



Evaluation of Fluorognost™ HIV-1 IFA

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Background

The Fluorognost™ HIV-1 Indirect Immunofluorescence Assay (IFA) is an *in vitro*, qualitative assay for the detection of antibodies to Human Immunodeficiency Virus Type 1 (HIV-1). Further details of the assay are given in Appendix 1. The test is intended to be used as an additional and specific test to detect antibodies to HIV-1 in human serum or plasma specimens. Fluorognost™ is used as a confirmatory test for specimens that are repeatedly reactive by EIA or may also be used as a screening test by trained personnel where EIAs are not available.

Within the UK, Western Blot (WB) testing is not routinely performed and is restricted to specimens with atypical serological profiles (weak or discordant), or where there are epidemiological factors which suggest supplementary tests are appropriate (see Appendix 2). At present, Fluorognost™ IFA is not routinely used within the UK and this study assessed the effectiveness of the assay on specimens from a UK reference laboratory, in particular as an alternative to WB.

Two operators underwent a compulsory two-day training session, and correctly identified members of an 18-member proficiency panel prior to commencing the study. Subsequent to the training session, a simplified laboratory protocol was produced by the manufacturer (see Appendix 3), plus supplementary details for processing of dried blood spots (DBS).

Specimen panel and methods

A panel of specimens of different types were tested, including routine HIV-1 positive and negative serum or plasma specimens (previously frozen), those which received Western Blot (Genelabs Diagnostics HIV 2.2) analysis (e.g. atypical serological profile or suspected seroconverters), plus HIV-1 positive and negative dried blood spot (DBS) specimens (Table 1). Details of how the patient HIV-status was derived is given in Appendix 2. Specimen status was anonymised prior to testing.

Table 1: Specimen panel

Category	Number
Anti-HIV-1 positive	102
Anti-HIV-1 negative	100
Anti-HIV-2 positive (commercial panel)	20
Anti-HIV-1 positive (Group O, commercial panel)	2
Required Western Blot test	69
Anti-HIV-1 positive dried blood spot	50
Anti-HIV-1 negative dried blood spot	50

Each specimen was tested singly using protocol 'Method B' (incorporating a counter-stain step; Appendix 1), and the results read independently by two trained readers. Positive specimens were scored as 'weak positive', 'positive', or 'strong positive'. Any ambiguous or discordant results were retested in duplicate. During the training session, it was noted that counterstaining with a 1:10 dilution gave clearer results than 1:30 and

hence on the trainer's suggestion this was used throughout the evaluation. Dried blood spot specimens from HIV-1 infected individuals were generated from EDTA blood, and both elution methods mentioned in the protocol were used interchangeably in the study (Appendix 1).

Modifications to the protocol

The microscope did not exactly match that stipulated in the kit insert but was agreed to be acceptable; briefly, the excitation filter was 450-490 nm not 500, and the barrier filter was 520 where 510 or 530 were listed (see Appendix 1 for further details). Following discussion with the trainer, pre-warming of reagents to room temperature for 30 min prior to use was not always performed, and tapping slides onto absorbent material rather than vacuum pump aspiration was used to remove liquid from the wells. A simplified laboratory protocol was provided post-training, including incubation time ranges (see Appendix 3). The number of freeze-thaw cycles of the serum/plasma specimens (recovered from -20°C storage) was not recorded, but is likely to be less than two (a maximum of two is specified in the kit insert). The manufacturer confirmed that this should not affect the results and suggested always, following dilution (five µl serum/plasma and 145µl PBS), performing a centrifugation step at 10,000g for three minutes following dilution using five µl serum/plasma and 145µl PBS to remove any particulate matter prior to use. Fluorescent images captured with a digital camera had a reduced intensity compared to direct microscope reading and hence were not used for scoring of results in this study.

Results

Results are summarised in Table 2. Further details are provided in Appendices 4 (specimens requiring repeat testing) and 5 (specimens giving false negative or indeterminate results).

