

METHOD AND KIT

The present invention relates generally to the fields of virology and immunology. In particular, it relates to the field of diagnostic assays. More particularly, the present invention provides a Point-Of-Care test (POCT) that can detect the serology markers of human immunodeficiency virus in a sample.

The Human Immunodeficiency Viruses (HIVs) are pathogenic retroviruses that cause HIV infection and the acquired immunodeficiency syndrome (AIDS), which remains one of the most important global public health threats. It was estimated that 35 million people worldwide are living with HIV, with 2.1 million newly infected; and 1.5 million of AIDS-related deaths (UNAIDS, 2013). HIV infection causes gradual failure of the immune system in human, leading to an increased susceptibility of the body to life-threatening opportunistic infections and cancers.

HIV was first discovered in two separate research groups independently in 1983. Two types of HIV have been identified and characterized: HIV-1 and HIV-2. Both types are transmitted by sexual contact, through contaminated blood, body fluids and from mother to child during pregnancy or breastfeeding. HIV-1 is the major cause of HIV infection and AIDS in the world. HIV-2 is less common and less infective. Studies of the geographic distribution of HIV infections reveal that HIV-1 is the predominant type worldwide, HIV-2 is largely concentrated in West Africa.

Prevention and treatment of AIDS depends on the accurate laboratory diagnosis of HIV infections, which is essential to identify HIV infected persons who could benefit from treatment and to reduce HIV transmission. HIV infections elicit immune responses and induce HIV specific antibodies in human. The serological methods, such as Enzyme-linked Immunosorbent Assay (ELISA), Colloidal Gold Immunochromatographic Rapid Test, and Western Blot (WB) are widely used in the laboratory diagnosis of HIV-1 and HIV-2 infection.

However, to date, there has been no rapid Point-of-Care screening test for the simultaneous and differential detection of HIV infections with multiple markers.

As such, the present invention is a qualitative immunochromatographic assay for the rapid *in vitro* detection and differentiation of antibodies to HIV-1 and HIV-2 in human serum, plasma, finger pricked whole blood or whole blood with anti-coagulants. It is intended for professional use as a rapid screening test for the presence of antibodies to HIV in patients and aid in the diagnosis of HIV-1/HIV-2 infection. The positive results should be further confirmed by more specific supplemental tests such as Western Blots.

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

Any document referred to herein is hereby incorporated by reference in its entirety.

In a first aspect of the present invention, there is provided a method for detecting a human immunodeficiency virus infection in a subject, the method comprising (a) providing human immunodeficiency virus antigens; (b) contacting the human immunodeficiency virus antigens with a biological sample obtained from the subject, the biological sample suspected of containing human antibodies against the human immunodeficiency virus antigens; (c) detecting the presence of a complex that forms between the human immunodeficiency virus antigen and the human antibody, wherein the method simultaneously and differentially detects the presence of antibodies in the sample to HIV-1 and HIV-2 antigens, and the presence of the complex is indicative of a HIV-1 and/or HIV-2 infection in the subject.

Preferably, the method further comprising (a) providing a solid support having a first end and a second end; (b) immobilising the human immunodeficiency virus antigens on the solid support, the antigens are immobilised separately and spaced apart from each other along the solid support; (c) immobilising a molecule on the second end of the solid support, the molecule capable of binding to the human antibody in the complex, the molecule further comprising at least one detectable labelled moiety; (c) loading the sample on the first end of the solid support, the sample contacting the antigens as it travels along the solid support in

a first direction along the solid support and towards the second end; and (d) loading a buffer on the second end of the solid support adjacent the molecule such that the buffer solution solubilise the molecule as it travels along the solid support in a second direction opposite the first direction, wherein the presence of the complex formed on the solid support is
5 detected by the labelled moiety of the molecule.

Preferably, the method further provides for an absorbent pad that is placed adjacent to or abuts the first end of the solid support, the absorbent pad and the solid support are separated by a separator, wherein removing the separator allows contact between the
10 absorbent pad and the solid support to enhance the flow of the buffer along the solid support in the second direction, for example to increase the flow rate of the buffer.

Preferably, the human immunodeficiency virus antigen may be a structural protein of human immunodeficiency virus particles and fragments thereof, or any glycoproteins, lipids
15 and carbohydrates derived from the human immunodeficiency virus. More preferably, the method comprises using all the human immunodeficiency virus antigens gp120, gp41, gp36 and p24.

Preferably, the human antibody is IgG.
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Any such binding method may be used that is known to a person skilled in immunoassay techniques.

Preferably, the molecule is a human immunodeficiency virus antigen.
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Alternatively, the molecule is an anti-human antibody. Preferably, the anti-human antibody is a goat anti-human IgG.

Preferably, the detectable labelled moiety is a gold conjugate.
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Preferably, the biological sample is selected from the group comprising of whole blood, plasma, and serum.

In a second aspect of the present invention, there is provided a kit for detecting a human immunodeficiency virus infection in a human subject, the kit comprising a solid support with human immunodeficiency virus antigens, wherein the human immunodeficiency virus antigens are gp120, gp41, gp36 and p24 and the antigens are immobilised on the solid support separately and spaced apart from each other.

Preferably, the solid support further comprising a molecule immobilised on it, the molecule capable of binding to a complex formed with the surface-bound human immunodeficiency virus antigens, the molecule further comprising at least one detectably labelled moiety. In an embodiment, a sample obtained from the subject is loaded on a first end of the solid support, and a buffer for solubilising the molecule is loaded on a second end of the solid support opposite the first end.

Preferably, the kit further comprises an absorbent pad adjacent the first end of the solid support, the absorbent pad and the solid support are separated by a separator, wherein removing the separator allows contact between the absorbent pad and the solid support to enhance the flow of the buffer along the solid support.

