



HIV PLUS (1/2/P24) ELISA TEST SYSTEM

INTENDED USE

The Reactiva Search's HIV PLUS is an enzyme immunoassay for the detection of Ig-antibodies to Human Immunodeficiency Virus (HIV) type 1 including group O, type 2 and p24 antigen in human serum or plasma samples. The assay is intended for the screening of blood units, for the diagnosis and the monitoring of HIV infection. The HIV is a member of Retroviridae family, Lentivirus genus. It is an enveloped virus with a nucleocapsid containing two molecules of RNA. The RNA template is retrotranscribed to cDNA and integrated into the host genome. The env, pol and gag genes code for the three major types of structural proteins: gp120 and gp41 envelope proteins, p24 capsid protein, p17 matrix protein and an enzymatic protein for transcription of RNA (reverse transcriptase). Two HIV genotypes designed HIV-1 and HIV-2 are originally identified in American and African patients with AIDS and AIDS related complex (1, 2). The HIV-1 is subdivided according to the divergence in the structure of env gene in M (major) group with subtypes A to J and in a further cluster of heterogeneous viruses named group O (outside the classic HIV-1) primary appearing in West Africa (3). The HIV infection is transmitted through sexual intercourse, contaminated needle, blood transfusion, maternal/newborn route. The pathogenesis is characterized by progressive depression of immune system with developing of opportunistic infections (virus, fungi, protozoa) and neoplasms such as Kaposi's sarcoma and malignant lymphoma. Serological diagnosis of HIV infection is usually based on the detection of antibodies by enzyme linked immunosorbent assay (Elisa). Since its introduction in 1985, the test have been improved in sensitivity and specificity until the recent introduction of p24 antigenemia detection, which could be the only HIV marker in the window phase of infection (4). The Elisa test, able to detect simultaneously HIV p24 antigen and HIV antibodies, allows to obtain an earlier diagnosis and reducing the risk of HIV transmission by blood donation (5-7).

PRINCIPLE OF THE TEST

The HIV PLUS kit is a fourth generation assay based on "direct sandwich" Elisa principle. The microplate wells are coated with HIV specific synthetic antigens derived from conserved DNA sequences encoding for immune-dominant antigenic determinants (HIV-1gp41 and HIV-2 gp36) and with monoclonal antibody (mab1) to p24 antigen. In the first incubation the diluted sample, dispensed into the well, reacts with the solid phase; antibodies to HIV and p24 antigen, if presents, are captured by the antigens and by the mab anti-p24. After washing, bound p24 antigen is detected in the second incubation by the addition of an anti-p24 mab2, HRP-labelled, directed against a different epitope from the mab linked to the solid phase. After further washing out all the other components of the sample, in the third incubation, HIV 1/2 antibodies bound to antigens of solid phase are detected by the addition of ENV HIV antigens labelled with horse radish peroxidase (HRP). The enzyme captured on the solid phase, acting on the Chromogen/Substrate solution, generates an optical signal that may be detected by an Elisa reader. Samples are pre-treated in the well with an Additive able to block interferences, particularly complement fractions that can change the correct titration of the antibodies.

MATERIALS AND COMPONENTS PROVIDED

Strip Microplate - Microplate of 8 x 12 strips of breakable wells activated with synthetic HIV antigens and with mab anti-p24. The microplates are sealed in an aluminum pouch in presence of desiccant bag.

Antigen Positive Control - Ready to use. Buffered solution of p24 recombinant protein. It contains 0.09 % sodium azide, 0.09 % Kathon as preservatives and Coomassie brilliant blue as coloring agent. Volume 0.5 ml

Antibody Positive Control - Ready to use. Buffered solution of human serum base reactive for antibodies to HIV. It contains 0.09 % sodium azide, 0.09 % Kathon as preservatives and Coomassie brilliant blue as coloring agent. Volume 0.5 ml

Note - The Positive Control has been inactivated with 1% tri (n-butyl) phosphate and 1% Triton X-100 at 30 °C for 4 hours by the manufacturer.

Negative Control - Ready to use. Buffered solution of human serum base not reactive for antibodies to HIV and for p24 antigen. It contains 0.09 % sodium azide, 0.09 % Kathon as preservatives and Coomassie brilliant blue as coloring agent.

