Internal Technical Report No. 201
Year of Publication : December 2002

COMMERCIALIZATION AND ASSESSMENT OF A RAPID HIV ANTIBODY DETECTION KIT

P. K. Ghosh*, D. Saha, N. V. Thaker, R. A. Karnik, S. G. Bhatt, R. P. Tiwari, R. S. Bagai, M. V. Christian and S. K. Vyas

Biotechnology Division, Cadila Pharmaceuticals Limited. Survey No. 342 Nani Kadi, Mehasana-382715. Gujarat, India.

Summary:

A rapid test for detecting HIV infection in blood, based on the principle of RBC agglutination was assessed and commercialized. The test is based on the principle of using recombinant anti-RBC monoclonal antibody (Mab) Fab molecules fused to immunodominant portions of the HIV epitopes of HIV-1 gp41, HIV-2 gp36 and HIV-1 p24 as reagents. The test reaction is visible to the naked eye and detects anti-HIV antibodies in 5 minutes or less. This kit was used to test a significant number of samples, its performance was compared to a popular ELISA based kit and the positives were confirmed with a popular Western Blot assay. From these studies, the kit was found to be highly sensitive and specific.

Key Words: HIV antibody detection, HIV fusion protein, RBC agglutination, rapid HIV test.
*Correspondence to: P K Ghosh, E-mail: **pghosh@cadilapharma.co.in**

INTRODUCTION:

The human immunodeficiency virus (HIV) pandemic has crossed astounding proportions with about 36 million presently infected and adding 15000 new cases every day, majority of them are in the developing countries (1). The correlates of protection are yet unknown. Detection of the disease becomes therefore the most significant first step towards protecting the society from further infection. Professionals including surgeons, gynecologists, dentists as well as persons involved in managing activities where they are exposed to human blood would be benefited if they knew the status of their clients with respect to HIV infection so as to enable them to take measures that adequately protect them from this infection (2). The ability to test for presence of antibodies against HIV proteins in human samples has helped the mankind to a large extent in containing the spread of the disease. The most commonly used assays for HIV diagnosis are the Enzyme Linked Immunosorbent Assays (ELISAs), which are time consuming, require specialized equipment and skilled technicians. The combination of ELISA assay with Western blot assay for confirmation is highly sensitive and specific for HIV detection; however it is very expensive and is time consuming (3). Therefore, rapid tests have become more desirable, especially for mass screening where simplicity and cost of the test is of considerable importance. This situation is especially relevant for developing countries where adequate skilled manpower is not available. It has been reported that often a sizeable percentage of people never return to the test center to collect their HIV test results (4). Hence, HIV tests that give results immediately are of importance to make available counseling and treatment to people who are diagnosed to be HIV positive. This would also aid significantly in HIV management. The importance of HIV rapid testing of pregnant women to assist infected mothers with adequate therapy and to provide them with counseling has also been understood. Hence, the need for rapid HIV detection tests has increased (5).

HIV rapid assay formats available includes particle agglutination, membrane immuno-concentration (flow through devices) and immuno-chromatographic strips (lateral flow). Particle agglutination tests using HIV antigen coated latex beads give results in 10 to 60 min, flow through devices require 5 to 15 min to perform the test, lateral flow strips take about 15 min while whole blood agglutination tests require 5 min or less to detect anti HIV-antibodies (6). HIV rapid tests are reported to show false positive results. Hence, HIV positive status is declared only after confirmatory testing using pre evaluated formats of ELISA and Western Blot assays (7). Western blot assays essentially detect and differentiate between antibodies directed against HIV proteins such as gag (p13, p18, p24, p55), pol (p51, p65) and envelope (gp160, gp120, gp41) (8). Assays to detect anti-HIV antibodies that depend upon the high specificity of interactions between antigen and their antibodies or their modified forms have been developed (9). One such assay utilizes Fab fragments of monoclonal antibodies (Mabs) that recognize and bind to the human red blood cell (RBC) surface proteins. These fragments are chemically conjugated with peptides of immunodominant epitopes of HIV protein that would capture anti-HIV antibodies to give a readable reaction. Reagents made from such molecules had good sensitivity and specificity for use in rapid diagnostic kits for HIV. The yields of such molecules are low and the procedures cumbersome, making them unsuitable for commercial applications (10).

The RBC-agglutination based rapid detection kit developed by M/s Cadila Pharmaceuticals Ltd. Ahmedabad, India uses genetically engineered fusion proteins to detect anti-HIV antibodies. The reagents of the kit contain Fab based bi-functional proteins for the detection of anti-HIV antibodies in whole blood, serum and plasma. These recombinant Fab fragments of the MAbs are specific for erythrocyte surface antigens, the Fd chain of which is fused to immunodominant epitopes of HIV-1 gp41, HIV-2 gp36 and HIV-1 p24 proteins. The kit uses three different MAbs to ensure universal reactivity with erythrocytes from all individuals. In presence of anti-HIV antibodies in the sample being tested, each arm of the antibody binds to the antigenic epitope on the Fab molecule which is bound to RBC at the other end resulting in a network that causes clumping (agglutination), visible to the naked eye. Use of several fusion proteins that recognize RBC surface proteins and have multiple HIV antigenic epitopes, enhances the probability of agglutination of RBCs from reactive samples many fold. The format has hence been named as *NEVA HIV* detection kit i.e. Naked (N) Eye (E) Visible (V) Agglutination (A) assay. The kit also supplies a Control Reagent that has the anti-RBC MAb molecules without the antigenic epitopes fused to them.

The inclusion of p24 antigenic epitopes in the system enhances earlier detection of anti-HIV antibodies for samples that are recently infected and are in the stage of sero-conversion i.e. in the window period when antibodies to envelope proteins of HIV are not yet adequate (11). Since *NEVA HIV* is an erythrocyte-agglutination assay, RBCs are required for the test reaction to occur. *NEVA HIV* can therefore be used directly with a drop of whole blood from a finger prick. If the sample to be tested is serum/plasma, it requires to be reconstituted using washed RBCs of O^{-ve} blood group.