Preferably, the molecule is an anti-human antibody. The anti-human antibody may be a goat anti-human IgG.

In alternative embodiments, the molecule may also be a human immunodeficiency virus antigen.

Preferably, the detectable labelled moiety is a gold conjugate.

Preferably, the solid support includes a nitrocellulose membrane.

Advantageously, the present method and kit allows for a quick and easy detection of a human immunodeficiency virus infection in a sample taken from a patient. In particular, the

method and kit can provide results that can differentiate whether a patient is HIV-1 and/or HIV-2 positive.

The present method and kit allows for increased sensitivity compared to conventional HIV screening tests and have proven early seroconversion detection.

In order that the present invention may be fully understood and readily put into practical effect, there shall now be described by way of non-limitative examples only preferred embodiments of the present invention, the description being with reference to the accompanying illustrative figures.

In the Figures:

Figure 1 is a schematic drawing of a kit according to an embodiment of the present invention;

Figure 2 is a schematic drawing of using a kit according to embodiment of the present invention;

Figure 3 is a schematic drawing showing possible results on a kit according to an embodiment of the present invention;

Figure 4 is a schematic drawing showing a cross section of the kit according to an embodiment of the present invention; and

Figures 5, 6 and 7 are charts showing the profiles of visible test lines.

Example

1. The kit

Referring to Figure 1, the present invention provides for a kit 5 for detecting a human immunodeficiency virus infection. In particular, the present kit is a Point-of-Care screening test kit that is capable for the simultaneous and differential detection of HIV infections (HIV 1 and/or HIV-2) with multiple markers. Such kits are specific, rapid and economical. The kit 5 includes a casing having an inlet Well A 20 for receiving a biological sample obtained from a subject, another inlet Well B 25 for receiving a chaser buffer (which will be described more later), and a reaction chamber 30. The reaction chamber 30 may be the portion of the kit 5 which includes a window allowing a user to see the results obtained when the assay method is carried out on the kit 5. The inlet 20 may also include a sample chamber for receiving the sample. The inlets 20, 25 and reaction chamber 30 are all in fluid communication with each other. Inlet 20 is opposite inlet 25. The kit 5 also includes any solid support structure 10 having human immunodeficiency virus antigens immobilised on it. The solid support 10 may be made of any suitable material, for example an absorbent material, that spans the inlets 20, 25 and includes the reaction chamber 30 where the human immunodeficiency virus antigens are immobilised. The front of the casing of the kit 5 adjacent the window may include markings showing the locations of the various test lines (and hence, human immunodeficiency virus antigens that are immobilised on the solid support structure 10). As such, the antigens are immobilised on the solid support in a "striped" and linear fashion as shown in Figure 1. These antigens may be immobilised on the nitrocellulose membrane using a Dispensing system, followed by a drying step. Next the "striped" membrane is subjected to blocking step with blocking solution containing Casein, Triton, PVP and 5X stabil coat for 1.5 min, followed by a drying step. The antigens may be immobilised by any suitable methods known to the skilled person

In an embodiment, and as shown in Figure 1, the human immunodeficiency virus antigens gp120, gp41, gp36 and p24 are immobilised on the solid support structure 10. This is shown on the markings adjacent the window of the kit 5. These 4 human immunodeficiency virus antigens (or test lines) are arranged separately and spaced apart in a linear fashion such that they line up along the length of the window of the kit 5. An indicator line 40 (in an embodiment, the indicator line may be blue) is shown on the solid support structure 10 to indicate when the sample has travelled across the immobilised gp36, p24, gp41, gp120 and protein A along the length of the solid support structure 10. The method of carrying out the

assay on the kit 5 will be described below. In addition to the indicator line 40, a control line 45 may be included. Advantageously, having all four human immunodeficiency virus antigens derived from HIV-1 and HIV-2 virus on a single strip of solid support structure 10 allows for an assay to be carried out to determine the serology of the HIV detected in a sample.

In an embodiment, the kit is an indirect solid-phase immunochromatographic assay that is based on a reverse flow technology disclosed in US Patent No. 6,316,205 (contents of which are incorporated herein) and may be used for the simultaneous and differential detection of antibodies to HIV-1 and HIV-2 in human serum, plasma, finger pricked whole blood or whole blood with anti-coagulants.

As shown in Figure 1, highly purified recombinant antigens gp120 and gp41 representing HIV-1, gp36 representing HIV-2 and p24 (reactive for both HIV-1 and HIV-2) immobilised and are striped on the support structure as 4 separate test lines. The support structure may be a nitrocellulose membrane.

The antigens may be obtained commercially and purified by chromatography.

Antigen	Manufacturer	Catalogue Number
Recombinant HIV-1 gp120 antigen	BioTech Africa	BA05-P
Recombinant HIV-1 gp41 antigen	BioTech Africa	BA01B-F
Recombinant HIV-1 p24 antigen	BioTech Africa	BA04-C
Recombinant HIV-2 gp36 antigen	BioLink International Inc	S00206

The kit may further include any molecule that is capable of binding to a complex that may be formed with the human immunodeficiency virus antigen immobilised on the membrane

and a human antibody against the human immunodeficiency virus antigen. In this example, the molecule may be an anti-human antibody. In an embodiment, the anti-human antibody is goat anti-human IgG. The goat anti-human IgG further conjugated to colloidal gold for detection of the bound complex. Alternatively, the molecule may be a human immunodeficiency virus, or part of it.

Figure 4 shows the kit of the present invention in greater detail, in particular it shows a cross section of it. For ease of description, the components shown are separated from each other. In the actual kit, these components are in contact with each other.