Volume 1.0 ml

Sample Diluent - Proteic solution for the dilution of samples that contains stabilizers, 0.09 % sodium azide, 0.09 % Kathon as preservatives and Coomassie brilliant blue as coloring agent. Volume 40.0 ml

Washing Solution - To dilute before use. Solution 25x concentrated that contains Imidazole buffer and surface-active agent. Volume 50.0 ml

Conjugate A (20x concentrated). Buffered proteic solution, that contains specific mab2 anti-p24 labelled with HRP, proteic stabilizers, 0.02% gentamicin sulfate and 0.09 % Kathon as preservatives. Volume 0.6 ml

Conjugate A Diluent - Buffered proteic solution, for the dilution of the concentrated Conjugate that contains specific stabilizers 0.02%gentamicin sulphate,0.09% Kathon as preservatives and Brilliant Blue Coomassie as coloring agent. Volume 12.0 ml

Conjugate B (20x concentrated). Buffered proteic solution, that contains goat anti-human F(ab)-Ig antibodies, labelled with HRP, proteic stabilizers, 0.02% gentamicin sulphate and 0.09 % Kathon as preservatives. Volume 0.6 ml

Conjugate B Diluent - Buffered proteic solution, for the dilution of the concentrated Conjugate that contains specific stabilizers 0.02%gentamicin sulphate,0.09% Kathon as preservatives and Red Ponceau as coloring agent. Volume 12.0 ml

Chromogen - To mix with Substrate. Solution of 3, 3', 5, 5' tetramethylbenzidine (TMB), activators and stabilizers, in a phosphate/citrate buffer.

Note - Store protected from light. Volume 7.0 ml

Substrate - To mix with Chromogen. Solution that contains hydrogen peroxide (H2O2), activators and stabilizers, in a phosphate/citrate buffer. Volume 7.0 ml

Stop Solution - Solution of 0.3 M sulphuric acid.

Note: Handle with care. Volume 10.0 ml

Note - All the materials of human origin have been controlled and certified by the supplier to be negative for HBsAg, HCV Ab and HIV Ab.

MATERIALS REQUIRED BUT NOT PROVIDED

- Micropipettes of 20, 100, 300 and 1000 µl with disposable tips.
- Vortex mixer and adsorbent papers.
- Distilled water.
- Timer.
- Incubator set at 37 ± 1 °C (dry or moist heat).
- Automatic or manual microplate washer able to aspirate and dispense volumes of 300 - 400 µl.
- Photometric microplate reader linear up to at least 2 OD and supplied with filters of 450 nm and 620 - 630 nm.
- Microplate Selaers

REAGENT PREPARATION

Washing Solution The concentrated solution to be diluted 25 x with distilled water before use.

Conjugate A Fifteen minutes before its use, dilute 1:20 the concentrated Conjugate A with its diluent.

Conjugate B Fifteen minutes before its use, dilute 1:20 the concentrated Conjugate B with its diluent

Example for 2 strips: 100 µl of concentrated Conjugate A + 1.9 ml of Conjugate A Diluent.

Chromogen/Substrate About 5 minutes before use, mix 1 volume of Chromogen with 1 volume of Substrate, in a disposable plastic container, according to needs. This solution is stable for 4 hours at room temperature protected from light.

Note - Don't use the product after the expiration date.

STORAGE AND STABILITY

1. The kit has to stored at 2 - 8 °C and used before the expiration date declared on the external label.
2. The pouch containing the microplate has to be brought to room temperature before opening. Take out from the frame only the strips necessary for the test programmed and store the remaining strips in the same pouch in presence of the desiccant bag. Close hermetically the pouch and store again at 2 - 8 °C. If stored properly, strips are stable for 2 months from opening.
3. The diluted Washing solution, at room temperature, is stable for 1 week.
4. The Chromogen/Substrate are stable until the expiration of the kit.
5. The other reagents can be used every time, if stored at 2 - 8 °C and handled carefully for avoiding contamination.