Specimens from HIV-1 infected patients

Anti-HIV-1 antibody positive serum/plasma specimens

The Fluorognost™ IFA correctly identified 101 of 102 straightforward anti-HIV-1 positive specimens, with one indeterminate result (a sensitivity of 99.0%). This indeterminate was an anti-HIV-1-antibody positive specimen from a possible seroconverter. Two specimens from HIV-1 Group O-infected patients were both IFA positive.

Dried blood spots

The sensitivity of IFA for DBS specimens was 96.0% (48/50); two specimens from suspected seroconverting patients gave indeterminate results (one anti-HIV-1 negative, and one anti-HIV-1 positive).

Western Blot-tested specimens

Of 51 specimens from HIV-1 infected patients tested using Western Blot (including specimens from five patients whose serology indicated dual HIV-1 and HIV-2 infection), 32 were IFA positive, 15 negative and four indeterminate. These included four, 14 and

three suspected seroconverter specimens, respectively. In comparison, the HIV-1 WB identified 40/51 positives. Within these were multiple specimens taken at different time points from a single patient with an atypical serological profile (four WB negative and one WB indeterminate); all of which were IFA negative. If these are counted as a single specimen, the IFA detected 32/47.

Specimens from HIV-2 infected patients

There were 23 specimens from HIV-1 negative/HIV-2 positive patients, of which 20 (86.9%) were IFA positive, two indeterminate and one negative. The version of the kit assessed is not intended to detect anti-HIV-2 antibodies.

Specimens from HIV-1 uninfected patients

The IFA specificity for straightforward anti-HIV-1 negative serum/plasma specimens was 98% (2/100 specimens gave indeterminate results), and of DBS 100% (50/50). For WB specimens (including 10 WB indeterminates), the IFA specificity was 100% (16/16).

Dried Blood Spots

The DBS tended to give higher levels of non-specific fluorescence than serum or plasma specimens, but only a small proportion (6%) required repeat tests (see Appendix 4). Two specimens from HIV-1 infected individuals were IFA negative, both from suspected seroconverters (one HIV-Ab negative, one unknown Ab status).

Repeat testing

Excluding technical errors, 42 specimens (of 393 specimens; 10.7%) required repeat testing, half of which were WB specimens (52.4%). Details of these are provided in Appendix 4.

Scoring of positives

The two readers scored positive results as 'weak positive', 'positive' or 'strong positive', and where both readers agreed this was recorded. However, the readers considered this too subjective; the HIV-1 infected well contained a 'mixed cell environment' of 40 to 60% of infected cells with uninfected cells and there were often score differences between the two readers. Interestingly, 87.5% (28/32) of specimens both readers assigned as 'strong' positive were from straightforward antibody positive specimens, compared to 34.5% of those recorded as 'weak' positive, which included anti-HIV-2-positive and Group O specimens.

Table 2a: Sensitivity (straightforward HIV-positive specimens)

Patient status	Specimen category	Total	IFA result			Sensitivity (%)
			Pos	Neg	Indet	
HIV-1 positive	HIV-1 Ab pos	102	101	0	1	99.0
	HIV-1 Ab pos (Group O)	2	2	0	0	100
	DBS	50	48	2	0	96.0
	TOTAL	154	151	2	1	98.1
HIV-2 positive	HIV-2 pos	20	17	1	2	85.0 [†]

Table 2b: Specificity (straightforward HIV-negative specimens)

Patient status	Specimen category	Total	IFA result			Specificity (%)
			Pos	Neg	Indet	
HIV-1 negative	HIV-1 Ab neg	100	0	98	2	98.0
	DBS	50	0	50	0	100
	TOTAL	150	0	148	2	98.6