Table 1 Schematic representation of the structure of Fab Fragments:

	Fragment	Description
1.	LC 1	LC fragment against RBC surface Ag 1
2.	LC 2	LC fragment against RBC surface Ag 2
3.	LC 3	LC fragment against RBC surface Ag 3
4.	Fd 1 | C	Fd fragment against RBC surface Ag 1 with c-myc tag (C)
5.	Fd 2 | **C**	Fd fragment against RBC surface Ag 2 with c-myc tag
6.	Fd 3 | **C**	Fd fragment against RBC surface Ag 3 with c-myc tag
7.	Fd 1 | gp 41 | **C**	Fd fragment against RBC surface Ag 1 fused with HIV-1 gp41 epitope and c-myc tag.
8.	Fd 3 | gp 41 | **C**	Fd fragment against RBC surface Ag 3 fused with HIV-1 gp41 epitope and c-myc tag.
9.	Fd 1 | gp 36 | **C**	Fd fragment against RBC surface Ag 1 fused with HIV-2 gp36 epitope and c-myc tag.
10.	Fd 3 | gp36 | **C**	Fd fragment against RBC surface Ag 3 fused with HIV-2 gp36 epitope and c-myc tag.
11.	Fd 2 | p 24 | **C**	Fd fragment against RBC surface Ag 2 fused with HIV-1 p24 epitope and c-myc tag.

Preparation of these molecules in quantities suitable for commercial applications and their formulation into kit reagents was standardized as described in the present paper. The advantages and shortcomings of *NEVA HIV* for HIV detection were studied in comparison with standard ELISA and Western Blot tests. The ease, simplicity and reliability of *NEVA HIV* test had been assessed.

METHODS:

The 11 clones of *E. coli* BL21 (λDE3) were obtained from the University of Delhi South Campus; the clones expressed the light (LC) and the heavy (Fd) fragments of the recombinant Fab molecules (12). For the Control reagent, the Fd fragments have only the c-myc tag while the Fd molecules comprising the HIV reagent are fusion proteins having antigenic epitopes of gp41, gp36 and p24 of HIV fused to the Fd fragment followed by the c-myc tag (Table 1).

The c-myc epitope tag at the C- terminus of the Fd molecules is a deca-peptide which aids in Fab protein detection during purification utilizing highly specific anti c-myc antibodies (Sigma-Aldrich, USA) (14). The amino acid sequences of the HIV antigenic epitopes and the c-myc tag are described in Table 2.

Expression of Fd and LC proteins:

The eleven clones, each expressing the constituent fragments for assembling the Fab-fusion proteins were individually grown in batches of 12 X 1L super broth medium containing Ampicillin (100 µg/mL) in specially designed baffled flasks at 30^{o} C and induced with 0.25 mM Isopropyl β-thiogalactoside (IPTG) for 3 hrs. The cell mass was harvested at 4^{o} C and lysed using Lysozyme in presence of NaCl and Triton X-100 to release the expressed protein Inclusion Bodies (IB). The IB was then given several washes of TE buffer (50 mM Tris; 20 mM EDTA) to obtain partially purified IB of the respective proteins. The protein content of the IB was estimated using Bradford assay at 595 nm (13) and stored at -70^{o} C for further use.

Preparation of Fab molecules:

The Control reagent of the kit contains three Fab molecules while the HIV reagent contains five Fab fusion proteins each of which were made by renaturing LC and Fd fragments in equimolar quantities (Table 3).

Equimolar quantities of the LC and Fd IB to make a total of 800 mg protein were thawed and denatured in presence of 6 M Guanidine Hydrochloride, reduced by addition of Dithioerythriotol (DTE) (1 mg DTE mg^{-1} IB protein) for 2 hours, mixed and centrifuged at 19000 rpm for 40 min at 4^{o} C. The supernatant containing denatured and reduced LC and Fd fragments was released in chilled 8 L Renaturation buffer (Arginine 500 mM, Glutathione-oxidized 0.9 mM, Tris-HCl 100 mM, EDTA 2 mM, pH 8.0) at 1 mL min^{-1}. The protein content of renatured sample was approximately 0.1 mg mL^{-1}. The renatured material was allowed to stand at 10^{o} C for 75 hr to allow folding and association of the LC and Fd molecules to form Fab protein.

Table 2 Sequence of HIV antigenic epitopes

	Antigenic Epitope	Size
1	HIV-1 gp 41: AVERYLKDQQLLGIWGCSGKLICTTAVPWNA	31 amino acids
2	HIV-2 gp36**:** GAIEKYLQDQARLNSWGCAFRQVCHTTVPWVNGA	34 amino acids
3	HIV-1 p24: RHMVHQAISPRTLNAWVKVIEEKAFSPEVIPMFTALSEGATPQDLNTMLNTVGGHQAAMQMLKETINEEAAEWDRVHPV HAGPIAPGQMREPRGSDIAGTTSTLQEQIGWMTNNPPIPVGEIYKRWIILGLNKIVRMYSPTSILDIRQGPKEGSGA	156 amino acids
4	c-myc tag: EQKLISEEDL	10 amino acids

For proteins P1, P3, P4, P6 and P7, the renatured sample, was dialyzed against 20 mM Tris; pH 8.0 in presence of decreasing concentrations of urea with buffer change every 12 hrs till the conductivity of dialyzed buffer become almost equal to the fresh buffer. In case of proteins P2 and P5, the sample was dialyzed against 20 mM Tris, pH 8.0 with decreasing Urea concentration with three changes every 12 hours followed by dialysis against 20 mM Diethanolamine (DEA) buffer; pH 9.5 with changes of dialysis buffer after every 12 hours, till the pH of the dialyzed buffer was same as that of the starting buffer. At the completion of dialysis, precipitates in the buffer were filtered out using a 1.2 µm Nitrocellulose membrane filter. The renatured and dialyzed protein was then subjected to purification to obtain pure Fab protein using multistep column chromatography procedures controlled by AKTA*explorer* (Amersham Pharmacia Biotech). The entire purification was carried out in a walk-in cooler at 2^{o}- 8^{o} C.

