WHO Prequalification of In Vitro Diagnostics Programme
PUBLIC REPORT

Product: Bioelisa HIV 1+2 Ag/Ab
Number: PQDx 0183-060-00

Abstract

Bioelisa HIV 1+2 Ag/Ab with product codes **3000-1172** and **300-1173**, manufactured by Biokit S.A., CE-mark regulatory version, was accepted for the WHO list of prequalified in vitro diagnostics and was listed on 2 March 2015. This public report was amended on October 2016.

Bioelisa HIV-1+2 Ag/Ab is a fourth generation enzyme immunoassay for the simultaneous qualitative detection of HIV p24 antigen and antibodies to HIV-1 (groups M and O) or HIV-2 in human serum or plasma specimens in clinical laboratories and as a first-line screening assay in blood centres.

All initially reactive specimens should be retested in duplicate to confirm the initial result.

The test kit contains:

	96 tests Product code 3000-1172	**480 tests Product code 3000-1173**
Coated Wells 2 x 8 wells coated with antigens representing epitopes of HIV-1 gp41 and HIV-2 gp36 and monoclonal antibodies against p24. Plates are sealed into aluminum pouch with desiccant. microwells coated with HIV antigens and monoclonal antibodies.	1 plate	5 plates
Negative control Diluted human serum negative for antibodies to HIV-1/2 and HIV p24 antigen. Contains 0.1% ProClinTM 300 as preservative. Ready to use.	1 bottle of 2 ml	1 bottle of 4 ml
HIV-1 positive control Diluted human serum containing antibodies to HIV-1 (Heat inactivated). Contains 0.1% ProClinTM 300 as preservative. Ready to use.	1 bottle of 2 ml	1 bottle of 4 ml
HIV-2 positive control	1 bottle of 2 ml	1 bottle of 4 ml

Diluted human serum containing antibodies to HIV-2 (Heat inactivated). Contains 0.1% ProClinTM 300 as preservative. Ready to use.		
HIV-1 p24 positive control Diluted human serum spiked with recombinant HIV-1 p24 antigen. Contains 0.1% ProClinTM 300 as preservative. Ready to use.	1 bottle of 2 ml	1 bottle of 4 ml
Sample diluent Biotinylated monoclonal antibodies against HIV p24 diluted in Tris-buffer. Contains 0.05% ProClinTM 300 as preservative and red dye. Ready to use.	1 bottle of 14 ml	1 bottle of 70 ml
Conjugate 1 Phosphate buffer containing HIV-1 and HIV-2 biotinylated antigens. Contains 0.05% ProClinTM 300 as preservative and blue dye. Ready to use.	1 bottle of 25 ml	1 bottle of 120 ml
Concentrated conjugate 2 (101x) Contains peroxidase labelled streptavidin. To be diluted 1:101 in conjugate diluent 2 before use.	1 bottle of 0.25 ml	1 bottle of 1.2 ml
Conjugate 2 Phosphate buffer containing protein stabilizers, 0.05% ProClinTM 300 as preservative and yellow dye.	1 bottle of 25 ml	1 bottle of 120ml
Substrate-TMB Contains 3,3', 5,5'-Tetramethylbenzidine (TMB) and hydrogen peroxide. Ready to use.	1 bottle of 20 ml	1 bottle of 100 ml
Stopping solution 0.8N sulphuric acid (H_2SO_4). Ready to use.	1 bottle of 12 ml	1 bottle of 60 ml
Concentrate washing solution (25x) Concentrate PBS buffer pH 7.4 (25x). Contains Tween-20 as detergent, <0.2% ProClinTM 300 as preservative To be diluted 1:25 in distilled or deionised water before use.	2 bottles of 50 ml	3 bottles of 100 ml
Resealable plastic bag For storage of unused strips.	1	1
Adhesive plate sealers To cover the microplate during incubations.	Not specified	Not specified

Storage: the test kit should be stored at 2 to 8 °C.

Shelf-life: 9 months.

Summary of prequalification status for Bioelisa HIV 1+2 Ag/Ab

	Initial acceptance	
	Date	**Outcome**
PQ status amended	14 October 2016	listed
Status on PQ list	2 March 2015	listed
Dossier assessment	21 August 2014	MR
Inspection status	18 April 2014	MR
Laboratory evaluation	16 December 2014	MR

MR: Meets Requirements
NA: Not Applicable

Bioelisa HIV 1+2 Ag/Ab was accepted for the WHO list of prequalified in vitro diagnostics on the basis of data submitted and publicly available information.

Background information

Biokit S.A. submitted an application for prequalification of Bioelisa HIV 1+2 Ag/Ab. Based on the established prioritization criteria, Bioelisa HIV 1+2 Ag/Ab was given priority for prequalification.

Product dossier assessment

Biokit S.A. submitted a product dossier for Bioelisa HIV 1+2 Ag/Ab as per the Instructions for compilation of a product dossier (PQDx_018 v1). The information submitted in the product dossier was reviewed by WHO staff and external experts (assessors) appointed by WHO in accordance with the internal report on the screening and assessment of a product dossier (PQDx_009 v2). Based on the product dossier screening and assessment findings, a recommendation was made to accept the product dossier for Bioelisa HIV 1+2 Ag/Ab for prequalification.

Manufacturing site inspection

A comprehensive inspection was performed at the site of manufacture (Barcelona, Spain) of Bioelisa HIV 1+2 Ag/Ab in March 2014 as per the Information for manufacturers on prequalification inspection procedures for the sites of manufacture of diagnostics (PQDx_014 v1). The inspection found that the manufacturer had a well implemented quality management system and good manufacturing practices in place that ensured the consistent manufacture of a product of good quality. The manufacturer's responses to the nonconformities found at the time of the inspection were accepted on 18 April 2014.

Laboratory evaluation

Bioelisa HIV 1+2 Ag/Ab was evaluated by WHO in the fourth quarter of 2014. From this evaluation, we drew the following conclusions:

Bioelisa HIV-1+2 Ag/Ab (Biokit S.A.) is an enzyme immunoassay for the detection of HIV-1/2 antibodies and HIV-1 p24 antigen in human serum and plasma specimens. A volume of 100 µl of specimen is needed to perform the assay. This type of assay requires laboratory equipment and cannot be performed in laboratories with limited facilities. Reading of the results must be performed with a spectrophotometer.

In this limited performance evaluation on a panel of 1119 specimens, we found an initial sensitivity (95% CI) of 100% (99.2 - 100%) and an initial specificity (95% CI) of 99.2% (98.2 - 99.8%) compared to the reference results. The final sensitivity (95% CI) was 100% (99.2 - 100%) and the final specificity (95% CI) was 99.4% (98.4 - 99.8%) compared to the reference results. Lot to lot variation observed was within the acceptance range.