In an embodiment, the solid support 10 is part of a strip which includes a sample pad 50, a test strip 55, and a gold conjugate pad 60. The sample pad 50 receives the sample from the inlet 20. The solid support 10, particular the test strip 55, includes the immobilised antigens 35 (test markers). The test strip 55 may be made from a material that is different to that of the sample pad 50 and gold conjugate pad 60. For example, the test strip 55 is made of a nitrocellulose membrane. This is the part of the strip that is shown in the window 30. The gold conjugate pad 60 has the molecule that is capable of binding to the antibody-antigen complex. This molecule has a gold conjugate for detection purposes. The gold conjugate pad 60 receives the buffer from the inlet 25. As such, the sample pad 50 is at a first end of the strip while the gold conjugate pad 60 is at a second end of the strip. First and second ends are opposite each other.

At the first end of the strip, i.e. the sample pad 50, is an absorbent pad 70. The absorbent pad 70 is placed beneath the sample pad 50. The absorbent pad 70 and sample pad 50 are separated by a separator 75, i.e. the "pull tab" as shown in the Figures.

In operation, a sample is received in inlet 20 and gets absorbed by the sample pad. The sample then travels along the strip in the direction A to the test strip 55 by capillary action where it comes into contact with the antigens that are immobilised on the test strip 55. If there are any HIV antibodies in the sample, anti-HIV antibodies will bind to the immobilised antigens and antibody-antigen complexes will be formed on test strip 55 at the relevant marker positions as indicated in 35. A buffer is then received in inlet 25 and re-solubilise the

molecule on the gold conjugate pad 60. When the separator 75 is removed by pulling the tab, the sample pad 50 and absorbent pad 70 comes into contact. This helps to draw the resolubilized gold conjugate and buffer from the gold conjugate pad 60 to travel the strip in a direction that is opposite that of the sample, i.e. Direction B. The binding between molecule and the antigen-antibody complex is detected by the labelled moiety.

A more detailed description on the workings of the kit will be described below.

2. Biological test sample collection

A biological test sample may be obtained from a subject (for example, a patient) that may be suspected of being infected with the human immunodeficiency virus (HIV). The biological sample taken from such patients may contain human antibodies against a human immunodeficiency virus antigen. The antibodies in the biological test sample (serum, plasma, finger pricked whole blood or whole blood with anti-coagulants) form antibody-antigen complexes with HIV specific recombinant antigens immobilized on nitrocellulose membrane in the test viewing window as the test sample migrates upward from the sample well.

Whole blood can be collected in anti-coagulant containing tubes and used as outlined in the assay procedure immediately or stored at 2°C to 8°C for not more than 72 hours before use.

The presence of 6 anti-coagulants, Acid Citrate Dextrose (ACD), Citrate-phosphate-dextrose (CPD), Ethylenediaminetetraacetic acid (EDTA), Potassium Oxalate (K-Oxalate), Lithium Heparin (Li Hep) and Sodium Citrate (Na Citrate) were found to have no effects on the performance of test kit.

Serum / plasma samples should be stored at 2°C to 8°C if the test is to be run within 7 days of collection or frozen at -20°C or colder if the test is to be delayed for more than 7 days. Clear, non-hemolyzed samples are preferred. Lipemic, icteric or contaminated (particulate or bacterial) samples should be filtered (0.45µm) or centrifuged before testing.

3. Assay procedure

The assay method for detecting a human immunodeficiency virus infection in a subject may be carried out on the kit 5. The method includes providing human immunodeficiency virus antigens; contacting the human immunodeficiency virus antigens with a biological sample
5 obtained from the subject that is suspected of containing human antibodies against the human immunodeficiency virus antigens; and detecting the presence of a complex that forms between the human immunodeficiency virus antigen and the human antibody. Advantageously, the present method simultaneously and differentially detects the presence of antibodies in the sample to HIV-1 and HIV-2 antigens. The presence of the complex is
10 indicative of a HIV-1 and/or HIV-2 infection in the subject. Preferably, the antigens target human IgG.

As such, as the kit was described earlier, the human immunodeficiency virus antigens are immobilised on a solid support structure 10. A sample (for example, serum, plasma or whole
15 blood) is loaded in Well A 20 of the kit 5. In the case where the sample is whole blood, chase buffer may be added to the Well A 20 right after the sample addition. The sample chamber, which may be part of or is in fluid communication with the Well A 20, is also in fluid communication with the reaction chamber 30 of the kit. As such, the sample will start travelling or wicking up the solid support structure 10 in a first direction A as shown in
20 Figure 2. Since the human immunodeficiency virus antigens are immobilised in a linear fashion, the sample will travel from the antigen immobilised nearest the Well A 20 to the antigen immobilised furthest the Well A 20 until it reaches the indicator line 40. This means that the sample has completely travelled the distance of the solid support structure 10 and has reacted with all the human immunodeficiency virus antigen test lines 35 and control line
25 45 in the reaction chamber 30. The buffer may contain sodium chloride, Sodium di hydrogen phosphate monohydrate, sodium azide, EDTA Na₂.2H₂O, Triton X-100 and 10% SDS.

Detecting the presence of any antibody-antigen complex formed on the solid support structure 10 is carried out by loading a chase buffer into the buffer chamber Well B 25. The anti-human
30 IgG gold conjugate in buffer chamber Well B 25 will be resolubilised by the chase buffer and travel along a buffer support 10 in a second direction (B) opposite the first direction (A). The anti-human IgG gold conjugate comprises any molecule that is capable of binding to the

human antibody in the complex. Such a molecule further comprising at least one detectable labelled moiety. The molecule may be an anti-human antibody. In alternative embodiments, the molecule may be a human immunodeficiency virus antigen or an anti-human antibody. In an embodiment, the anti-human antibody may be a goat anti-human IgG. The moiety for detection may be a gold conjugate. Other types of detection systems such as biotin/avidin may be used.