Purification of Fab protein:

Fab-fusion proteins P1, P3, P4, P6, P7 and P8 were purified on cation exchanger resin SP Sepharose FF (Pharmacia) packed in XK 50/20 column (Pharmacia). The protein sample to be purified was brought to pH 5.0 by addition of 5% acetic acid and the solution allowed to stand for 16 hrs at 4^{o} C. Precipitates formed were removed by filtration through 1.2 µm filter. The sample was loaded on pre-equilibrated SP-Sepharose FF (Pharmacia) column in 20 mM Acetate buffer; pH 5.0, at flow rate 10.0 mL/min. For purifying Fab proteins against Ag 1, the proteins bound to the column were eluted by applying a gradient of 0 mM to 500 mM NaCl in 20 mM Acetate buffer (pH 5.0) over 5 column volumes at a flow rate of 12.0 mL min^{-1}. The eluted protein was collected as 12 mL fractions. For purifying Fab proteins against Ag 3, a gradient of 100 mM to 600 mM NaCl in 20 mM Acetate buffer was applied. The elution was monitored at 280 nm and the fractions containing the protein were analyzed by 10% SDS PAGE and agglutination assay employing anti c-myc antibody (12). Fractions containing the protein were pooled for further purification. Salt concentration of the pool was adjusted to 2 M by addition of NaCl and the pH was adjusted to 7.2 by adding 1M Tris solution. The processed SP pool was then loaded on a pre-equilibrated hydrophobic interaction chromatography column, XK16/20 containing Toyo-butyl gel (Tosohaas, Germany), in 20 mM Phosphate buffer; 2 M NaCl, pH 7.2. Proteins of Control reagent (P1, P2 and P3) do not bind to the Toyobutyl gel and are collected in the flow through during loading of the column while the aggregates remain bound to the column. HIV reagent proteins bind to the column and were eluted by applying steps of decreasing salt concentration. Proteins with gp41 antigenic epitope (P4, P6) were eluted with 50 mL of 1.5 M NaCl buffer, followed by 100 mL of 0.6 M NaCl buffer. Proteins with gp36 antigenic epitopes (P7, P8) were eluted with 50 mL 1.2 M NaCl buffer followed by 100 mL of 0.5 M NaCl buffer. The fractions containing the protein peak or the flow through (in case of control proteins) were pooled and treated with Iodoacetamide 50mM final concentration. The sample was then loaded on a pre-equilibrated Sephadex G25 column, XK 26/70, 450 mL column volume for HIV reagent proteins and on Sephadex G25 column, XK 50/70, 1000 mL column volume for Control reagent proteins in 20 mM Tris, pH 8.5 to remove the NaCl and Iodoacetamide. The protein eluted from the gel filtration column was collected and loaded at a flow rate of 4 mL min^{-1} on anion exchange Q- Sepharose column, XK 16/20, 25 mL column volume pre-equilibrated with 20 mM Tris buffer, pH 8.5. Applying a linear gradient of 0 mM to 350 mM NaCl concentration buffer over 8 column volumes eluted the protein. Fractions containing Fab molecules as observed in the gel profile and activity as observed from the agglutination assay were pooled. The protein thus purified was subjected to a final polishing step using Sephacryl S200 gel filtration column, XK 50/100, 1700 mL column volume, in 20 mM Phosphate buffered saline. After the fractions containing the proteins were analyzed by the gel profile and activity was observed from the agglutination assay fractions containing purified Fab molecules were pooled. The protein content of the pool was estimated using Bradford Assay (13) and the protein was stored at -70^{o} C after addition of stabilizer.

The P2 and P5 proteins were purified from the renatured sample at pH 9.5, on pre-equilibrated anion exchange matrices of Q Sepharose FF (Pharmacia) in XK 50/20 column (Pharmacia), 200 mL column volume, in 20 mM DEA buffer; pH 9.5. The column was eluted by applying a gradient of 0 mM to 500 mM NaCl concentration buffer; pH 9.5 over 3 column volumes buffer at a flow rate of 12 mL min^{-1} and the eluted protein was collected as 12 mL fractions. This enabled rest of the large quantities of unwanted *E. coli* proteins to be removed. The procedures adopted were published earlier (14). The fractions containing the protein peak (OD 280 nm) were analyzed and pooled for further purification. The pooled sample was then dialyzed against 10 mM acetate buffer; pH 5.0 to bring the pH of the sample to approximately 5.0 and to decrease its conductivity. The dialyzed sample was then filtered and loaded on pre-equilibrated SP-Sepharose FF (Pharmacia) gel on XK 26/20 column, 70 mL column volume, in 20 mM acetate buffer; pH 5.0, at flow rate 4.0 mL min^{-1}. The column was eluted by applying a gradient of 0 mM to 500 mM NaCl in 20 mM Acetate buffer; pH 5.0, over 5 column volumes buffer, at a flow rate of 4.0 mL min^{-1} and the eluted protein was collected as 8 mL fractions. The fractions were pooled and the salt concentration of the pool was adjusted to 3 M by addition of NaCl and the pH was adjusted to 7.2 by adding 1M Tris solution. The processed SP pool was then loaded on pre-equilibrated hydrophobic interaction chromatography column, XK16/20, 25-30 mL Column volume, in 20 mM phosphate buffer; 3 M NaCl, pH 7.2, containing Toyobutyl gel (Tosohaas, Germany).