Table 2c: Western Blot specimens

Patient status	Specimen category	Total	IFA result			Sensitivity (%)
			Pos	Neg	Indet	
HIV-1 positive	WB HIV-1 pos	35	27	4 ^a	4	77.1
	WB HIV-1 neg	5	0	5 ^b	0	0.0
	WB HIV-1 indet	6	0	6 ^c	0	0.0
	WB HIV1+2 pos	5	5	0	0	100
	TOTAL	51	32	15	4	62.7
HIV-2 positive	WB HIV-2 pos	3	3	0	0	100
			Pos	Neg	Indet	Specificity (%)
HIV-1 negative	WB HIV-1 neg	5	0	5	0	100
	WB HIV-1 indet	10	0	10	0	100
	TOTAL	15	0	15	0	100

Ab: antibody; Pos: positive; Neg: negative; Indet: indeterminate; DBS: dried blood spot †: the assay is for the detection of HIV-1 and not HIV-2. a: includes 2 specimens from a single patient taken at different time points. b: includes 4 specimens from a single patient at different time points, with highly atypical antibody evolution; this patient also had a WB indet/IFA neg result (c).

Technical appraisal

The two-day training session comprised a short presentation followed by practical sessions, and was clear and comprehensive. No DBS specimens were assessed but the protocol modifications required for this were discussed. During the course of the study, the time taken to perform the IFA decreased as the operators became more confident in reading the results.

While comprehensive, the kit insert was inconvenient for use as a laboratory protocol due to a small typeface and a large amount of text; the protocol provided post-training (Appendix 2) was much clearer. The results sheets provided had very little space for results entry if comments were required, plus there was no provision for recording the temperatures and incubation times of the run.

The slide packaging was not easy to open and the potential of damaging the well surfaces was a concern; a perforated side edge, for example, would help in this. The glass coverslips were provided in an open packet, but were not easy to remove and no provision was made for breakages of these fragile items which did occur during the evaluation; this could also pose a potential safety concern. Coloured lids on the reagent vials meant their identification was quick and easy, and the box containing the kit provided a convenient humidity chamber for use in the assay.

Conclusions

The Fluorognost™ HIV-1 IFA showed a good sensitivity for the detection of anti-HIV-1 in straightforward anti-HIV-1 antibody positive serum/plasma specimens (99%), including representatives of the genotypically diverse Group O (100%), and also worked well for DBS (96% sensitivity). This assay has been proposed as an alternative to Western Blot for confirmation of HIV and as such was compared to WB results. As described earlier and summarised in Appendix 2, this subset of specimens represents those most challenging to diagnose and not standard second-line confirmatory specimens. Of 51 specimens from HIV-1 positive patients receiving WB testing (including five dual HIV-1 and HIV-2 positive individuals), 78.4% were WB-positive, compared to 62.7% positive by IFA. However, IFA correctly assigned 100% of specimens from HIV-uninfected patients compared to 33.3% by WB which had a large number of indeterminate results. Considering the results in terms of a correct assignment of HIV-1 infected or uninfected status based on the use of WB or IFA as a confirmatory test, therefore, IFA was slightly better than WB (71.2% vs 68.2%).

Many of the specimens from HIV-1 infected individuals which were not detected by the IFA were from patients who were confirmed or suspected seroconverters (76.2%), or who had atypical serological profiles and included some which were anti-HIV-1-antibody-negative by alternative methods. Of note, despite being for the detection of anti-HIV-1, cross-reactivity allowed 20/23 HIV-2 specimens to be detected using the IFA.

The IFA is relatively time consuming at approximately 3 hours for 23 specimens, but this is comparable to WB (a typical EIA takes the same amount of time for a run of 96 tests). Further, the IFA requires a fluorescent microscope – equipment becoming less common in laboratories with the increasing emphasis on molecular diagnostics and automation. In summary, as a confirmatory test for HIV-1, the IFA is of limited use for seroconversion specimens, but is approximately equivalent in performance to WB overall, being slightly less sensitive for the detection of positives but better at resolving negatives.

Acknowledgements

We would like to thank members of the Virus Reference Department for providing specimens, testing and specialist advice, and the Quality Assurance Laboratory for access to their laboratory, microscope and technical support.