The sample pad and absorbent pad are separated by a separator (pull tab), and removing the separator allows the contact of sample pad and absorbent pad, which facilitate the flow downwards in direction B. The presence of the complex is detected by detecting the moiety. In an embodiment, the separator may be a material that is impermeable. In the examples, the separator is the tab 15 shown in Figure 1.

The bound antibody-antigen complexes are subsequently detected by goat anti-human IgG gold conjugate carried by a chase buffer that flows downward (direction B) giving a pink-purplish colour. The control line 45 may contain Protein A captures human IgG from the patient's sample, subsequently binds with the anti-human IgG gold conjugate. The appearance of control line 40 serves to validate the proper addition of human specimens and migration of sample and chase buffer, as well as the re-solubilised anti-human IgG gold conjugate. Protein A or recombinant Protein A may be obtained from *Staphylococcus aureus* Cowan I that is expressed in Bacillus. 1mg of Recombinant Protein A binds to at least 10mg of human IgG.

The gold conjugate is re-solubilised when 3 drops added to the oval well (Well B). The gold conjugate is sprayed and dry on the gold conjugate pad 60. Once chase buffers added, the buffer will dissolve the gold conjugate and the gold conjugate in liquid format will flow downwards (direction B).

After pull-tab pulled, 1 drop of chase buffer was used to make contact between sample pad and absorbent pad to facilitate the flow downwards. The drop of chase buffer connects the sample pad portion of the solid support to the absorbent pad.

The kit 5 is allowed to warm to room temperature ($25^{\circ}\text{C} \pm 3^{\circ}\text{C}$) before running the assay for optimal binding. For best results, the assay is conducted at room temperature ($25^{\circ}\text{C} \pm 3^{\circ}\text{C}$).

The assay method is diagrammatic represented in Figure 2.

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The following are examples of the assay procedures using various samples.

(a) Assay for serum/plasma

- 10 25 μl serum/plasma sample is added to square well [A]. Sample will start wicking up the nitrocellulose membrane. When the sample reaches the blue indicator line (the portion of the nitrocellulose membrane that is furthest away from well [A] in test strip 55), 3 drops of chase buffer is added into oval well [B]. Having wells of different shapes help to differentiate the wells for different purposes. For example, well A is square, while well B is oval. Square well for addition of specimens and oval well for addition of chase buffer.
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- The “HIV” Pull Tab 15 is pulled until resistance is felt. Another 1 drop of chase buffer is added into the square well [A]. The results are read at 20 minutes but before 25 minutes. “Pull Tab” is to separate two stage of flow. Before pull-tab is pulled, it functions as separator to avoid contact between absorbent pad and sample pad, and thus the specimens flow upwards (direction A) by capillary reaction; after pull-tab 75 is pulled, the absorbent pad contacts with sample pad and facilitate the flow of gold conjugate downwards (i.e. the direction where the anti-human IgG gold conjugate flows towards well A - Direction B).
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25 (b) Assay for whole blood

- 20 μl of whole blood sample is added to square well [A], followed by 1 drop of chase buffer to the same well. This is required to facilitate the flow rate of the whole blood specimens. Whole blood contains red blood cells aggregates and other elements and tend to flow slower as compared to serum and plasma.
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The sample will start wicking up the membrane. When the sample reaches the blue indicator line (the portion of the nitrocellulose membrane that is furthest away from well [A] in test window 10), 3 drops of chase buffer is added into oval well [B].

- 5 The “HIV” Pull Tab 15 until resistance is felt. Another 1 drop of chase buffer is added into the square well [A]. The results are read at 20 minutes but before 25 minutes.

4. Interpretation of results

- 10 With reference to Figure 3, a test is HIV-1 positive if it meets any of the following criteria:

- (i) Control Line (C) appears with visible gp120 test line (with or without visible gp41 and/or p24 test lines);
- (ii) Control Line (C) appears with visible gp41 test line (with or without visible gp120 and/or p24 test lines); and
- (iii) Control Line (C) appears with visible gp120 & gp41 test lines (with or without visible p24 test line).

- 20 A test is HIV-2 positive if Control Line (C) appears with visible gp36 test line (with or without visible p24 test line)

A test is HIV-1 and HIV-2 positive if it meets any of the following criteria:

- (i) Control Line (C) appears with visible gp120 & gp36 test lines (with or without visible gp41 and/or p24 test lines);
- (ii) Control Line (C) appears with visible gp41 & gp36 test lines (with or without visible gp120 and/or p24 test lines); and
- (iii) Control Line (C) appears with visible gp120 & gp41 & gp36 test lines (with or without visible p24 test line).

A test is HIV positive (untypable) if Control Line (C) appears with visible p24 test line only.

A test is negative if Control line (C) appears and Test line(s) are not visible.

A test is invalid if Control line (C) is absent. The assay should be repeated using a new device.