For eight seroconversion panels, bioelisa HIV-1+2 Ag/Ab detected on average 1.25 specimens earlier than the benchmark assay (Enzygnost Anti-HIV 1/2 Plus [Siemens Healthcare Diagnostics]) and on average 0.625 specimens earlier than Vironostika HIV Ag/Ab (bioMérieux) EIA.

For the mixed titer panel, bioelisa HIV-1+2 Ag/Ab detected all specimens. For the HIV-1 p24 antigen panel, bioelisa HIV-1+2 Ag/Ab correctly classified all specimens. For the HIV culture supernatant panel, bioelisa HIV-1+2 Ag/Ab detected all HIV-1 and HIV-2 specimens.

For the 1st International Reference Panel for anti-HIV [NIBSC code 02/210], bioelisa HIV-1+2 Ag/Ab detected all subtypes tested (HIV-1 A, HIV-1 B, HIV-C, HIV-1 CRF01_AE, HIV-1 O and HIV-2). For the HIV-1 p24 antigen standard [NIBSC code 90/636], bioelisa HIV-1+2 Ag/Ab detected to 0.39 international units. In contrast, Vironostika HIV Ag/Ab (bioMérieux) detected 12.5 international units.

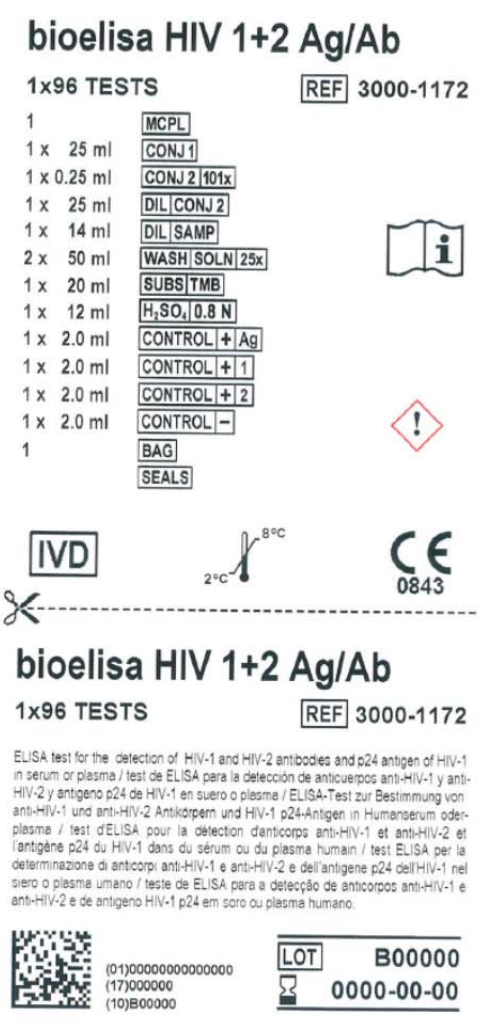
In this study, 0.09% of the results were recorded as indeterminate (equivocal). The invalid rate was 0 %.

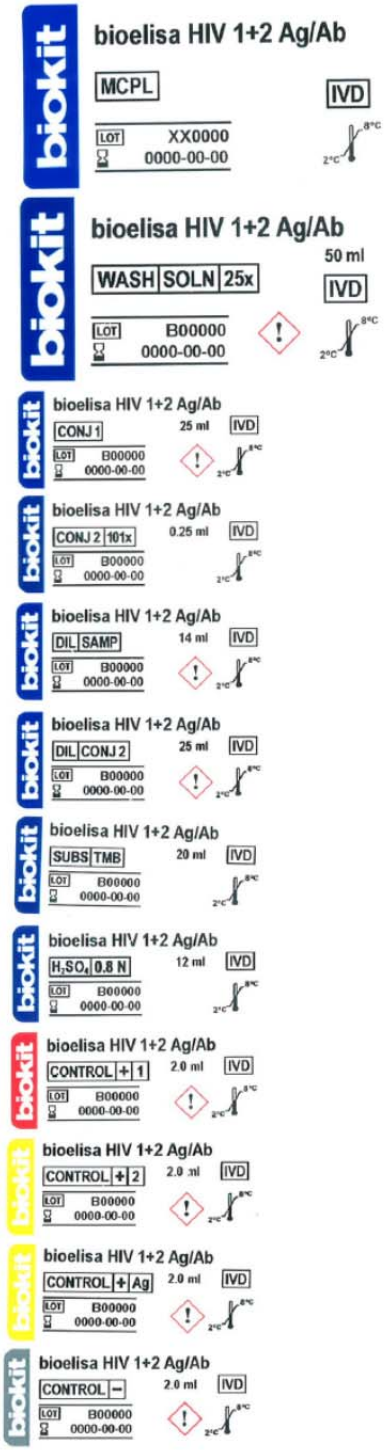
Labelling

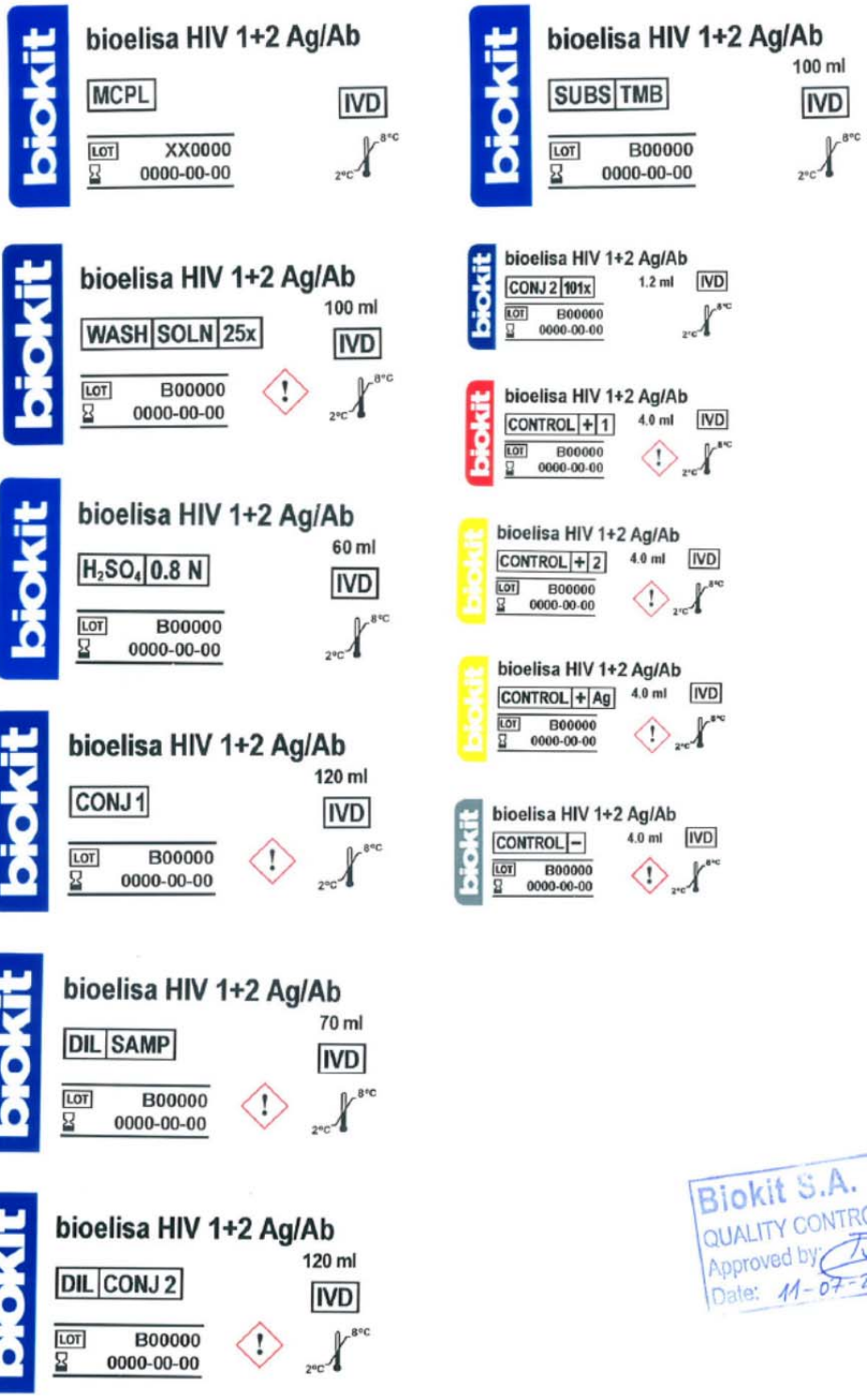
1. **Labels**
2. **Instructions for use**

