use the reagents according to the working procedures for clinical laboratories.
- The reagents and samples should be discarded according to the local regulations in force.
- All patient samples should be handled as capable of transmitting infection. Controls are inactivated; however, they should be handled as infectious material.
- Control sera have been tested and found non-reactive to Hepatitis B surface antigen (HBsAg) and antibodies to Hepatitis C virus (HCV). However, they should be handled as potentially infectious material.

(*)Include AIP N.V., Belgium biomaterial

- All materials used to perform the test should be treated in order to ensure the inactivation of pathogenic agents. The recommended method is autoclaving for 1 hour at 121ºC. Liquid waste may be disinfected with sodium hypochlorite (5% final concentration) for at least 60 minutes.
- Do not exchange reagents from different kits and lots.
- Do not use foreign reagents.
- Microtitration plates must be incubated in incubator. Do not use water bath. Do not open the incubator during the process.
- Avoid the contact of the microtitration plates with hypochlorite fumes from the biohazard disposal containers or other sources, as hypochlorite affects the reaction.
- Avoid contact of the skin and mucous membranes with sulfuric acid (Stopper) and DMSO (TMB). H315+H320: Causes skin and eye irritation. H314 Causes severe skin burns and eye damage. P262 Do not get in eyes, on skin, or on clothing. P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P302 + P352 IF ON SKIN: Wash with plenty of soap and water. P280 Wear protective gloves/protective clothing/eye protection/face protection.- Rinse with plenty of water in case of reagent contact with the skin or eyes.
- Avoid any spilling of liquids or spraying.
- Do not pipette by mouth. Use disposable gloves and eye protection during handling of the samples and test reagents.

REAGENT PREPARATION

Wash Buffer: at low temperature the reagents components may precipitate. In such case, heat the solution at 37ºC until complete dissolution. To obtain ready to use wash buffer, dilute Concentrated Wash Buffer (25x) with distilled or deionized water. Example: 40 ml in 960 ml for one microtitration plate.

Conjugate 2: in a labelled tube, dilute concentrated Conjugate 2 (100x) in Conjugate 2 Diluent. Example: 200 ul in 20 ml for one microtitration plate.

Substrate: in a labelled tube, dilute concentrated TMB (100x) in TMB Diluent. Example: 200 ul in 20 ml for one microtitration plate. Concentrated TMB is dissolved in DMSO. Since the melting point of DMSO is 18ºC, the TMB must reach room temperature (20-25ºC) and homogenized thoroughly before using.

Homogenize the TMB concentrated solution before dilution.

Coated microtitration plate, Sample Diluent, Conjugate 1, Stopper, Positive Control, Negative Control and p24 Positive Control: ready to use.

STABILITY AND STORAGE INSTRUCTIONS

Provided Reagents are stable at 2-10ºC until the expiration date shown on the box. Do not freeze.

It is important that all the material used for the preparation be clean.

Concentrated Wash Buffer and Stopper: may be stored at 2-25ºC.

Wash Buffer: once diluted it is stable for 3 months at 2-25ºC.

Diluted Conjugate 2: once prepared it is stable for 24 hours at 2-25ºC.

Coated microtitration plate: provided in vacuum-sealed pouches with desiccant. Do not open the pouch before performing the test or until it has reached room temperature, otherwise the contents can get moistened. Unused well strips should be kept in the pouch with the desiccant, sealed and stored at 2-10ºC.

Substrate: once prepared, it should be used within 3 hours stored at 2-25ºC and protected from light. It should be discarded in case any coloration is observed.

SAMPLE

Non-diluted serum or plasma

a) Collection: obtain the sample in the usual way.

b) Additives: not required for serum. If plasma is used, any anticoagulant commonly used for transfusions can be employed.

c) Known interfering substances: do not use contaminated, hyperlipemic or hemolyzed sera or plasma. No interferences are observed by bilirubin up to 30 mg/dl, ascorbic acid up to 50 mg/dl, lipemic samples up to 1453 mg/dl triglycerides or hemolyzed samples up to 15 mg/dl hemoglobin. Samples containing particles should be clarified by centrifugation.

d) Stability and storage instructions and transport: sample should be stored refrigerated (2-10ºC). If test is not performed within 72 hours, it should be frozen at -20ºC. Avoid repeated freezing and thawing. This may cause erroneous results. Plasmas should be rapidly thawed by heating for a few minutes at 40ºC (to limit fibrin precipitation).

If samples must be transported, they should be packed according to local regulations for the shipment of infectious materials.

PROCEDURE

1- Bring the reagents and the samples to room temperature 30 minutes before starting the test.

2- Prepare the required Wash Buffer volume.

3- Remove the amount of wells required for the test and replace the microtitration plate in its sealed pouch with desiccant, store immediately at 2-10ºC.