5 Performance of the present HIV Rapid Test

Total Diagnostic Performance

Diagnostic Parameter	Performance of the present HIV Rapid Test	95% Confidence Interval
Sensitivity (n=801)	100.00%	99.54% to 100.00%
Specificity (n=2057)	99.12%	98.62% to 99.48%
Positive Predictive Value	97.80%	96.55% to 98.69%
Negative Predictive Value	100.00%	99.82% to 100.00%

- 10 A total of 801 anti-HIV positive specimens and 2057 anti-HIV negative specimens collected from various countries were tested on the present HIV Rapid Test at MP Biomedicals Asia Pacific Pte Ltd- R&D Laboratory or ICDDR, Bangladesh (Clinical evaluation). As shown in Table above, the present HIV Rapid Test demonstrated 100 % sensitivity (95% CI : 99.54% to 100.00%) and 99.12% specificity (95% CI: 98.62% - 99.48%). The >99% accuracy based on the
- 15 tested population (n = 2858) indicating the good performance of the present HIV Rapid Test. The positive predictive value and negative predictive value for the present HIV Rapid Test were found to be 97.80% and 100% respectively.

Sensitivity

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Study	Category	Total Sample Size	Diagnostic Sensitivity of the present HIV Rapid
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			Test
In-house Study	HIV-1 positive	516	100.00% (516/516)
	HIV-2 positive	122	100.00% (122/122)
	HIV positive (untypable)	3	100.00% (3/3)
Clinical Evaluation	HIV positive	160	100.00%(160/160)
Total		801	100.00%(801/801)

A total of 801 specimens comprised of 516 anti-HIV 1 specimens, 122 anti-HIV-2 positive, 3 anti-HIV positive (untypable) and 160 anti-HIV positive specimens were tested on the present HIV RT. The anti-HIV positive specimens were collected from various countries, including Vietnam, USA, China, Africa, Thailand, India, West Africa, Bangladesh, etc. 160 anti-HIV positive specimens were tested at clinical site and the remaining 641 specimens were tested at MP Biomedicals –R&D laboratory.

The present HIV Rapid Test detected all 801 specimens as positive, demonstrating 100% sensitivity (95 % CI: 99.54% - 100%).

Specificity

Study	Category	Total Sample Size (n)	Diagnostic Specificity of the present HIV Rapid Test
In-house study	Blood Donors /Healthy Donors	1184	99.32% (1176/1184)
In-house study	Hospitalized / Clinical	193	100.00% (193/193)
Clinical evaluation	Clinical	222	98.20% (218/222)
In-house study	Cross-reactive	100	99.00% (99/100)

Study	Category	Total Sample	Diagnostic Specificity of the
In-house study	Interference	103	100.00%(103/103)
In-house study /Clinical evaluation	Pregnancy	235	97.87%(230/235)
Clinical evaluation	Anti <i>E-coli</i> Positive	20	100.00% (20/20)
Total		2057	99.12% (2039/2057)

A total of 2057 specimens comprised of seven categories (healthy donors, cross-reactive, interference substances, hospitalized, clinical, anti *E-coli* positive, pregnancy specimens) were tested on the present HIV Rapid Test at MP Biomedicals-R&D laboratory or International Centre for Diarrhoeal Disease Research (ICDDR), Bangladesh (clinical site).

1184 blood donors or healthy donors specimens were tested and the present HIV Rapid Test demonstrated overall 99.32% (1176/1184) specificity on this category of samples.

Total 193 specimens comprised of 173 hospitalized and 20 clinical patients (from Sera Care Life Sciences, HIV Antigen Mixed Titer performance panel PRA201, The WHO international standard – NISBC Code 02/210 and National Reference for conformation reagent of HIV antibody, China) negative with anti-HIV were tested on the present HIV Rapid Test. All specimens were non-reactive on the present HIV Rapid Test, resulting in 100.00% (193/193) specificity.

222 clinical anti-HIV negative specimens collected by ICDDR, Bangladesh were tested on the present HIV Rapid Test at ICDDR, Bangladesh. 218 specimens were non-reactive on the present HIV Rapid Test, resulting in 98.20% specificity for this category of specimens.

100 specimens positive with other infectious disease were tested on the present HIV Rapid Test to verify the analytical specificity of the present HIV Rapid Test. Out of 100 specimens positive with other infectious diseases, 1 Rheumatoid Factor positive specimen was found to

be positive with the present HIV Rapid Test, resulting in the 99.00% (99/100) specificity for this category of specimens. On the other hand, the present HIV Rapid Test demonstrated 100% specificity using 103 specimens with potentially interference substances, such as icteric, hemolyzed, triglyceride, lipemic, autoimmune disease, antinuclear antibody, etc.

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In addition to 145 pregnancy specimens tested in-house, 90 pregnancy specimens were also tested at ICDDR, Bangladesh, resulting in total 235 pregnancy specimens. Five specimens were found to be reactive with the present HIV Rapid Test, which results in 97.87% specificity.

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As some of the recombinant HIV antigens (gp41, p24 and gp36) used in the present HIV Rapid Test were expressed in *E. coli*, the potential cross-reactivity with anti-*E.coli* positive human specimens were examined. 20 anti-*E.coli* positive human specimens were tested on the present HIV Rapid Test at ICDDR, Bangladesh. The present HIV Rapid Test did not show cross-reactivity with all 20 anti-*E.coli* positive samples, resulting in 100% specificity (20/20).

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In overall, the present HIV Rapid Test demonstrated 99.12% total diagnostic specificity using 2057 specimens, with 95% CI as 98.62% to 99.48%.

20 Differentiation of HIV-1 and HIV-2

Serotype	Total Sample Size	Accuracy of differentiation by the present HIV Rapid Test
HIV-1	530	97.55% (517/530)
HIV-2	132	93.94% (124/132)

The present HIV Rapid Test detects and differentiates between anti HIV-1 and anti HIV-2 antibodies, by four separate test lines (gp120, gp41, p24 and gp36) on the test devices.

Highly purified recombinant antigens gp120 and gp41 representing HIV-1, gp36 representing HIV-2 and p24 reacts with both HIV-1 and HIV-2.