1. Labels

For product code 3000-1172 (96 tests/kit)






biokit
bioelisa HIV 1+2 Ag/Ab
MCPL
IVD
LOT XX0000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
SUBS TMB
100 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
WASH SOLN 25x
100 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONJ 2 101x
1.2 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONTROL + 1
4.0 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
H2SO4 0.8 N
60 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONTROL + 2
4.0 ml
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LOT B00000
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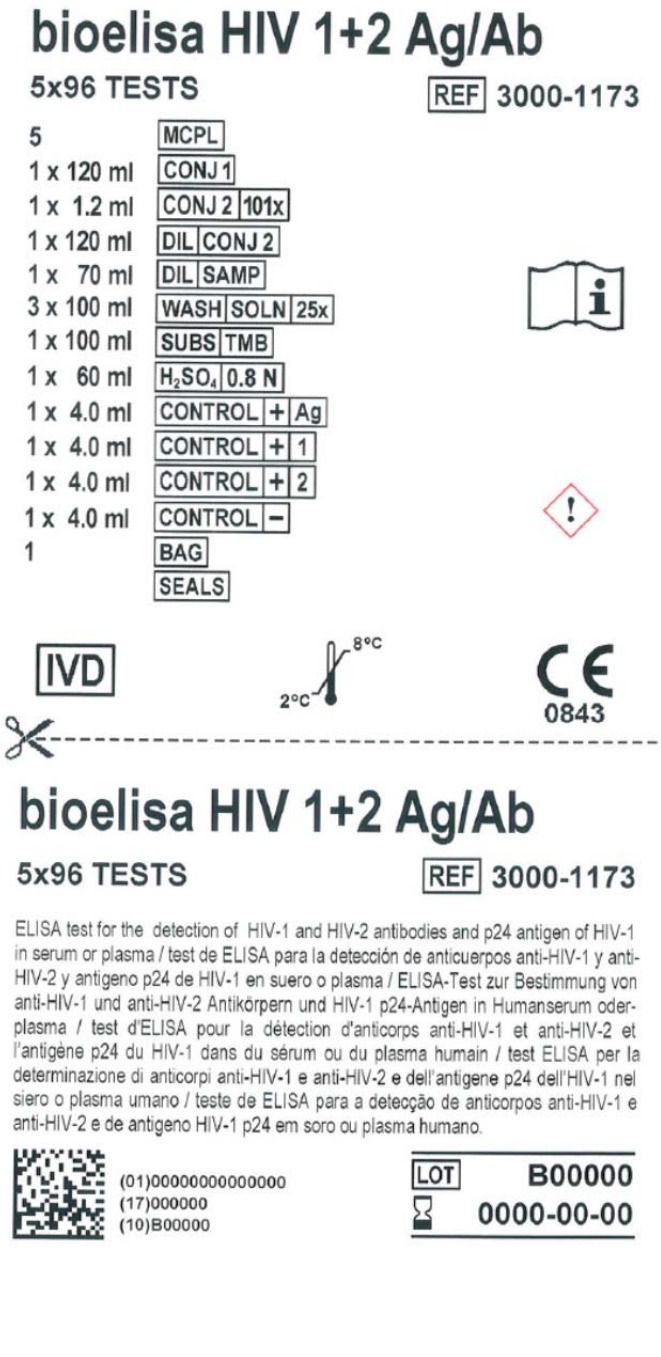
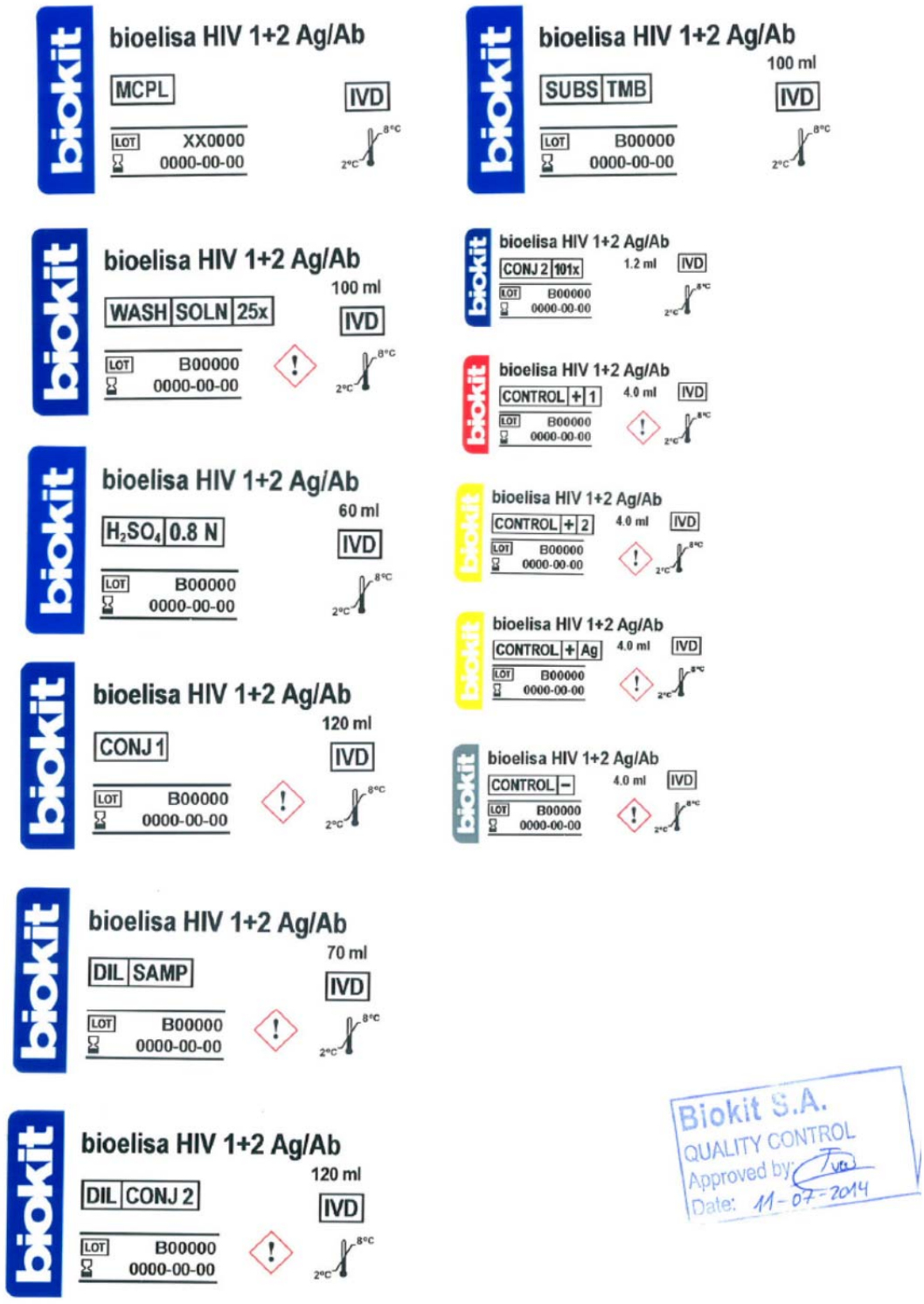
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONJ 1
120 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONTROL + Ag
4.0 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONTROL –
4.0 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
DIL SAMP
70 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
DIL CONJ 2
120 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
Biokit S.A.
QUALITY CONTROL
Approved by:
Date: 11-07-2014