Protein P2 was collected in flow through during loading. Protein P5, bound to the Toyobutyl column was eluted by applying 100 mL of 0.3 M NaCl buffer. Both the proteins were purified further after adding iodoacetamide, by desalting chromatography using Sephadex G25 column and a final polishing step of Sephacryl S200 (gel filtration) chromatography. The concentration of the purified protein was estimated and the protein stored after stabilization.

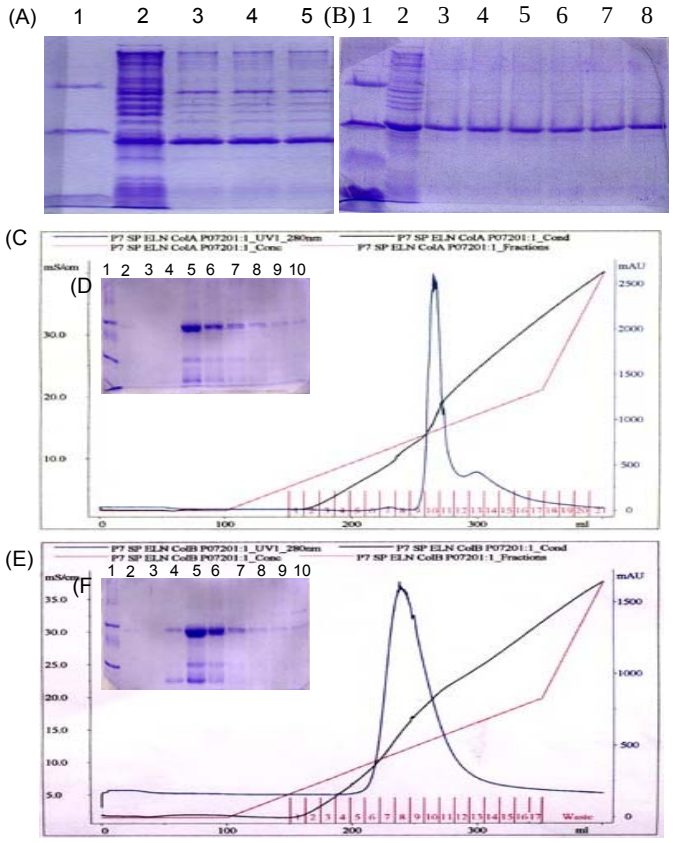

Figure 1: **(a)** Expression of LC 1 (12.5% reducing SDS PAGE); Lane: 1- Molecular weight marker (low), 2-Total cell pellet, 3, 4, 5- partially purified IB pellet. **(b)** Expression of Fd 1- gp 36 (SDS PAGE); Lane: 1- Molecular weight marker (low), 2-Total cell pellet, 3, 4, 5, 6, 7, 8- partially purified IB pellet. **(c)** Chromatogram for P7 Fab protein purification using SP Sepharose FF Column A. **(d)** 10 % SDS PAGE of SP Sepharose fractions containing peak; Lane: 1- Molecular weight marker (medium), 2- Sample before loading on SP Seharose column, 3- Flowthrough, 4 - Fxn 9, 5 – Fxn 11, 6 – Fxn 13, 7 – Fxn 15, 8 – Fxn 17, 9 – Fxn 19, 10 – Fxn 21. **(e)** Chromatogram for P7 Fab protein purification using SP Sepharose FF Column B. **(f)** 10 % SDS PAGE of SP Sepharose fractions containing peak; Lane: 1- Molecular weight marker (medium), 2- Sample before loading on SP Seharose column, 3- Flowthrough, 4 - Fxn 6, 5 – Fxn 8, 6 – Fxn 10, 7 – Fxn 12, 8 – Fxn 14, 9 – Fxn 15, 10 – Fxn 16. Table below gives the agglutination titers of the column fractions.

Samples	Agglutination Titer of SP Sepharose fractions (A)	Agglutination Titer of SP Sepharose fractions (B)
Control	>128	>128
Before column	4	4
Flow Through	0	0
Fraction	32 (9)	16 (6)
	>128 (11)	>128 (8)
	128 (13)	>128 (10)
	128 (15)	128 (12)
	32 (17)	32 (14)
	32 (19)	16 (15)
	16 (21)	16 (16)

Formulation of the kit reagents:

The proteins P1, P2 and P3 were formulated into a Control reagent for the kit and the proteins P4, P5, P6, P7 and P8 were formulated into the HIV reagent of the kit to detect anti- HIV antibodies against HIV-1 and HIV-2.

Assessment of the sensitivity and specificity of the test:

Subjects and HIV tests:

Serum, plasma and blood samples were obtained from a random population of the Gujarat and Rajasthan region. The identity of the subjects was kept undisclosed and results of the test were not disclosed to individuals and used only for assessing the performance of the test. The samples were tested with *NEVA HIV*,