SAFETY PRECAUTIONS

- All the reagents contained in the kit are for in vitro diagnostic use only.
- Do not use the kit or reagents after the expiration date stated on labels.
- Do not mix reagents of different lots.
- Procedures should be performed carefully in order to obtain reliable results and clinical interpretations.
- Bring all the reagents to room temperature for at least 60 minutes, before the test is started.
- Avoid any contamination of reagents when taking them out of vials. We recommend to use automatic pipettes and disposable tips. When dispensing reagents, do not touch the wall of microplate wells with tips, in order to avoid any cross-contamination.
- In the washing procedure, use only the Washing Solution provided with the kit and follows carefully the indications reported in the "Washing Instructions" section of this insert.
- Ensure that the Chromogen/Substrate does not come in contact with oxidizing agents or metallic surfaces; avoid any intense light exposure during the incubation step or the reagent preparation.
- Put the reagents in a glass or plastic disposable container, washed with sulphuric acid 1N, then with deionized water, before use.
- Samples and materials potentially infective have to be handled with care as they could transmit infection. All objects come in direct contact with samples and all residuals of the assay should be treated or wasted as potentially infective. Best procedures for inactivation are treatments with autoclave at 121°C for 30 minutes or with sodium hypochlorite at a final concentration of 2.5 % for 30 minutes. This last method can be used for the treatment of the liquid waste after that I has been neutralized with NaOH.
- Avoid any contact of liquids with skin and mucous membrane. Use always protective talk-free gloves, glasses and laboratory coats, according to the safety regulations.
- Some reagents of the kit contain sodium azide which may be toxic if ingested. Sodium azide may react with copper and lead piping to form highly explosive salts. On disposal, flush with large quantities of water.

TECHNICAL PRECAUTIONS

- At least 1 hour before use bring all the reagents necessary to the test to room temperature and mix carefully the liquid reagents supplied on vortex (in particular the Controls, the Conjugate and the Chromogen/Substrate) avoiding foaming. Take out from the frame only the strips necessary for the test programmed and store the remaining strips in the same pouch in presence of the desiccant bag.
- Distribution and incubation times should be the same for all the wells; avoid long interruptions among the different steps of the assay.
- It is suggested to eliminate the excess of washing solution from wells by blotting them gently on a paper adsorbent pad.
- The colour developed in the last incubation is stable for maximum 1 hour in the dark.
- We recommend reading the microplate at 450 nm (reading filter) and subtracting the blank at 620 - 630 nm (blinking filter). Blank the reader on A1 well.

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WASHING INSTRUCTION

A good washing procedure is essential to get correct and reliable analytical results. In case of manual washing, it is suggested to carry out 5 cycles, first dispensing and then aspirating 300 µl/well per cycle. Usually 5 cycles of automatic washing of 300 µl/well per cycle are sufficient to remove false positives and high background values. It is suggested to use an Elisa automatic microplate washer, qualified and properly serviced. Anyhow, we recommend to calibrate the washing system on the kit itself so to match the declared analytical performances. Any case, potentially infective wastes from microplate washing have to be inactivated with Na-hypochlorite at 2.5% final concentration for 30 minutes. All these materials have to be discarded according to the law as potentially infective wastes.

SPECIMEN COLLECTION AND STORAGE

Either fresh sera or plasma (EDTA, Heparin, Citrate) can be used for the assay. If not used immediately, they can be stored at 2 - 8 °C for 1 week. In case of longer storage freeze them at - 20 °C. Samples should be clear. If the samples are turbid, could be contaminated by microorganism, insofar it recommends to centrifuge them at 2000 rpm x 20 minutes at room temperature or filtrate on 0.22 µm filters. The samples that, after the above said procedure, did not became clear, can not be used.

TEST PROCEDURE

At least 1 hour before use bring all the reagents necessary to the test to room temperature and mix carefully the liquid reagents supplied on vortex (in particular the Controls, the Conjugate and the Chromogen/Substrate) avoiding foaming. Do not dilute Controls as they are ready to use.