For product code 3000-1175 (5 x 96 tests/kit)




biokit
bioelisa HIV 1+2 Ag/Ab
MCPL
IVD
LOT XX0000
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2°C 8°C
bioelisa HIV 1+2 Ag/Ab
SUBS TMB
100 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
WASH SOLN 25x
100 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONJ 2 101x
1.2 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONTROL + 1
4.0 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
H2SO4 0.8 N
60 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONTROL + 2
4.0 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONJ 1
120 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONTROL + Ag
4.0 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
CONTROL −
4.0 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
DIL SAMP
70 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
bioelisa HIV 1+2 Ag/Ab
DIL CONJ 2
120 ml
IVD
LOT B00000
0000-00-00
2°C 8°C
Biokit S.A.
QUALITY CONTROL
Approved by:
Date: 11-07-2014

2. Instructions for use

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READ HIGHLIGHTED CHANGES

bioelisa HIV-1+2 Ag/Ab

3000-1172 1 x 96 TESTS
3000-1173 5 x 96 TESTS (480)

ELISA test for the detection of antibodies to Human Immunodeficiency Viruses (HIV) type 1 (group M, O) or type 2 and HIV-p24 antigen in human serum or plasma samples in clinical laboratories and as a first-line screening assay in blood centers.

Summary

Serological evidence of infection with HIV may be obtained by testing for presence of HIV antigens or antibodies in serum or plasma of individuals suspected for HIV infection. Antigen can generally be detected during both acute phase and the symptomatic phase of AIDS. The antibodies to HIV-1 and/or HIV-2 can be detected throughout virtually the whole infection period, starting at or shortly after the acute phase and lasting till the end stage of AIDS. HIV fourth generation ELISA allows the simultaneous determination of HIV p24 antigens and HIV-1/HIV-2 antibodies reducing therefore the diagnostic window and allowing an early diagnosis of acute HIV infection. Apart from sexual transmission, the principal route of infection with HIV is blood transfusion. HIV can be present in both cellular and cell-free fractions of human blood. Therefore, all donations of blood or plasma should be tested due to the risk of HIV transmission through contaminated blood. This can be effectively achieved by testing for antibodies to HIV and p24 antigen by using a highly sensitive ELISA test.

Principle

bioelisa HIV-1+2 Ag/Ab is a fourth generation enzyme immunoassay for the simultaneous qualitative detection of HIV p24 antigen and antibodies to HIV-1 (groups M and O) or HIV-2. Antigens representing epitopes of HIV-1 gp41 and HIV-2 gp36 are coated onto microplate wells together with monoclonal antibodies against HIV p24. In the first step of the immunoassay, serum or plasma samples are added to these wells along with the sample diluent containing biotinylated anti-p24 antibodies. If specific HIV-1 and/or HIV-2 antibodies are present in the sample, they will form stable complexes with the HIV antigens attached to the wells. If p24 antigen is present in the sample, it will bind simultaneously to the antibodies in the well and to the biotinylated antibodies present in the sample diluent. The microwells are then washed to remove unbound serum proteins. A set of biotinylated antigens are added to the wells, these antigens will bind to the specific captured antibodies. After a second wash to remove unbound conjugate, a second conjugate of Streptavidin-HRP is added to the wells. In wells containing the "sandwich" immunocomplex, the colourless Chromogen is oxidised by the bound conjugate to a blue coloured product. The blue colour changes to yellow after stopping the reaction with sulphuric acid. The intensity of colour can be measured and is proportional to the concentration of anti-HIV 1/2 antibodies or HIV p24 antigen present in the sample. Wells containing negative samples remain colourless.

Components

1. MCPL MICROPLATE:
   12 x 8 wells coated with antigens representing epitopes of HIV-1 gp41 and HIV-2 gp36 and monoclonal antibodies against p24. Plates are sealed into aluminum pouch with desiccant.

2. CONTROL + 1 HIV-1 POSITIVE CONTROL:
   Diluted human serum containing antibodies to HIV-1 (Heat inactivated). Contains 0.1% ProClin™ 300 as preservative. Ready to use.

3. CONTROL + 2 HIV-2 POSITIVE CONTROL:
   Diluted human serum containing antibodies to HIV-2 (Heat inactivated). Contains 0.1% ProClin™ 300 as preservative. Ready to use.

4. CONTROL + Ag HIV POSITIVE CONTROL p24:
   Diluted human serum spiked with recombinant HIV-1 p24 antigen. Contains 0.1% ProClin™ 300 as preservative. Ready to use

5. CONTROL – NEGATIVE CONTROL:
   Diluted human serum negative for antibodies to HIV-1/2 and HIV p24 antigen. Contains 0.1% ProClin™ 300 as preservative. Ready to use.