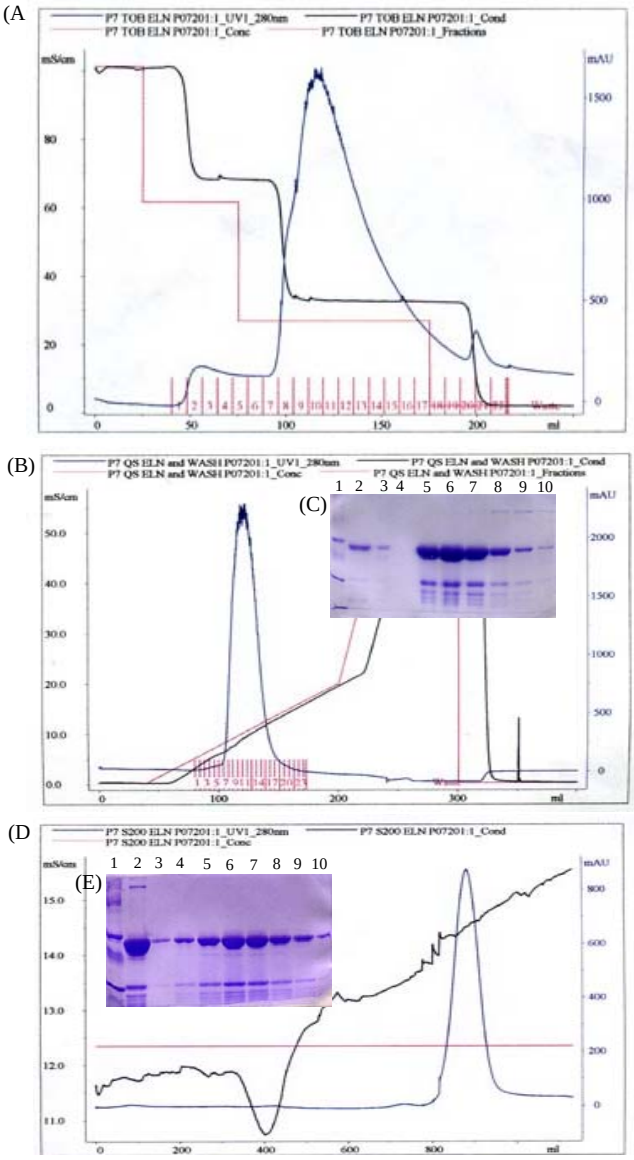

Figure 2: **(a)** Chromatogram for P7 Fab protein purification by Hydrophobic Interaction Chromatography (Toyobutyl). **(b)** Chromatogram for P7 Fab protein purification by anion exchange chromatography (Q Sepharose) **(c)** 10 % SDS PAGE of Q Sepharose fractions containing peak; Lane: 1- Molecular weight marker (medium), 2- Pool of fractions of SP Seharose chromatography, 3- Flow through, 4 - Fxn 8, 5 – Fxn 10, 6 – Fxn 12, 7 – Fxn 14, 8 – Fxn 16, 9 – Fxn 18, 10 – Fxn 20. **(d)** Chromatogram for P7 Fab protein purification by gel filtration (Sepharyl S200). **(e)** 10 % SDS PAGE of Sephacryl S200 fractions containing peak; Lane: 1- Molecular weight marker (medium), 2- Pool of fractions of Q Sepharose 3 – Fxn 8, 4 - Fxn 10, 5 – Fxn 12, 6 – Fxn 15, 7 – Fxn 18, 8 – Fxn 21, 9 – Fxn 23, 10 – Fxn 25. Table below gives the agglutination titers of the column fractions.

Samples	Agglutination Titer of Q Sepharose fractions (A)	Agglutination Titer of Sephacryl S200 fractions (B)
Control	>128	>128
Before column	32	>128
Flow Through	0	-
Fraction	32 (8)	8 (8)
	>128 (10)	16 (10)
	>128 (12)	64 (12)
	>128 (14)	>128 (15)
	128 (16)	>128 (18)
	128 (18)	128 (21)
	128 (20)	32 (23)
	-	8 (25)

and a commercially available ELISA assay (Biotest ELISA) as Reference Standard. The samples found to be reactive by these assays were tested by Western blot kit HIV BLOT 2.2 (Genelabs

Diagnostics), to confirm the HIV status of the sample. The results obtained were used to evaluate the sensitivity and specificity of the *NEVA HIV* test.

Table 3 Description of composition of Fab molecules of the reagents.

	REAGENT PROTEIN	CONSTITUENT PROTEINS	
		LC	Fd
CONTROL REAGENT PROTEINS			
1.	P1	LC 1	Fd 1
2.	P2	LC 2	Fd 2
3.	P3	LC 3	Fd 3
HIV REAGENT PROTEINS			
4.	P4	LC 1	Fd 1-gp41
5.	P5	LC 2	Fd 2-p24
6.	P6	LC 3	Fd 3-gp41
7.	P7	LC 1	Fd 1-gp36
8.	P8	LC 3	Fd 3-gp36

The *NEVA HIV* test was performed by placing a drop of diluent reagent each on the glass slide having two spots marked “Control” and “HIV”. Then a drop of Control reagent was placed on the “Control” spot and a drop of the HIV reagent was placed on the “HIV” spot. A drop of sample to be tested (reconstituted with O-Negative RBC in case of serum/plasma sample) was then placed on each of the spots. The reaction contents were mixed and the results were read for development of an agglutination reaction and recorded.

All the assays were performed using commercially available kits according to the manufacturers instructions given in the product inserts. Each of the samples was subjected to repeated analysis by the assays to confirm the result reported.

Samples:
A total of 2823 samples of serum, plasma and whole blood were tested for the evaluation of NEVA HIV kit.

Analysis of data:
The results of the assays on the samples were analyzed by comparison with the ‘Reference Standard’ test (ELISA) and the confirmatory test (WB). The sensitivity and specificity of *NEVA HIV* kit was calculated from these data.

RESULTS AND DISCUSSION:
Expression of Fab fragments:
The BL21 clones on culturing and induction expressed the LC and Fd fragments as insoluble IB that are inert were partially purified on the subsequent washing steps (Figure 1 a, b). The IB pellet had approximately 70%- 80% of IB protein.

Preparation of Fab fragments:
The Fab fragments, LC and Fd obtained in the form of IB were denatured and reduced so that their protein chains open up and they can refold into functional moieties in suitable environment. They were appropriately mixed in equimolar quantities so that an approximately equal number of LC and Fd molecules exist in the renaturing system and they mutually interact to form functional Fab molecules (Table 3). The LC and Fd chains of the antibody Fab molecules have high affinity for each other and they bind by disulphide bond formation (15). This property aids in the making of Fab molecules.