4- In the microtitration plate wells to be used dispense the Sample Diluent, then the sample (S), the Negative Control (NC) by triplicate, the Positive Control (PC) by duplicate and the p24 Positive Control (p24), according to the following scheme:

	S	PC	p24	NC
Sample Diluent	100 ul	100 ul	100 ul	100 ul
Positive Control	-	100 ul	-	-
p24 Positive Control	-	-	100 ul	-
Negative Control	-	-	-	100 ul
Sample	100 ul	-	-	-

To avoid evaporation, cover the plate with an adhesive tape.

5- Incubate for 60 minutes at 37ºC.

6- After incubation, completely discard the liquid from each well. Wash 5 times according to Wash instructions (See Washing procedure).

7- Add ready to use Conjugate 1:

Conjugate 1	200 ul	200 ul	200 ul	200 ul

To avoid evaporation, cover the plate with an adhesive tape.
8- Incubate Conjugate 1 for 30 minutes at 37°C. In parallel, prepare diluted Conjugate 2.
9- After incubation discard Conjugate 1 from each well by aspiration or inverting and tapping the microtitration plate several times onto absorbent paper. DO NOT WASH THE MICROTITRATION PLATE BETWEEN INCUBATION WITH CONJUGATE 1 AND CONJUGATE 2.
10- Add diluted Conjugate 2:

Diluted Conjugate 2	200 ul	200 ul	200 ul	200 ul

To avoid evaporation, cover the plate with an adhesive tape.
11- Incubate for 30 minutes at 37°C.
12- Prepare the Substrate.
13- After incubation, discard Conjugate 2 washing 5 times according to wash instruction (see Washing procedure).
14- Add the Substrate:

Substrate	200 ul	200 ul	200 ul	200 ul

15- Incubate for 30 minutes at 18-25°C. Keep the microtitration plate protected from light and then add:

Stopper	50 ul	50 ul	50 ul	50 ul

16- Read the absorbance of the solution in the wells in spectrophotometer at 450 nm or bichromatically at 450/600-650 nm.

Note: bichromatic reading is recommended. In case the reading is monochromatic, perform a reagent blank (but omitting the addition of Diluted Conjuate 2) that will have to be subtracted from all sample values.

STABILITY OF FINAL REACTION
The reaction color is stable for 5 to 30 minutes, thus the results must be read within that period of time.

WASHING PROCEDURE
The wells are washed with 350 ul diluted wash buffer. When filling the wells make sure not to spill. The wash solution should be in contact with the wells for 30 to 60 seconds. Make sure no residual liquid remains after the final washing step. Perform double aspiration to remove excess buffer. If after such procedure, it still persists, invert the plate onto absorbent paper and tap it several times. Otherwise, erroneous results may be obtained.

Note: the washing procedure is crucial for the test result. If excess wash buffer remains in the wells, or if the wells are not completely filled, erroneous results may be obtained. Do not let the wells air dry during the procedure. Automatic washers should be rinsed with distilled or deionized water at the end of the day to avoid obstructions due to the presence of salt in the wash buffer.

SUMMARY OF THE PROCEDURE

STAGE	PROCEDURE	PRECAUTIONS/OBSERVATIONS
Wash buffer	Prepare Wash solution	Dissolve salt crystals
Sample Diluent	Add 100 ul Sample Diluent in each well	
Samples	Add 100 ul S, PC, NC and p24	Color change is observed when adding the sample and controls
Incubation	Cover the wells and incubate for 60 minutes at 37°C	In incubator
Washing step	Wash each well with 350 ul Wash buffer (5 times)	Time of contact of the Wash solution from 30 to 60 seconds. Completely remove the residual liquid from the wells
Conjugate 1	Add 200 ul Conjugate 1	
Incubation	Cover the wells and incubate for 30 minutes at 37°C	In incubator
Preparation	Conjugate 2	During Conjugate 1 incubation, prepare Conjugate 2
Conjugate 2	Remove Conjugate 1 and dispense 200 ul Conjugate 2	Do not wash between Conjugate 1 and diluted Congate 2
Incubation	Cover the wells and incubate for 60 minutes at 37°C	
Preparation	Substrate	During Conjugate 2 incubation, prepare Substrate
Washing step	The same as above	
Substrate	Add 200 ul Substrate	
Incubation	18-25°C for 30 minutes	Mantein the wells protected from light
Stop	Add 50 ul Stopper	
Reading	Read in spectrophotometer	Read within 5 to 30 minutes

ASSAY VALIDATION CRITERIA

The assay is considered valid if the following conditions are simultaneously met:

1- The readings of at least 2 of the 3 Negative Controls should be less than or equal to 0.100 O.D. Any Negative Control not meeting this condition should be excluded.