6. DIL SAMP SAMPLE DILUENT:
   Biotinylated monoclonal antibodies against HIV p24 diluted in Tris-buffer. Contains 0.05% ProClin™ 300 as preservative and red dye. Ready to use.

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7. CONJ 1 CONJUGATE 1 READY TO USE:
Phosphate buffer containing HIV-1 and HIV-2 biotinylated antigens. Contains 0.05% ProClin™ 300 as preservative and blue dye. Ready to use.

8. DIL CONJ 2 CONJUGATE DILUENT 2:
Phosphate buffer containing protein stabilizers, 0.05% ProClin™ 300 as preservative and yellow dye.

9. CONJ 2 101X CONCENTRATE CONJUGATE 2 (101X):
Contains peroxidase labelled streptavidin. To be diluted 1:101 in conjugate diluent 2 before use.

10. SUBS TMB SUBSTRATE-TMB:
Contains 3,3', 5,5'-Tetramethylbenzidine (TMB) and hydrogen peroxide. Ready to use.

11. WASH SOLN 25x CONCENTRATE WASHING SOLUTION:
Concentrate PBS buffer pH 7.4 (25x). Contains Tween-20 as detergent, <0.2% ProClin™ 300 as preservative
To be diluted 1:25 in distilled or deionised water before use.

12. H_2SO_4 0.8N STOPPING SOLUTION:
0.8N sulphuric acid. Ready to use.

13. SEALS ADHESIVE SEALS:
To cover the microplate during incubations.

14. BAG RESEALABLE BAG:
For storage of unused strips.

Precautions
bioelisa HIV-1+2 Ag/Ab is intended for IN VITRO diagnostic use.
For professional use only.

WARNING: The Negative Control, HIV-1 Positive Control, HIV-2 Positive Control, HIV p24 Positive Control, Sample Diluent, Conjugate 1, Conjugate Diluent 2 and Concentrate Washing Solution contain <0.2% ProClin™ 300 as preservative.

Hazard statements
H317: May cause an allergic skin reaction.
Precautionary statements
P280: Wear protective gloves/protective clothing/eye protection/face protection.
P302+P352: IF ON SKIN: Wash with plenty of soap and water.
P333+P313: If skin irritation or rash occurs: Get medical advice/attention.
P363: Wash contaminated clothing before reuse.
P501: Dispose of contents/container in accordance with local/regional/national/international regulations.

WARNING: POTENTIALLY BIOHAZARDOUS MATERIAL.
All human source material used in the preparation of this product was found to be negative for the presence of HCV antibodies, *Treponema pallidum* antibodies, hepatitis B surface antigen and HIV 1/2 antibodies (except for the Positive Controls 1 and 2), using a commercial licensed method. Nevertheless, because no test method can offer complete assurance of the absence of infectious agents, this product should be handled with caution:

- Avoid contact of reagents with the eyes and skin. If that occurs, wash thoroughly with water.
- Wear gloves.
- Do not pipette by mouth.
- Do not smoke.

- Dispose all used materials in a suitable biohazardous waste container. Remains of samples, controls, aspirated reagents and pipette tips should be collected in a container for this purpose and autoclaved 1-hour at 121°C or treated with 10% sodium hypochlorite (final concentration) for 30 min before disposal. (Remains containing acid must be neutralised prior to the addition of sodium hypochlorite).

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Handling instructions:

- Adjust washer to the plate used (flat bottom) in order to wash properly.
- Do not mix reagents from different lots.
- Do not use reagents after the expiration date.
- Do not use the reagent if you observed any change in appearance of components included in the kit.
- Extreme care should be taken to avoid microbial contamination and cross contamination of reagents.
- Use a new pipette tip for each specimen and each reagent.
- Do not exchange reagents from different lots or use reagents from other commercially available kits. The components of the kit are precisely matched for optimal performance of the tests.
- CAUTION - CRITICAL STEP: Allow the reagents and specimens to reach room temperature (20-25°C) before use. Shake reagent gently before use. Return at 2-8°C immediately after use.
- The enzymatic activity of conjugate 2 might be affected by dust and reactive chemical substances like sodium hypochlorite, acids, alkalis etc. Do not perform the assay in the presence of these substances.
- It is very important to keep the Substrate-TMB solution in a well-sealed container and avoid light exposure.
- Soaps and/or oxidising agents remaining in containers used for the Substrate-TMB solution can interfere with the reaction. If glass containers or re-used plastic containers are used, they should be washed with 1N sulphuric or hydrochloric acid, rinsed well with distilled water and dried before use. We recommend using disposable plastic containers.

Storage and stability

The components will remain stable through the expiration date shown on the label if stored at 2-8°C. The bag containing the microplate should be brought to room temperature before opening to avoid condensation in the wells. Once opened the bag, the microplate wells are stable for 3 months at 2-8°C in the plastic bag tightly sealed, along with the silicagel. Discard any microplate sealed without the silicagel. Once diluted, the washing solution is stable for 6 days only if stored at 2-8°C. Do not use diluted washing solutions if stored overnight at room temperature. Once diluted, the conjugate 2 is stable for 30 days at 2-8°C. Store the Substrate-TMB solution in the dark.

Available packaging

- 1 microplate kit (1x96 tests), REF 3000-1172.
  Contains: 1 microplate, 1 x 2 mL negative control, 1 x 2 mL HIV-1 positive control, 1 x 2 mL HIV-2 positive control, 1 x 2 mL HIV-1 p24 positive control, 1 x 14 mL sample diluent, 1 x 25 mL conjugate 1 ready to use, 1 x 0.250 mL concentrate conjugate 2 (101x), 1 x 25 mL conjugate 2 diluent, 1 x 20 mL substrate-TMB, 1 x 12 mL stopping solution, 2 x 50 mL concentrate washing solution, 1 resealable bag and adhesive seals.

- 5 microplates kit (5 x 96 tests), REF 3000-1173.
  Contains: 5 microplates, 1 x 4 mL negative control, 1 x 4 mL HIV-1 positive control, 1 x 4 mL HIV-2 positive control, 1 x 4 mL HIV-1 p24 positive control, 1 x 70 mL sample diluent, 1 x 120 mL conjugate 1 ready to use, 1 x 1.2 mL concentrate conjugate 2 (101x), 1 x 120 mL conjugate 2 diluent, 1 x 100 mL substrate-TMB, 1 x 60 mL stopping solution, 3 x 100 mL concentrate washing solution, 1 resealable bag and adhesive seals.

Material required not provided

- Distilled or deionised water.
- Disposable gloves and timer.
- Appropriate waste containers for potentially contaminated materials.
- Disposable V-shaped troughs.
- Dispensing system and/or pipette (single or multichannel) and disposable pipette tips.
- Absorbent tissue or clean towel.
- Dry incubator or water bath, 37 ± 1°C.
- Microplate reader with a 450 nm filter. Reference filter of 620 nm or 630 is advisable.
- Manual or automated wash system.