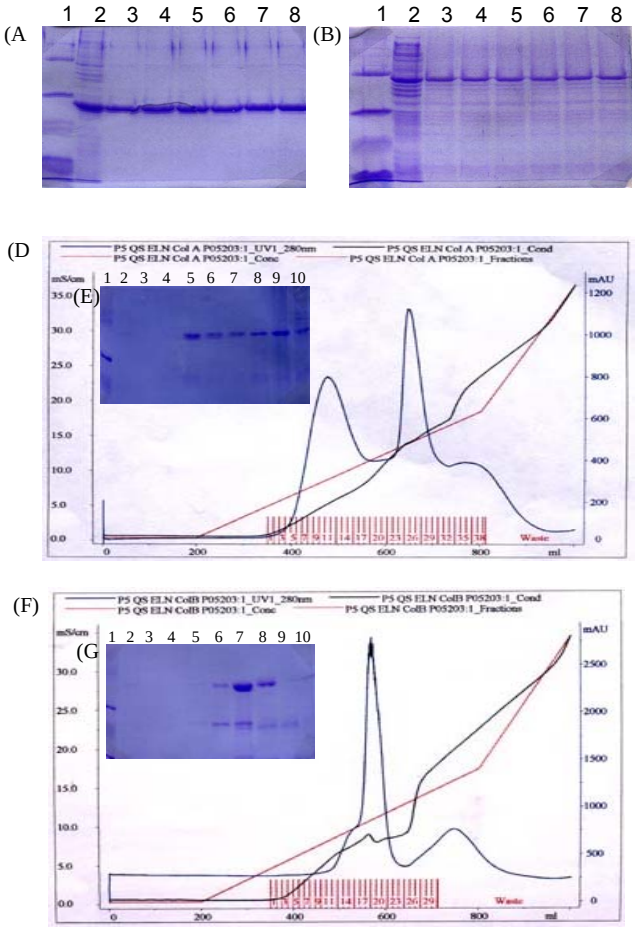

Figure 3: (a) Expression of LC 2 (12.5% reducing SDS PAGE); Lane: 1- Molecular weight marker (low), 2-Total cell pellet, 3, 4, 5, 6, 7, 8- partially purified IB pellet. **(b)** Expression of Fd 2- p 24 (SDS PAGE); Lane: 1- Molecular weight marker (low), 2-Total cell pellet, 3, 4, 5, 6, 7, 8 - partially purified IB pellet. **(c)** Chromatogram for P5 Fab protein purification using Q Sepharose FF Column A. **(d)** 10 % SDS PAGE of Q Sepharose fractions containing peak; Lane: 1- Molecular weight marker (medium), 2- Sample before loading on Q Seharose column, 3- Flow through, 4 - Fxn 5, 5 – Fxn 11, 6 – Fxn 16, 7 – Fxn 19, 8 – Fxn 23, 9 – Fxn 27, 10 – Fxn 30. **(e)** Chromatogram for P5 Fab protein purification using Q Sepharose FF Column B. **(f)** 10 % SDS PAGE of Q Sepharose fractions containing peak; Lane: 1- Molecular weight marker (medium), 2- Sample before loading on Q Seharose column, 3- Flow through, 4 - Fxn 12, 5 – Fxn 14, 6 – Fxn 16, 7 – Fxn 18, 8 – Fxn 20, 9 – Fxn 22, 10 – Fxn 24.

Purification of Fab Protein:
The renatured material contains Fab molecules; aggregates of Fab, LC and Fd, free LC and Fd molecules as well as *E. coli* proteins that have to be removed to purify the Fab protein of interest. Also, the renaturation system has high concentration of chemicals that create oxidizing-reducing conditions. These chemicals were removed from the system gradually so as to maintain the conformation of the Fab molecules by slow dialysis. The pH adjustment step also removed non-Fab protein. The Fab protein was further purified using a multistep chromatography procedure. The class of proteins comprising P1, P3, P4, P6, P7& P8 had similar purification steps. Proteins P2 & P5 had another set of purification protocol. Details of the purification chromatograms and gel as well as agglutination analysis of the fractions obtained during chromatographic purification of the protein P7 from the first group and protein P5 of the second group have been presented. Figure 1 (a)-(f) & 2 (a)-(f) depicts the subsequent purification of protein P7 and Figure 3 (a)-(f) & 4 (a)-(f) depicts the purification of protein P5. The starting material for the preparation of Fab molecule inclusive of equal molecules of LC and Fd proteins was 800 mg. The final yields of the purified Fab proteins are given in Table 4.