1. Leave the A1 well empty for blanking operations. Dispense 100 µl of Negative Control in triplicate, then 100 µl of Positive Control. Dilute 1:50 all the sample into disposable dilution vials dispensing 200 µl of Sample Diluent and 4 µl of sample. Mix on vortex, and then dispense 100 µl of diluted sample into each microplate well. In automatic, it is also possible to dispense 100 µl of Sample Diluent and 2,0 µl of sample directly into each microplate well.
 2. Cover the microplate with the plate sealer and incubate strips for 60 min at 37 °C.
 3. Peel out the plate sealer and wash the microplate according to instructions. Dilute the necessary quantity of concentrated Conjugate A in its Diluent
 4. Add 100 µl of the diluted Conjugate A to all the wells, but A1.
 5. Cover the microplate with the plate sealer. Then incubate the microplate sealed for 60 minutes at 37 °C.
 6. Peel out the plate sealer and wash the microplate according to instructions. Prepare the necessary quantity of concentrated Conjugate B in its Diluent
 7. Add 100 µl of the diluted Conjugate B to all the wells, but A1.
 8. Cover the microplate with the plate sealer. Then incubate the microplate sealed for 30 minutes at 37 °C.
 9. Peel out the plate sealer and wash the microplate according to instructions. Prepare the necessary Chromogen/Substrate solution (1065/1065.X).
 10. Add 100 µl of Chromogen/Substrate to all the wells, A1 included.
 11. Incubate the microplate for 15 minutes at room temperature, protected from light.
 12. Block the enzymatic reaction by adding 100 µl Stop Solution to all the wells, A1 included. Read the microplate at 450 nm and 620 - 630 nm blanking the instrument on A1 well.
- Note - Read the microplate within 10 minutes after the dispensing of the Stop Solution.

ASSAY SCHEME

At least 1 hour before use bring all the reagents necessary to the test to room temperature and mix carefully the liquid reagents supplied on vortex (in particular the Controls, the Conjugate and the Chromogen/Substrate) avoiding foaming. Do not dilute Controls as they are ready to use.

Position	Controls/Samples
A1	Blank
B1+C1+D1	Negative Control
E1	Antigen Positive Control
F1	Antibody Positive Control
G1.....H12	Samples

Reagents	Blank (A)	Controls	Samples
Controls	-	100 µl	-
Sample Diluent	-	-	100 µl
Sample			2,0 µl
Cover with the sealer and incubate for 60 minutes at 37 °C			
Peel out the sealer and wash 5 cycles with 300 µl/well per cycle Dilute the necessary quantity of the concentrated Conjugate A.			
Conjugate A		100 µl	100 µl
Cover with the sealer and incubate for 60 minutes at 37 °C			
Peel out the sealer and wash 5 cycles with 300 µl/well per cycle Dilute the necessary quantity of the concentrated Conjugate B.			
Conjugate B		100 µl	100 µl
Cover with the sealer and incubate for 30 minutes at 37 °C			
Peel out the sealer and wash 5 cycles with 300 µl/well per cycle			
Chromogen/Substrate	100 µl	100 µl	100 µl
Incubate for 15 minutes at room temperature in the dark			
Stop Solution	100 µl	100 µl	100 µl
Blank the reader on A1 well. Read at 620 - 630 nm for measuring the microplate background, then at 450 nm.			
Note - Read the microplate within 15 minutes after the dispensing of the Stop Solution.			

VALIDITY OF THE ASSAY

The assay is considered valid if:

- the OD 450 nm of the A1 blank well is < 0.100. Higher values are index of Chromogen/Substrate contamination;
- after blanking on A1, the OD 450 nm mean value of the Negative Control (NC) is < 0.200. Abnormal values may be observed when the washing instrument does not work correctly or the washing procedure has not been adapted to the assay as described in the proper section;
- the OD 450 nm value of the Antigen Positive Control and Antibody Positive Controls > 0.500. Lower values can be result when the storage temperature was not optimal or with a not correct operative procedure.

In case that the above data do not match the correct values, before repeating the test check carefully the expiration date of the kit, the performances of the instruments used for the assay and the procedure of distribution of Controls and samples.

CALCULATION OF RESULTS

If the validity of the assay is confirmed, calculate the Cut-off (Co) value through the following formula:

$$\text{Cut-off} = \text{NC mean} + 0.200$$

$$\text{Grey-zone} = \pm 10 \%$$

Example of calculation
 Negative Control mean 0.061 OD 450 nm
 Antigen Positive Control 1.918 OD 450 nm
 Antibody Positive Control 0.844 OD 450 nm
 Cut-off = 0.061 + 0.200 = 0.261

Grey-zone = 0.235 - 0.287

Sample 1 0.054 negative
 Sample 2 0.243 grey zone
 Sample 3 1.256 positive

Samples with OD value below the lower limit of the grey-zone are reported as negative. No further testing is required. Samples with an OD value within or exceeding the upper limit of the grey-zone are reported as initially reactive. The samples should be retested in duplicate.