Sample collection

Use fresh serum with or without Serum separator tube (SST), EDTA plasma, Acid-citrate-dextrose (ACD) plasma, Lithium heparin plasma, Sodium heparin plasma, Sodium citrate plasma, Potassium oxalate/sodium fluoride plasma, CPD and CPDA plasma. Other anticoagulants should be evaluated before use. Samples can be stored at 2-8°C for 8 days. For longer periods, samples should be frozen (-20°C). Avoid repeated freezing and thawing (maximum 4 freeze-thaw cycles). Specimens showing visible particulate matter should be clarified by centrifugation. Serum or plasma samples should not be heat inactivated, since that may cause incorrect results.

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Automatic processing
Automated or semi-automated assay may be used with different instruments. It is very important to validate any automated system to demonstrate that the results obtained for samples are equivalent to the ones obtained using the manual assay. It is recommended that the users validate periodically the instrument. If there is any difficulty in the programming and setting of Biokit automatic processors, please contact your distributor.

PROCEDURE
Previous operations
Allow all the reagents to reach room temperature (20-25°C) before running the assay.
Gently mix all liquid reagents before use.
Check the washing solution concentrate for the presence of salt crystals. If crystals have formed, resolubilize by warming at 37°C until crystals dissolve. Use distilled or deionized water.
Negative and Positive controls should be treated as the samples.

Dilute the washing solution 1:25 in distilled water. If the entire plate is used, add 40 mL of concentrate washing solution (25x) to 960 mL of deionised water. If the entire plate is not used, prepare the proportional volume of solution.

Dilute the concentrated conjugate 2 1:101 with the conjugate diluent 2 according to table 1. For the 1 plate packaging, if the entire plate is to be used, add 250 µL of concentrate conjugate directly to the bottle containing 25 mL of conjugate diluent. **Mix gently**.

TABLE 1

Strips required		1	2	4	6	8	10	12
Conjugate diluent 2	mL	2.0	4.0	8.0	12.0	16.0	20.0	25.0
Concentrate conjugate 2	µL	20	40	80	120	160	200	250

Assay procedure
1. Use only the number of strips required for the test. Reserve 7 wells for blank and controls. One well as Blank (e.g. A1), three wells as negative control (e.g. B1, C1, D1) and, at least, one well of each of the positive controls: positive control HIV-1 (e.g. E1), positive control HIV-2 (e.g. F1) and positive control HIV p24 (e.g. G1).

2. Add 100µL of sample diluent to **all** the wells except the Blank.

3. Add first 100 µl of samples, and last 100 µl of negative and each of the positive controls into their respective wells.

4. Cover the plate with an adhesive seal, mix gently and incubate at 37°C ± 1°C for 60 minutes with a tolerance range of 58 – 65 minutes

5. Remove and discard the adhesive seal. Aspirate the contents of the wells and fill them completely (approximately 350 µL) with the diluted washing solution. Repeat the process of aspiration and washing 4 more times, **5 (five) cycles in total**. Ensure that each column of wells soaks for at least 15 seconds before the next aspiration cycle.

6. Transfer 200 µL of conjugate 1 into each well, except the one reserved for the substrate blank. Avoid bubbles upon addition.

7. Cover the microplate with an adhesive seal and incubate at 37°C ± 1°C for 30 minutes with a tolerance range of 28 – 35 minutes.

8. Remove and discard the adhesive seal. Aspirate and wash the wells as in the previous washing step for **3 (three)** wash cycles.

9. Transfer 200 µL of working conjugate 2 into each well, except the one reserved for the substrate blank. Avoid bubbles upon addition.

10. Cover the microplate with an adhesive seal and incubate at 37°C ± 1°C for 30 minutes with a tolerance range of 28 – 35 minutes.

11. Remove and discard the adhesive seal. Aspirate and wash the wells as in step 5, for **5 (five)** wash cycles.

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12. Add 150 µL of Substrate-TMB solution to each well, including the blank.

13. Incubate at room temperature (20-25°C) for 30 minutes with a tolerance range of 28 – 35 minutes, protected from direct light exposure

14. Stop the reaction by adding 100 µL of stopping solution in the same sequence and time intervals as for the Substrate-TMB.

15. Blank the reader at 450 nm with the blank well and read the absorbance of each well within 30 minutes. It is recommended to read in bichromatic mode using a 620 - 630 nm reference filter.

Quality control
Results of an assay are valid if the following criteria are accomplished:

1. The Absorbance value of the blank well must be ≤ 0.100.
2. The Absorbance value of the positive controls must be ≥ 0.900 after subtracting the blank. If not the run should be repeated.
3. Each individual absorbance value of the negative control must be ≤ 0.120 after subtracting the blank. If one of the negative control values does not meet the Quality Control criteria, it should be discarded and the mean value calculated again using the remaining two values. If two or more negative control Absorbance values do not meet the Quality Control Range specifications, the test is invalid and must be repeated.

Results
1. Subtract the blank from each of the valid negative controls. Calculate the mean absorbance of the negative control (NCx). Calculate the cut-off value by adding 0.170 to the mean absorbance of the negative control.

   Cut-off = NCx + 0.170

   Example: NCx = 0.011 cut-off value = 0.011 + 0.170 = 0.181

2. Divide the absorbance of the sample by the cut-off value.

   Positive: ratio absorbance/cut-off ≥ 1.0
   Negative: ratio absorbance/cut-off < 0.9
   Equivocal: ratio absorbance/cut-off ≥ 0.9 < 1.0

Interpretation of results
Negative Results: Specimens with an absorbance to cut-off ratio lower than 0.9 are considered non reactive which indicates that no anti-HIV 1 and/or HIV-2 antibodies or HIV p24 antigen have been detected with **bioelisa HIV-1+2 Ag/Ab** kit.

Positive Results: Specimens giving an absorbance equal to or greater than the cut-off value are considered initially reactive, which indicates that anti-HIV 1/2 antibodies or HIV p24 antigen have probably been detected using **bioelisa HIV-1+2 Ag/Ab.** All initially reactive specimens should be retested in duplicate to confirm the initial result.

Equivocal: Specimens with an absorbance to cut-off ratio between 0.9 and 1.0 are considered equivocal and retesting of these specimens in duplicate is required to confirm the initial results.

- If, after retesting the initially reactive samples, both wells report negative results (S/CO < 0.9), these samples should be considered as non-repeatable positive (or, false positive) and recorded as negative.

- If, after retesting in duplicate, one or both wells report positive results, the final result from this ELISA test should be recorded as repeatedly reactive. Repeatedly reactive specimens can be considered positive for antibodies to HIV 1/2 or p24 antigen, therefore the patient is probably infected with HIV1 or HIV2.

- After retesting in duplicate, samples with values close to the cut-off value should be interpreted with caution and considered as equivocal, or uninterpretable for the time of testing.