Example: calculation of the Negative Control mean

Control	Absorbance
1	0.019
2	0.016
3	0.016
Total	0.051

NC = Total Abs / 3 = 0.051 / 3 = 0.017

2- The readings for the Positive Controls should be greater than or equal to 0.800 O.D.

Example: Positive Control reading

Control	Absorbance
1	1.968
2	2.012

3- The reading for the Positive Control for p24 should be greater than or equal to 0.400 O.D.

Note: In case one of the above conditions is not met, repeat the assay.

Cut-off

Calculate the Cut-off value adding 0.180 to the Negative Control mean.

Example: Negative Control mean = 0.017

Cut-off value = 0.180 + 0.017 = 0.197

INTERPRETATION OF RESULTS

Non-reactive samples: are those which absorbance value is lower than the Cut-off value.

Initially reactive samples: are those whose absorbance value is greater than or equal to the Cut-off value. They should be repeated by duplicate using the original sample. If both duplicates are negative, the sample is considered non-reactive for HIV. If at least one duplicate is positive, the sample is considered repeatedly reactive and contains p24 antigen and/or antibodies to HIV-1 or HIV-2. Repeatedly reactive results should be verified by a confirmatory method; according to the current the legal regulations.

A sample initially reactive may be non-reactive in both repetitions. This may be due to:

- Cross contamination of a non-reactive well with high titer of antibodies and/or antigens to anti-HIV.
- Sample contamination during dispensation, lack of precision in sample and/or conjugate or TMB dispensation into the well, tip reutilization.
- Well contamination with hypochlorite or other oxidizing agents.
- Non coagulated samples with rest of fibrin or fibronectin.

In certain cases a non-reactive sample may produce a falsely reactive reaction, both in the initial analysis as in its repetitions. Some probable causes of this effect may be:

- Contamination of a non-reactive sample by a strongly reactive one.
- Sample contamination during collection, processing or storage.
- Presence of interfering substances, such as autoantibodies, antibodies to any of the components of the reagent, drugs, etc.
- Substrate contamination with Conjugate 2.
- Use of hemolyzed samples, incompletely coagulated serum, plasma containing fibrin or samples with microbial contamination. This type of sample may yield falsely reactive results.
- Ineffective dispensation and/or aspiration of the wash solution (obstructed system).
- Insufficient aspiration with residual wash solution in the wells after the last washing step.
- Bubble formation in the wells during the reading procedure.

PROCEDURE LIMITATIONS

See Known interfering substances under SAMPLE.

Do not use pooled samples.

Do not use other body fluids such as saliva, cerebrospinal fluid or urine.

The colorimetric method verifying the sample load is unsuitable to verify accuracy of the volumes distributed, it only validates the presence of the sample.

Occasionally, when performing bichromatic readings, negative absorbance values may be obtained, that do not invalidate the determination. This is produced by the fact that some samples give readings lower than the Reagent Blank.

SPECIFIC PERFORMANCE FEATURES

Clinical Sensitivity

a) Clinical sensitivity with commercial seroconversion panels (BBI, Boston Biomedica, Inc.)

BBI Panels		**HIV Ag/Ac ELISA 4ª Generación Results**	**Western Blot Results (according to BBI)**
Panel name	**Sample collection days**	**Time (days) in which sample becomes reactive**	
Panel BBI 916 P	0 4 9 15 30 35	15	30
Panel BBI 924 X	0 2 8 10 26 33 35 40	26	35 (indet)
Panel BBI 925 Y	0 1018 22 44 49	44	44 (indet)
Panel BBI 926 Z	0 2 7 9 27 32	7	27
Panel BBI 927 AB	0 28 33 35 40	28	35
Panel BBI 929 AD	0 4 14 18 21 25 28	18	25-28
Panel BBI 931 AF	0 2 7 9 15 28 33 35 42	28	33
Panel BBI 935 AJ	0 10 16 21 24 28 43	28	43
Panel BBI 944 AT	0 2 7 9 14 16	7	14 (indet)
Panel BBI 945 AU	0 3 7 13 15 20	13	20
Panel BBI 946 AV	0 4 7 11	7	ND

Panel BBI 948 AX	0 18 20 23	23	Non reactive
Panel BBI 954 BD	0 2 7 10 14 17 21	21	Non reactive
Panel BBI 955 BE	0 3 7 12 14	7	All indeterminate samples
Panel BBI 956 BF	0 40 42 47 50	47	Non reactive
Panel BBI 957 BG	0 7 9 14 16 23 28	23	All indeterminate samples
Panel BBI 958 BH	0 2 7 9 15 17	9	Non reactive
Panel BBI 959 BI	0 7 9 14 19 21 26	7	14