- Initially reactive samples that do not react in both of duplicate repeat tests are reported as negative.
- Initially reactive samples that are confirmed reactive or grey zone have to be submitted to additional more specific tests (confirmatory tests).
- Repeatedly reactive samples not confirmed positive are considered false-reactive samples.
- Repeatedly "grey zone" samples confirmed positive are considered positive.
- Repeatedly "grey zone" samples not confirmed positive are considered indeterminate. In such case the repetition of test with a new sample taken 2-4 weeks is recommended.

PERFORMANCE CHARACTERISTICS

Sensitivity The clinical sensitivity was assessed examining 18 BBI and 2 BCP seroconversion panels. In the table is reported the ability of HIV PLUS kit to detect antibodies to HIV in comparison with a registered assay. In 16 seroconverter subjects a concordance in the detection of the first serum sample HIV positive have been observed between two kits; in 2 subjects HIV PLUS kit detects HIV antibodies before than licensed assay; in other 2 subjects licensed kit detects antibodies to HIV before than HIV PLUS. The discordances ranged from 2 to 7 days.

Panel ID	First reactive bleed (days)		Panel ID	First reactive bleed (days)	
	HIV PLUS	Ref. kit		HIV PLUS	Ref. kit
PRB904	92	92	PRB946	>11	>11
PRB912	0	0	PRB947	11	11
PRB914	0	0	PRB948	>23	>23
PRB917	65	65	PRB950	21	28
PRB919	9	9	PRB951	19	19
PRB922	0	0	PRB952	15	17
PRB938	9	9	PRB955	14	14
PRB940	11	11	PRB959	14	14
PRB941	21	18	BCP65907	28	24
PRB945	15	15	BCP68106	29	29

The analytical sensitivity (for antigen) was assessed examining the HIV-1 Antigen p24 Sensitivity Panel PRA801 (BBI):

Panel member ID	W.H.O. Standard mIU/ml	DuPont Standard pg/ml	REACTIVA HIV PLUS
PRA801-01	>2000	>200	Reactive
PRA801-02	1600	140	Reactive
PRA801-03	970	85	Reactive
PRA801-04	483	42	B.L.
PRA801-05	250	21	Not Reactive
PRA801-06	125	10	Not Reactive
PRA801-07	60	5	Not Reactive
PRA801-08	25	2	Not Reactive
PRA801-09	<10	<2	Not Reactive
PRA801-10	Negative	Negative	Not Reactive

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The sensitivity was also evaluated examining 405 patients with HIV-1 and 100 with HIV-2 infection as confirmed by Western Blotting and/or PCR positivity. All samples sera were examined with HIV PLUS kit (sensitivity 100%). The non-B subtype distribution of 49 out of 405 HIV1 positive samples has shown in the following table:

Subtype of tested samples	No. samples tested
A	16
C	12
D	5
F	4
G	6
H	3
J	3
Total	49

The HIV PLUS shows an high level of sensitivity; the kit was able to detects HIV infection independently from HIV genotype.

Specificity The specificity of HIV PLUS testing 5000 samples from unselected blood donors was 99.8% and 100% examining 224 hospitalized patients HIV negative with a licensed reference kit. A total of 102 potentially cross-reactive samples including HBsAg or anti-HCV positive samples, samples from multiparous females, autoimmune patients, hypergammaglobulinemic, lipemic, haemolytic and icteric, and subjects RF positive have been examined. All samples were negative when tested with HIV PLUS (specificity 100%).

Specimen	No. examined	False positive	Specificity
Blood donors sera	5000	8	99.8 %
Hospitalized patients sera	224	0	100 %
Potentially cross-reactive sera	102	0	100 %

Reproducibility Replicates of HIV antibody negative, low positive and high positive sera have been examined. The results within assays are reported in the table.

Specimen	No. replicates	Intra-assay	
		SD	CV%
Negative	24	0.006	15.7
Low +	36	0.044	10.7
High +	36	0.097	4.2

LIMITATION OF THE PROCEDURE

Highly lipemic, icteric, hemolysed samples or repeatedly defrost samples and therefore subject to contamination, should not be used as they can give false results in the assay.

PROCEDURE AUTOMATION

This procedure can be used with an automatic device under customer's responsibility and providing he validates the results with an adequate method. For more information, please contact the automatic device manufacturer.

PRECAUTIONS IN USE

The use of the laboratory reagents according to Good Laboratory Practice (GLP) is recommended

WASTE MANAGEMENT

Please, refer to local legal requirements.

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PRESENTACIÓN:

CONT. 96 TEST CODIGO: RSET067-2