- Follow-up, confirmation and supplementary testing of any positive specimen with other analytical system (e.g. WB, PCR) is required. Clinical diagnosis should not be established based on a single test result. It should integrate clinical and other laboratory data and findings.

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Limitations of the procedure

- Optimal assay performance requires strict adherence to the assay procedure described. Deviation from the procedure may lead to aberrant results.
- As in all sensitive immunoassays, there is the possibility that non-repeatable positive results occur.
- Positive results must be confirmed with another available method and interpreted in conjunction with the patient clinical information.
- Negative results obtained with **bioelisa HIV-1+2 Ag/Ab** are considered negative for antibodies to HIV-1/2 and p24 antigen and further testing is not required.
- The prevalence of the marker will affect the assay's predictive values.
- This assay cannot be used to test pooled (mixed) plasma. **bioelisa HIV-1+2 Ag/Ab** has been evaluated only with individual serum or plasma specimens.
- **bioelisa HIV-1+2 Ag/Ab** is a qualitative assay and the results cannot be used to measure concentrations of antibodies to HIV or p24 antigen. This assay cannot distinguish between infections with HIV-1 and HIV-2.
- A negative result does not exclude the possibility of exposure or infection with HIV.

Expected results

The number of positive results depends on the disease incidence in the geographic area and the type of tested population. In the world, the HIV incidence in people older than 15 years varies from 0.1% in Australia, New Zeeland and Asia Pacific, 0.3% in Western Europe, North Africa and Middle East, 0.5% in East Europe, Central Asia and Latin America, 0.6% in North America and South Asia and 9% in sub-Saharan Africa. In the same country, incidence also varies enormously according to the tested population. In Western Europe, HIV antibodies prevalence in blood bank donations varies from 0 to 5 cases over 100,000 donations, while the incidence among prisoners, sex workers and drug abusers may easily reach 20% in these risk populations.

Performance characteristics

Analytical Sensitivity

The limit of detection has been estimated below 0.5 IU/ml by testing the WHO HIV p24 Antigen, First International Reference NIBSC code 90/636 with two different lots during the internal evaluation. Additionally, the limit of detection has been also estimated below 12.5 pg/ml by testing BIORAD HIV-1 Antigen Standard 72217 with two different lots during the internal evaluation.

Sensitivity

- A total of 537 HIV-1 positive samples were evaluated with **bioelisa HIV 1+2 Ag/Ab**, among them: 28 HIV-1 *fresh-same-day* collected samples, 85 HIV-1 and 4 HIV-1 Subtype O samples in an European reference lab; 380 HIV -1 samples were evaluated at Biokit and 40 HIV-1 non B subtype samples (and at least 3 samples of each HIV-1 subtype) were evaluated in another European reference lab. **bioelisa HIV 1+2 Ag/Ab** detected all 537 samples. The sensitivity found was100% (537/537).

- A total of 115 HIV-2 positive samples were evaluated with **bioelisa HIV 1+2 Ag/Ab**, among them: 27 HIV-2 samples in an European reference lab and 88 HIV-2 samples were evaluated at Biokit. **bioelisa HIV 1+2 Ag/Ab** detected all 115 samples. The sensitivity found was 100% (115/115).

- A total of 61 HIV-1 p24 Ag positive samples were evaluated also with **bioelisa HIV 1+2 Ag/Ab**, among them: 15 samples in an European reference lab, 16 samples were evaluated in another European reference lab and 30 samples at Biokit. **bioelisa HIV 1+2 Ag/Ab** detected all 61 samples. The sensitivity found was 100% (61/61).

- Additional sensitivity studies were carried out by testing a total of 31 seroconversion panels at Biokit and a European reference lab. Results obtained were comparable to the most sensitive commercial method for HIV 1+2 antigen and antibody detection.

- 16 HIV Seroconversion Panels from BBI/Seracare and Zeptometrix were evaluated at Biokit. **bioelisa HIV-1+2 Ag/Ab** identified correctly all the positive specimens.

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Seroconversion Panel	**bioelisa HIV Ag/Ab**	HIV Ag/Ab Assay	HIV Ab Assay	HIV p24 antigen	NAT
	First sample detected positive in the panel				
BB1-PRB965-n=6	2	2	4	2	1
ZEPT-12007-n=9	4	4	5	ND	4
BBI-PRB954-n=7	6	6	7	6	4
BBI-PRB932-n=9	4	ND	4	4	4
ZEPT-9017-n=11	1	4	3	1	1
ZEPT-9014-n=7	1	1	3	1	1
ZEPT-9012-n=8	5	6	7	5	1
ZEPT-9032-n=14	8	8	7	8	1
ZEPT-6243-n=10	7	7	8	7	1
ZEPT-9022-n=9	7	8	9	7	1
ZEPT-9018-n=11	9	9	10	9	1
BBI-PRB939-n=9	6	ND	8	6	5
BBI-PRB956-n=5	4	4	5	4	2
BBI-PRB945-n=6	3	4	4	3	1
BBI-PRB955-n=4	2	2	4	2	1
BBI-PRB958-n=6	3	3	5	3	1

- In the previous table it can be observed the first sample detected as positive in each panel evaluated with **bioelisa HIV-1+2 Ag/Ab** in comparison with 4th Generation assays (Ag/Ab Assays), 3rd Generation assays (Ab Assays), assays for HIV p24 antigen and HIV RNA NAT assays. **bioelisa HIV-1+2 Ag/Ab** showed excellent sensitivity to detect early antigen or antibodies in 16 HIV Seroconversion Panels evaluated.

- Another study was conducted to assess the sensitivity in 32 *early seroconversion samples*. These type of samples are characterized to be positive to the presence of HIVp24 Ag and/or positive by NAT (Nucleic Acid Testing), not detected by all HIV 3rd generation methods and to be negative or Indeterminate by Western-Blot methods. **bioelisa HIV 1+2 Ag/Ab** detected all 32 samples. The sensitivity found was 100% (32/32).

- Finally, 50 cell culture supernatans from HIV cell cultures, including different HIV-1 subtypes and HIV-2 were evaluated in a European Reference Lab. **bioelisa HIV 1+2 Ag/Ab** detected all 50 samples. The sensitivity found was100% (50/50).

Specificity

The specificity was evaluated by testing a total of 6465 unselected blood donors' samples at three different sites.

- In the first site 3045 unselected samples including 684 first donors were tested. From this total, 4 samples were reactive. The specificity obtained in this study was 99.87% (3041/3045).

- In the second site 500 unselected samples from Blood Bank were tested. All 500 samples were negative. The specificity was 100% (500/500)

- An internal evaluation of 2920 unselected samples from Blood Bank was performed. From this evaluation, 6 samples were reactive. The specificity obtained in this study was 99.79%. (2914/2920)

A detailed summary of these specificity studies is shown in the table below.