As shown in Table above, the accuracy of the present HIV Rapid Test in differentiating HIV-1 and HIV-2 was evaluated based on 516 anti HIV-1 positive and 122 anti HIV-2 positive specimens.

Out of 638 specimens (3 HIV Positive (untypable) specimens were not applicable for the analysis of differentiation accuracy), there were 44 specimens detected positive for HIV-1 and HIV-2 antibody on the present HIV Rapid Test. These specimens were further tested on MPD HIV Blot 2.2 and MPD HIV-2 Blot 1.2 to determine the HIV serotype. Specimen showed positive results on MPD HIV Blot 2.2 was considered as HIV-1, whilst specimen showed positive results on MPD HIV-2 Blot 1.2 was considered as HIV-2. Specimen found positive with both MPD HIV Blot 2.2 and MPD HIV-2 Blot 1.2 was considered as dual infection (HIV-1 & HIV-2 positive). 10 samples (from 516 HIV-1 category) & 14 samples (from 122 HIV-2 category) were tested positive with both MPD HIV Blot 2.2 and MPD HIV-1 Blot 1.2. Thus, these 24 specimens were classified as HIV-1 and HIV-2 co-infection for the calculation, resulting in 506 anti HIV-1 positive and 108 anti HIV-2 positive and 24 anti HIV-1& anti HIV-2 positive.

Of 506 anti HIV-1 positive specimens, 1 specimen was detected as positive (untypable), 493 specimens were detected as positive for HIV-1 antibody and 12 specimens were detected as positive for HIV-1 and HIV-2 antibody on the present HIV Rapid Test.

Of 108 anti-HIV-2 specimens, 100 specimens were correctly detected as positive for HIV-2 and 8 specimens were found to be reactive with both HIV-1 test lines and HIV-2 test lines on the present HIV Rapid Test.

Based on the results, the accuracy of the differentiation of the present HIV Rapid Test was determined. Out of 506 HIV-1 specimens, 493 specimens were considered accurately detected for HIV-1 serotype (with reactive gp41 and/or gp120 test lines). On the other hand, out of 108 HIV-2 specimens, 100 specimens showed reactive HIV-2 (gp36) test line and

considered correctly differentiated for HIV-2 serotype. The 24 HIV-1 & HIV-2 positive specimens determined by MPD HIV Blot 2.2 and MPD HIV-2 Blot 1.2 were showing concordant results with reactive HIV-1 and HIV-2 markers on the present HIV Rapid Test. Thus, the 24 samples were included in both HIV-1 and HIV-2 differentiation accuracy analysis respectively.

Hence, the overall differentiation accuracy by the present HIV Rapid Test was found to be 97.55% (517/530) for HIV-1 and 93.94% (124/132) for HIV-2.

Detection of HIV-1 and HIV-2 subtypes

Serotype^	Total Sample Size	Performance of MULTISURE HIV Rapid Test
HIV-1	89*	100% (89/89)
HIV-2	4	100% (4/4)

* Including Group M (Subtype A, B, C, D, E, F, G, H, J, K, BC, C/E, CRF01_AE, CRF02_AG, CRF02_AB, E/F, G/CRF02, H/A1, K/CRF09, CRF01/CRF15) & Group O

^ Source of panels:

1. HIV worldwide Performance Panel WWRB301, Sera Care Life Sciences
2. HIV worldwide Performance Panel WWRB304, Sera Care Life Sciences
3. HIV worldwide Performance Panel WWRB305, Sera Care Life Sciences
4. WHO International Standard-NIBSC Code 02/210, NIBSC
5. National Reference for Confirmation Reagent of HIV Antibody, China

Out of 93 serotype specific specimens, 85 anti-HIV 1 specimens comprised of various subtypes from Group M and 4 anti-HIV 1 specimen was from Group O; the remaining 4 specimen was from HIV-2. As shown in Table above, various subtypes from Group M tested on MULTISURE HIV Rapid Test included subtype A, B, C, D, E, F, G, H, J, K, BC, C/E as well as

circulating recombinant forms (CRF) derived from recombination between viruses of different subtypes (i.e.CRF01_AE, CRF02_AG,CRF02_AB, E/F, G/CRF02, H/A1, K/CRF09 & CRF01/CRF15).

- 5 The present HIV Rapid Test detected all available HIV-1 Group M subtypes, the HIV Group O subtype and HIV-2 as positive.

Interpretation on MULTISURE HIV Rapid Test	Sample size	Profile of visible test lines	Occurrence	Percentage (%)	
HIV-1 Positive	495	gp41	22	22/495	4.44%
		gp120	1	1/495	0.20%
		gp41 & p24	22	22/495	4.44%
		gp120 & gp41	167	167/495	33.74%
		gp120 & p24	3	3/495	0.61%
		gp120 & gp41 & p24	280	280/495	56.57%
HIV-2 Positive	101	gp36	72	72/101	71.29%
		gp36 & p24	29	29/101	28.71%
HIV-1 & HIV-2 Positive	44	gp41 & gp36	8	8/44	18.18%
		gp120 & gp41 & gp36	4	4/44	9.09%
		gp120 & p24 & gp36	1	1/44	2.27%
		gp41 & p24 & gp36	14	14/44	31.82%
		gp120 & gp41 & p24 & gp36	17	17/44	38.64%
Positive (untypable)	1	p24	1	1/1	100.00%

Table 1: Profile of visible test lines on MULTISURE HIV Rapid Test

The profiles of visible test lines for all positive samples were examined and outlined in the above Table 1. 56.57% of HIV-1 positive results showed visible gp120, gp41 and p24 test lines and 33.74% of the positive results showed visible gp120 and gp41 test lines. There are a few samples (4.44%) found to be positive with single gp41 test line and 4.44% of the samples showed visible gp41 and p24 test lines. One sample was found to be positive with single gp120 test line (Figure 5).