ND: non determinated; indet: indeterminate

b) Clinical Sensitivity in BBI Performance Panels:
In a study performed on different international commercial panels, the following results were obtained:
PRZ204 (HIV 1/2 Combo Performance Panel): 14 out of 14 reactive samples were detected.
PRZ205 (HIV 1/2 Combo Performance Panel): 14 out of 14 reactive samples were detected.
PRB204 (Anti-HIV 1 Mixed Titer Performance Panel): 23 out of 23 reactive samples were detected.
PRB108 (Anti-HIV 1 Low Titer Performance Panel): 14 out of 14 reactive samples were detected.

c) Clinical Sensitivity in Panels of reactive anti-HIV samples:
In a study performed on 540 samples from patients with HIV-1 and HIV-2 infection, confirmed by different methods, all of the samples were found reactive with the HIV Ag/Ac ELISA 4ª Generación kit.

No. of samples	Sensitivity
361 [hospitals] 203 [risk population]	100%
153 reactive samples and 50 non reactive	100%
26 HIV-2 samples	100%

Sensitivity of p24 antigen
BBI PRA801 panel (HIV Antigen Sensitivity Panel) consists of cultured serial dilutions of HIV-1 infected cells. The concentration of p24 antigen is determined by the standards: WHO: HIV-1 p24 Antigen 90/636, DuPont HIV p24 and Sanofi HIV Antigen, detecting up to dilution number 9 (p24 concentration < 2 pg/ml, according to BBI) with HIV Ag/Ac ELISA 4ª Generación kit.

Clinical Specificity
In a study performed on 3250 sera and plasma samples from two different centers, tested using the HIV Ag/Ac ELISA 4ª Generación kit, 99.94% (3229/3231) non reactive samples were obtained. Out of the 21 initially reactive samples, 19 were reactive, confirmed by other methods.

Specificity
In a study performed on 561 samples from an elderly population tested using the HIV Ag/Ac ELISA 4ª Generación kit, a specificity of 99.82% was obtained.
A possible cross-reactivity was evaluated, assaying samples from 266 individuals with different clinical conditions that may be the cause of unspecific reactions for the HIV Ag/Ac ELISA 4ª Generación test. These conditions include pregnant women, hemodialyzed patients, patients with autoimmune diseases or infectious diseases other than HIV (Syphilis, HTLV, Hepatitis B, Hepatitis C, CMV, Adenovirus, others). Only one sample from the autoimmune disease group showed unspecific reactivity. For this population the specificity was 99.62%.

Precision
The inter-assay and inter-lot reproducibility were evaluated. The assays were performed with samples having different reactivity levels.

	OD/CO Mean	Intra-assay C.V.	Inter-assay C.V.	Inter-lot C.V.
Sample 1	2.44	9.19 %	10.88 %	13.44 %
Sample 2	1.5	10.24 %	13.2 %	12.67 %
(+) Control	15.09	2.12 %	2.13 %	2.52 %
(-) Control	0.074	14.68 %	13.58 %	23.42 %

n = 144

PROCEDURE LIMITATIONS
- The variability of HIV-1 and HIV-2 viruses does not exclude the possibility of HIV-1 or HIV-2 infection. Not any known method may offer complete security.
- All ELISA techniques may show falsely reactive results.
- A non-reactive result does not exclude the possibility of HIV infection.
- A reactive result should be confirmed by other test.

WIENER LAB. PROVIDES
- Kit for 96 tests (Cat. Nº 1723451).
- Kit for 192 tests (Cat. Nº 1723551).

REFERENCES

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SYMBOLS

Policubeta	Sensib.

Coated microtitration plate

Diluyente	Muestra

Sample diluent

Conjugado	1

Conjugate 1

Conjugado	2 Conc.

Concentrated Conjugado 2

Conjugado	2 Diluy.

Conjugado 2 Diluent

TMB

TMB

TMB	Diluy.

Diluyente de TMB

Stopper

Stopper

Buf. Lavado	Conc.

Concentrated Wash Buffer

Control	+

Positive Control

Control	p24

Positive p24 Control

Control	-

Negative Control

The following symbols are used in the packaging for Wiener lab. diagnostic reagents kits.

CE This product fulfills the requirements of the European Directive 98/79 EC for "in vitro" diagnostic medical devices

EC REP Authorized representative in the European Community

IVD "In vitro" diagnostic medical device

Contains sufficient for <n> tests

Use by

Temperature limitation (store at)

Do not freeze

Biological risks

Volume after reconstitution

Cont. Contents

LOT Batch code

Manufactured by:

Harmful

Corrosive / Caustic

Irritant

Consult instructions for use

Calibr. Calibrator

CONTROL Control

CONTROL + Positive Control

CONTROL – Negative Control

REF Catalog number

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