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	Evaluation 1 (3045 sera)			
	Initial Reactive	Repeated Reactive	False Positive	Specificity %
Serum	6	4	4	99.87 (95% C.I.: 99.66 - 99.96)
	Evaluation 2 (500 sera)			
	Initial Reactive	Repeated Reactive	False Positive	Specificity %
Serum	0	0	0	100 (95% C.I.: 98.89 - 100)
	Internal Evaluation (1000 sera + 1920 plasmas)			
	Initial Reactive	Repeated Reactive	False Positive	Specificity %
Plasma	8	6	6	99.69 (95% C.I.: 99.32 - 99.89)
Serum	1	0	0	100 (95% C.I.: 99.44 - 100)
Total	9	6	6	99.79 (95% C.I.: 99.55 - 99.93)

- In addition 200 samples from hospitalized patients have been tested and compared to a reference test. 194 samples were found negative by both tests and six samples were reactive. The six samples were also reactive with other ELISA tests. A specificity of 100% was obtained in this study (194/194).

Precision

Intra-assay reproducibility:

The coefficient of variation obtained for the absorbance values of a HIV-1 positive sample assayed in a minimum of 40 replicates was 3.42%, 5.79% and 4.71% in three lots studied.

The coefficient of variation obtained for the absorbance values of a HIV-2 positive sample assayed in a minimum of 40 replicates was 6.39%, 5.91% and 8.38% in three lots studied.

The coefficient of variation obtained for the absorbance values of a HIV-1 p24 positive sample assayed in a minimum of 40 replicates was 4.08%, 3.44% and 3.71% in three lots studied.

Inter-assay reproducibility:

Four positive samples of different levels were tested in 15 different assays. The coefficients of variation obtained for the ratios absorbance/Cut-off of the four samples were 4.87%, 5.37%, 7.49% and 7.54%.

Interferences

Interference by addition

No interference has been found for haemoglobin (500 mg/dl), bilirubin (20 mg/dl), human Albumin (5 g/dl) and triglycerides (500 mg/dl).

Cross-reactivity

To evaluate possible interferences, 125 potential cross-reacting specimens were analyzed. Among those samples, 4 samples (RF+), 4 samples positive for anti-nuclear antibodies (ANA), 5 samples Mononucleosis, 89 samples from other related infectious diseases and 23 samples from pregnant women. No evidence of cross reactivity was found in the samples evaluated. The 2 anti-Toxoplasma IgM samples that tested positive were also positive by other HIV assays.

Potential interfering samples = 125	
RF (Rheumatoid factor) - 4	Elevated IgG - 4
ANA (anti-Nuclear Antibodies) - 4	Elevated IgM - 5
Mononucleosis - 5	HSV 1 IgG (Herpes Simplex Virus) - 5
Pregnant women- 23 (including 7 multiparous)	HSV 2 IgG (Herpes Simplex Virus) - 4
Syphilis Ab - 6	anti-CMV IgG (Cytomegalovirus) - 5
anti-EBV (Epstein-Barr Virus) - 3	anti-CMV IgM (Cytomegalovirus) - 7
anti-Rubella (Rubella Virus) - 4	anti-HTLV (Human T-lymphotropic Virus) - 4
anti-VZV IgG (Varicela Zoster Virus) - 2	anti-HCV (Hepatitis C Virus) - 8
anti-VZV IgM (Varicela Zoster Virus) - 2	anti-*E.coli* (*Escherichia coli*) - 5
anti-Toxoplasma IgG (*T. gondii*) - 10	HBsAg (Hepatitis B Antigen) - 8
anti-Toxoplasma IgM (*T. gondii*) - 7	

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bioelisa: Troubleshooting guide

Problem	Possible causes	*Solution*
1. Controls out of validation.	1a. Incorrect temperature, timing or pipetting.	*Check procedure. Repeat assay.*
	1b. Improper preparation of reagents, error of dilution, reagents not well mixed.	*Check procedure. Repeat assay.*
	1c. Cross-contamination of controls.	*Pipette carefully. Do not interchange caps. Repeat assay.*
	1d. Incorrect reading filter.	*Check that the wavelenght of the filter used is 450 nm. If no reference 620 - 630 nm is used, absorbance increases approximately 0.050.*
	1e. Interference in the optical pathway.	*Check the reader. Clean or dry the bottom of microplate. Check for air bubbles. Repeat reading.*
	1f. Used components from different lots.	*Do not use components from different lots as they are adjusted for each batch released.*
	1g. Expired reagents.	*Check the kit expiry date. Use a non- expired kit.*
2. No colour or only a light colour developed at the end of the assay.	2a. One or more reagents not added or added in wrong sequence.	*Check procedure. Repeat assay.*
	2b. Inactive conjugate. Wrong dilution of concentrate conjugate 2. Improper conservation.	*Check for contamination. Check procedure. Repeat assay.*
	2c. Inactive microplate: Improper conservation.	*Always keep unused strips in the bag very well closed, with the desiccant inside. Repeat assay.*
	2d. Inactive Sustrate-TMB: Improper conservation. The container used affects Substrate-TMB stability, cross-contamination with the stopping solution.	*Use disposable containers or wash with acid or ethanol and rinse with deionised water before re-use. Check procedure. Repeat assay.*
	2e. Too cold reagents.	*Let reagents reach room temperature before use.*

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bioelisa: Troubleshooting guide

Problem	Possible causes	*Solution*
3. Too much colour in all microplate wells.	3a. Contaminated or oxidised Substrate-TMB solution.	*Check that substrate is colourless, discard if blue. Use acid or ethanol washed or disposable containers. Repeat assay.*
	3b. Contaminated or improperly prepared reagents.	*Check for contamination: turbid aspect. Check dilutions. Repeat assay.*
	3c. Contaminated washing solution (1x).	*Check the quality of distilled or deionised water used for dilution. Repeat assay.*
	3d. Insufficient washing or washing not consistent: filling volume and/or aspiration insufficient or not uniform. Insufficient number of washing cycles, contaminated device.	*Check the washing device. Fill wells with washing solution close to the top, aspirate completely. Increase the number of wash cycles.*
	3e. Using of a washing solution from other manufacturer.	*Use only the washing solution supplied with the kit.*
4. Poor reproducibility or high number of non-repeatable reactive samples.	4a. Washing problems.	*See 3c, 3d, 3e.*
	4b. Uncalibrated pipettes or tips not well fitted. Improper pipetting.	*Use only calibrated pipettes, with well fitted tips and pipette carefully, without bubbles and splashing. Repeat assay.*
	4c. Reagents too cold or not well mixed before using.	*Equilibrate reagents to room temperature and mix thoroughly before using.*
	4d. Air currents over the microplate during incubations.	*Keep the microplate protected from air currents.*
	4e. Too long time for addition of samples and/or reagents. Inconsistency in time intervals. Air bubbles.	*Develop consistent and uniform technique.*
	4f. Interference in the optical pathway.	*See 1e.*