Majority (71.29%) of the HIV-2 positive results showed single visible gp36 test line (Figure 6). The remaining 28.71% of the test results showed visible p24 and gp36 test lines.

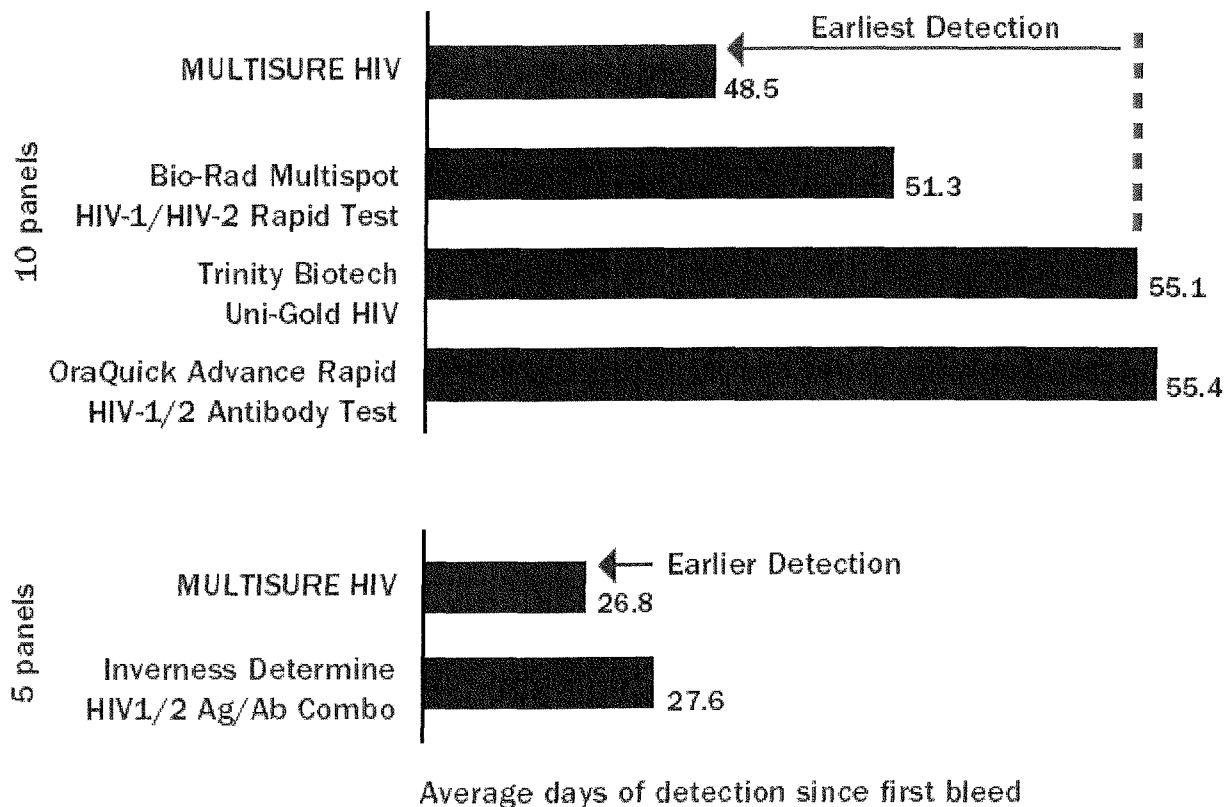
Among all 44 tests interpreted as positive with HIV-1 and HIV-2 antibody by the present HIV Rapid Test, 31.82% were found to be reactive with gp41, p24 & gp36 test lines, 18.18% were positive with gp41 and gp36 test lines and 9.09% were positive with gp120, gp41 and gp36 test lines. 38.64% samples demonstrated full profile with 4 visible test lines. Small percentage (2.27%) of the tests showed reactive gp120, p24 and gp36 test lines (Figure 7).

Reactivity with sero-conversion panels (Comparative performance with commercial Rapid Test kits)

Diagnostic Kits Description	Comparative performance of MULTISURE HIV Rapid Test with Inverness Determine HIV 1/2 Ag/Ab Combo		Comparative performance of MULTISURE HIV Rapid Test with Biorad Multispot HIV Rapid Test, Oraquick HIV 1/2 Rapid Ab and Trinity BioTech Uni Gold Recombinant HIV-1			
	MULTISURE HIV Rapid Test	Inverness Determine HIV 1/2 Ag/Ab Combo	MULTISURE HIV Rapid Test	Biorad Multispot HIV Rapid Test	Oraquick HIV 1/2 Rapid Ab	Trinity BioTech Uni Gold Recombinant HIV-1
Average days of detection since 1 st bleed (Sum of days of detection/ Total number of panels tested)*	26.8	27.6	48.5	51.3	55.4	55.1
Performance (Sensitivity)	38.89% (14/36)	30.56% (11/36)	41.18% (49/119)	35.29% (42/119)	28.57% (34/119)	28.57% (34/119)
Number of panels used for comparative performance	5	5	10	10	10	10

*In the case where an assay is negative over all seroconversion follow up bleeds, two additional days are assigned on the last bleed for calculation.

Table 2. Comparative performance of MULTISURE HIV Rapid Test with commercial Rapid Test kits on sero-conversion panels.