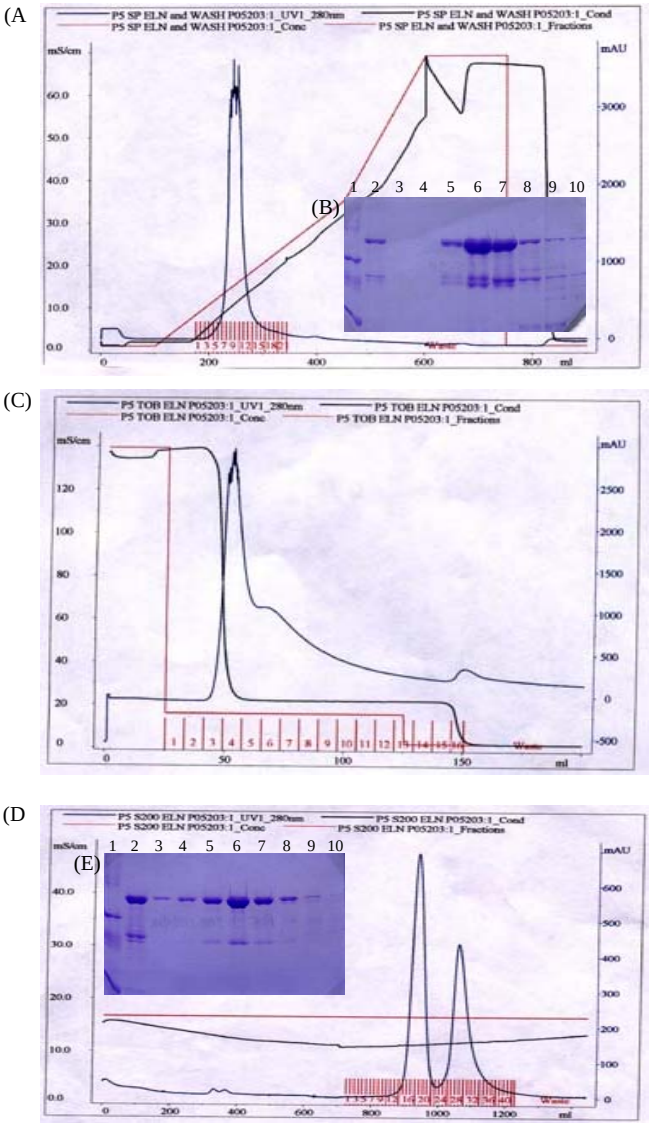

Figure 4: **(a)** Chromatogram for P5 Fab protein purification by cation exchange chromatography (SP Sepharose FF) **(b)** 10 % SDS PAGE of SP Sepharose fractions containing peak; Lane: 1- Molecular weight marker (medium), 2- Pool of fractions of Q Sepharose chromatography, 3- Flow through, 4 - Fxn 5, 5 – Fxn 7, 6 – Fxn 9, 7 – Fxn 11, 8 – Fxn 13, 9 – Fxn 15, 10 – Fxn 17. **(c)** Chromatogram for P5 Fab protein purification by Hydrophobic Interaction Chromatography (Toyobutyl). **(d)** Chromatogram for P5 Fab protein purification by gel filtration (Sephacryl S200) **(e)** 10 % SDS PAGE of Sephacryl S200 fractions containing peak; Lane: 1- Molecular weight marker (medium), 2- Pool of fractions of SP Sepharose, 3 – Fxn 14, 4 - Fxn 15, 5 – Fxn 16, 6 – Fxn 18, 7 – Fxn 20, 8 – Fxn 21, 9 – Fxn 22, 10 – Fxn 23. Table below gives the agglutination titers of the column fractions.

Samples	Agglutination Titer of SP Sepharose fractions (A)	Agglutination Titer of Sephacryl S200 fractions (B)
Control	>128	>128
Before column	>128	>128
Flow Through	0	-
Fraction	0 (5)	32 (14)
	>128 (7)	64 (15)
	>128 (9)	128 (16)
	>128 (11)	>128 (18)
	>128 (13)	>128 (20)
	32 (15)	64 (21)
	32 (17)	16 (22)
	-	0 (23)

The yields were found to be of the order that would make preparation of proteins economically viable. Also, repeated purified batches of the proteins resulted in constant yields of the purified proteins. Renaturation of LC and Fd proteins into Fab molecules is an important reaction in the process of preparation of Fab protein. The process of releasing LC and Fd proteins in the renaturing buffer was standardized and automated to get better yields of the proteins. It was observed that a Fab protein that was bound the RBC surface Antigen 2 did not react to give a reaction with the Bradford reagent. The assay was therefore not used for the estimation of the purified proteins P2. They were quantified using OD 280 in a UV Spectrophotometer using a quantified protein P3.

Table 4 Yields of purified Fab proteins

	Fab Protein	Yield (mg)	% Yield*
1	P1	75.8	9.5
2	P2	41.6	5.2
3	P3	136.4	17.0
4	P4	51.0	6.4
5	P5	15.0	1.9
6	P6	50.0	6.25
7	P7	32.5	4.0
8	P8	40.7	5.1

* (800 mg starting material)

Assessment of the sensitivity and specificity of the test:

The *NEVA HIV* kit reagents were formulated and filled in vials that dispense ~25 µL drop of the reagents and used to perform the tests. The assay procedure required dispensing drops of the reagents onto glass slides provided with the kit. Samples from a random population collected and stored in different centers across the country, a mixed titre anti-HIV panel sera comprising of 50 members from BBI, representative samples of HIV-1 O subtype from BBI, Inc. were also used in the study. A statistically significant number of 2823 samples were tested in total to determine the sensitivity and specificity of the NEVA HIV kit. Each of the samples was tested thrice with each of the Cadila *NEVA HIV* kit and the BIOTEST anti-HIV TETRA ELISA kit ('Reference Standard' produced by Biotest AG, Germany) twice. Such a large number of samples assessed aided in a broad-spectrum study of samples that exhibited unique results pattern. Samples positive by both these test were tested for confirmation with Western Blot kit, the Genelabs Diagnostics HIV Blot 2.2. Sensitivity and specificity of the *NEVA HIV* kit was calculated using these results as given in Table 5. Sensitivity was calculated as (Number of True Positives) / (Number of True Positives + Number of False Negatives) x 100 (16). The specificity was determined by (Number of True Negatives) /(Number of True Negatives + Number of False Positives) x 100 (16). A True Positive test was defined as a Positive result obtained with either of the assays and confirmed with the WB test with interpretation as per the WHO criterion. A false positive test was defined as a test that is confirmed to be negative according to the WHO criterion by WB test. The sensitivity of the *NEVA HIV* test was calculated to be 99.9% while its specificity was 98.5%.

There was one sample that the Reference Standard test failed to detect as positive which was found to be positive by *NEVA HIV* kit and confirmed to be positive by WB. *NEVA HIV* kit is found to be a highly rapid test as compared to ELISA and had specificities and sensitivities comparable to ELISA. It had also been found by

Table 5 Results of Kit tests on samples evaluated by NEVA HIV, Biotest Anti-HIV Tetra ELISA and confirmed by WB HIV Blot 2.2

Total number of samples	2823
Number of True Positives (TP)	698
Number of True Negatives (TN)	2091
Number of False Positives (FP)	33
Number of False Negatives (FN)	01

another group of investigators who were screening the HIV status of pregnant women that, the *NEVA HIV* kit had the calculated sensitivity of 94.1% and specificity of 96.4% which was derived from the evaluation of 1258 pregnant women and that the values were comparable to or even better than those of other rapid tests, such as Oraquick (sensitivity 90.3%, specificity 99.9%, Orasure Technologies, Inc., USA) and HIV-1/2 Determine (sensitivity 88.6%, specificity 99.9%, Abbott Laboratories, USA) evaluated simultaneously by them (17). *NEVA HIV* kit requires no specialized equipments for interpretation of results. This makes *NEVA HIV* kit extremely ideal for use in resource poor settings.

CONCLUDING REMARKS:

Finding the presence of antibodies specific to HIV antigens conveniently monitors the onset and prognosis of HIV infection. In this context antibodies to envelope antigens of the virus as well as to gag and pol protein antigens are positively correlated with the infection, although a positive reaction for an analyte does not necessarily imply HIV infection; reactive samples need to be confirmed by using Western Blot tests. There are some limitations of the testing formats (18), however such tests have been recognized as diagnostic procedures in the sero-surveillance programmes or in routine screening. ELISA formats have been used for a long time as more reliable, but as the time required by ELISA procedures are considerable, alternative methods are also being developed the world over. Membrane based flow through ELISA formats for HIV detection are reasonably faster but they are usually expensive. Many particle based agglutination assays are simple to perform but they lack sensitivity. The present format of RBC agglutination based assay is highly sensitive and specific.

REFERENCES:

(1) Esparza, J. Osmanov, S. Pattou-Markovic, C. Tour'e, C. Chang M-L. and Nixon, S. Past, Present and future of HIV vaccine trials in developing countries. Vaccine 20 (2000); pp. 1897 – 1898.

(2) HIV Antibody Assay: HIV InSite Knowledge Base Chapter Niel Constantine, PhD, University of Maryland, School of Medicine. Feb 1998.

(3) H.L. Francis, M. Kabeya et.al, 1988. Comparison of sensitivities and specificities of Latex Agglutination and an Enzyme-Linked Immunosorbent Assay for Detection of Antibodies to the Human Immunodeficiency Virus in African sera. A. J. Cli. Micro. Nov 1988: pp. 2462-2464.

(4) Centers for Disease control and Prevention. HIV counseling and Testing in Pubicly Funded Sites: 1993-1994 Summary Report. Atlanta: U.S.Department of Health and Human Services: 1996.

(5) Deborah Allen, Arthur Ammann, et.al. Revised Recommendations for HIV screening of Pregnant Women. Perinatal Counseling and Guidelines Consultation. MMWR Recommendations and Reports. November 9, 2001/50(RR19); pp.59-86

(6) Bernard M. Branson . Assessing Diagnostic Technologies Marketed to less Intusrialized countries. J. Int. Physicians in AIDS Care 28-30 (2000)

(7) Steven L. Sivak, Gray P. Wormser. Predictive Value of a Screening Test for Antibodies to HTLV-III. Am J Clin Pathol 1986, 85:700-703.

(8) Juan I. Esteban,Chang-Chih Tai, John W. D.Kay, J.Wai-Kuo Shih, Anne J. Bodner , Harvey J. Alter. Importance of Western Blot Analysis In Predicting Infectivity of Anti-HTLV-III/ LAV Positive Blood. The Lancet 16 Nov 1985.

(9) Kemp.etal, 1988, Pastan et.al, 1992, Brinkmann and Pastan et.al. 1994, Neuberger et.al.1984, Williams and Neuberger, 1986).

(10) Kim.M.Wilson, Michael Gerometta, Dennis B. Rylatt, Peter G. Bundesen, Dale A. MacPhee, Carmel J. Hillyard and Bruce E. Kemp. Rapid whole blood assay for HIV-1 seropositivity using a Fab-peptide conjugate. J. Immunological Methods, 138 (1991), pp.111-119.

(11) Schupbacb J. Hallero ,Vogt M,et.al. Antibodies to HTLV III in swiss patients with AIDS and pre-AIDS and ingroups at risk for AIDS. N. Eng J. Med.1985, pp. 265-70.

(12) Amita Gupta, Sanjay Gupta, Vijay K. Chaudhary, Recombinant fusion protein for haemagglutination based rapid detection of antibodies to HIV in whole blood. Journal of Immunological methods 256, (2001), pp. 121-140.

(13) Bradford, M. "A Rapid and Sensitive Method for the Quantitation of Protein. Utilizing the Principle of Protein-Dye Binding". Anal. Biochem (1976) 72: pp. 248-254.

(14) Amita Gupta, Vijay K. Chaudhary, Expression, purification, and characterization of a anti-RBCFab-p24 fusion protein for hemagglutination-based rapid detection of antibodies to HIV in whole blood, Protein Expr. Purif. 26 (2001), pp. 162-170.

(15) Kolodziej PA, Young RA. Epitope tagging and protein surveillance Methods Enzymol 1991;194: 508-19.

(16) Toman K. Sensitivity, specificity and predictive value of diagnostic test (1981). Bulletin of the International Union against Tuberculosis. 1981, 56, 18-28.

(17) Bhore, A.V.; Sastry, J.; Patke, D.; et al. (2003). Sensitivity and specificity of rapid HIV testing of pregnant women in India. *International Journal of STD & AIDS.* Vol. 14, No. 1, January, pp. 37-41.

(18) Ghosh P. K. and Sharma, N. C. (2000) Antibody detection: Current Approaches. J. Sci. Indus. Res. 59, 919-931.