The comparative performance on seroconversion sensitivity of the present HIV Rapid Test was evaluated against various other types of Rapid Test kits that are available. The results of commercial Rapid Test diagnostic kits were extracted from COA of seroconversion panels from Zeptometrix Corporation and SeraCare Life Sciences. Referring to Table 2, in 5 seroconversion panels, the present HIV Rapid Test and Inverness Determine HIV 1/2 Ag/Ab Combo kit detected all 5 seroconversion panels as positive.

The average days of detection since first bleed of the present HIV Rapid Test are comparable with Inverness Determine HIV 1/2 Ag/Ab Combo kit, which were 26.8 days and 27.6 days respectively (For the calculation for the average days of detection, in case where an assay is negative over all seroconversion follow up bleeds, two additional days are assigned on the last bleed for the calculation). The overall sensitivity of the present HIV Rapid Test, 38.89%, was better than Inverness Determine HIV 1/2 Ag/Ab Combo kit (30.56%).

As shown in Table 2, the average days of detection since first bleed for the present HIV Rapid Test, Biorad Multispot HIV Rapid Test, Oraquick Advance HIV 1/2 Rapid Ab and Trinity BioTech Uni Gold Recombinant HIV-1 were determined based on 10 seroconversion panels.

On average, the present HIV Rapid Test showed the earliest average days of detection since first bleed (48.5 days), followed by Biorad Multispot HIV Rapid Test (51.3 days), Trinity BioTech Uni Gold Recombinant HIV-1 (55.1 days) and Oraquick Advance HIV 1/2 Rapid Ab (55.4 days). Similarly, the present HIV Rapid Test demonstrated the highest reactivity (41.18%) as compared to other rapid diagnostic kits (28.57% - 35.29%).

Whilst there has been described in the foregoing description preferred embodiments of the present invention, it will be understood by those skilled in the technology concerned that many variations or modifications in details of design or construction may be made without departing from the present invention.

Claims

- 5 1. A method for detecting a human immunodeficiency virus infection in a subject, the method comprising:
- (a) providing human immunodeficiency virus antigens;
- (b) contacting the human immunodeficiency virus antigens with a biological sample obtained from the subject, the biological sample suspected of
10 containing human antibodies against the human immunodeficiency virus antigens;
- (c) detecting the presence of a complex that forms between the human immunodeficiency virus antigen and the human antibody,
- wherein the method simultaneously and differentially detects the presence of
15 antibodies in the sample to HIV-1 and HIV-2 antigens, and the presence of the complex is indicative of a HIV-1 and/or HIV-2 infection in the subject.
2. The method according to claim 1, the method further comprising:
- (a) providing a solid support having a first end and a second end;
- 20 (b) immobilising the human immunodeficiency virus antigens on the solid support, the antigens are immobilised separately and spaced apart from each other along the solid support;
- (c) immobilising a molecule on the second end of the solid support, the molecule capable of binding to the human antibody in the complex, the molecule
25 further comprising at least one detectable labelled moiety;
- (c) loading the sample on the first end of the solid support, the sample contacting the antigens as it travels along the solid support in a first direction along the solid support and towards the second end; and
- (d) loading a buffer on the second end of the solid support adjacent the
30 molecule such that the buffer solution solubilise the molecule as it travels along the solid support in a second direction opposite the first direction,

wherein the presence of the complex formed on the solid support is detected by the labelled moiety of the molecule.

3. The method according to claim 2, further providing an absorbent pad adjacent the first end of the solid support, the absorbent pad and the solid support are separated by a separator, wherein removing the separator allows contact between the absorbent pad and the solid support to enhance the flow of the buffer along the solid support in the second direction

4. The method according to any one of the preceding claims, wherein the human immunodeficiency virus antigen is any one selected from: structural proteins of human immunodeficiency virus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the human immunodeficiency virus.

5. The method according to any one of the preceding claims, wherein the human immunodeficiency virus antigens are gp120, gp41, gp36 and p24.

6. The method according to any one of the preceding claims, wherein the human antibody is IgG.

7. The method according to claim 2, wherein the molecule is a human immunodeficiency virus antigen.

8. The method according to claim 2, wherein the molecule is an anti-human antibody.

9. The method according to claim 8, wherein the anti-human antibody is a goat anti-human IgG.

10. The method according to claim 2, wherein the detectable labelled moiety is a gold conjugate.

11. The method according to any one of the preceding claims, wherein the biological sample is any one selected from the group comprising: whole blood, plasma, and serum.
12. A kit for detecting a human immunodeficiency virus infection in a human subject, the
5 kit comprising:
(a) a solid support with human immunodeficiency virus antigens,
wherein the human immunodeficiency virus antigens are gp120, gp41, gp36 and p24, and the antigens are immobilised on the solid support separately and spaced apart from each other.
- 10 13. The kit according to claim 12, wherein the solid support further comprising a molecule immobilised on it, the molecule capable of binding to a complex formed with the surface-bound human immunodeficiency virus antigens, the molecule further comprising at least one detectably labelled moiety.
- 15 14. The kit according to claim 13, wherein a sample obtained from the subject is loaded on a first end of the solid support, and a buffer for solubilising the molecule is loaded on a second end of the solid support opposite the first end.
- 20 15. The kit according to claim 14, further comprising an absorbent pad adjacent the first end of the solid support, the absorbent pad and the solid support are separated by a separator, wherein removing the separator allows contact between the absorbent pad and the solid support to enhance the flow of the buffer along the solid support
- 25 16. The kit according to claim 13, wherein the molecule is an anti-human antibody.
17. The kit according to claim 16, wherein the anti-human antibody is a goat anti-human IgG.
- 30 18. The kit according to any one of claims 13 to 17, wherein the detectable labelled moiety is a gold conjugate.

19. The kit according to any one of claims 12 to 18, wherein the solid support includes a nitrocellulose membrane.