Appendix 1: Brief assay description

The Fluorognost™ HIV-1 IFA detects HIV-1 antibodies. Immortalised human T-cells expressing HIV-1 antigens (fixed on the slide) specifically bind HIV-1 antibody, and detection is achieved by the subsequent binding of anti-human (rabbit) immunoglobulin conjugated to fluorescein isothiocyanate (FITC conjugate) which fluoresces on exposure to UV light. The degree and pattern of fluorescence seen in HIV-infected cells compared to uninfected cells determines the HIV status of the sample.

The protocol can be used in three ways: Method A (standard); Method B (incorporating a counterstaining step to aid examination of more difficult specimens such as seroconverters, or to assist those new to the IFA); and Method C (Method A, followed by a counterstaining step post-slide reading). Method B was used in this evaluation. For elution of DBS, two alternative procedures are offered in the protocol: 250µl of working PBS solution is added to ¼" disks from the DBS and either eluted overnight at 2-8°C or for 2 hours at room temperature with vigorous agitation. No difference in the results obtained using these two methods was observed in this study.

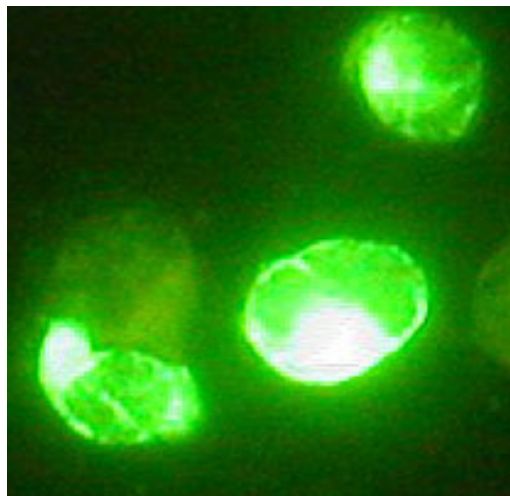


Figure 1: Distinctive apple-green polar cap staining pattern in an HIV-1 positive specimen.

Source: <http://www.fluorognost.com/>

General	
Assay name	Fluorognost™ HIV-1 IFA
Manufacturer/UK agent	Sanochemia Pharmazeutika AG
Product number	401593
Number of tests in one pack	50 (also available as 25)
Specimen volume (for dilution)	5 µl

Presentation	
Assay type	HIV-1 Indirect Immunofluorescence Assay (IFA)
HIV-1 IFA slide	Five wells each of inactivated HIV-1 infected (1i-5i) and uninfected (1-5) immortalised T-cells expressing HIV-1 antigens
Concentrated diluent	Phosphate Buffered Saline (PBS)
Concentrated FITC conjugate	Concentrated solution of fluorescein isothiocyanate conjugated with (rabbit) anti-human IgG
Mounting media	PBS-buffered glycerol in dispenser bottle
Negative serum control	Heat inactivated serum; non-reactive for HIV-1, HIV-2, HBVsAg, HCV Ab
Positive serum control	Heat inactivated HIV-1 antibody positive human serum; non-reactive for HIV-2, HBVsAg, HCV Ab
Concentrated counter staining solution	1% Evans Blue solution
Glass coverslips	

Stages[†]	
Make PBS diluent working stock	Dilute with distilled water to make 1 litre
Construct humidity chamber	5 min
Sample/control dilution	1:30 in PBS diluent
Vortex, centrifuge @ 10000 g	3 min
Add sample/control to slide wells	
Incubate sample (37°C)	30 min (±10 min)
Wash (PBS)	10 min (± 5 min)
Dilute and add FITC	1:10 in PBS diluent
Incubate FITC conjugate (37°C)	30 min (±10 min)
Wash (PBS)	10 min (± 5 min)
Dilute and add counter stain	
Incubate counter stain (RT)	5 min
Add mounting media and coverslip	5 min
Average reading time per slide	Approx 5 min
Approx test time per 5 slides (25 wells; single reader)	175 min

†: Standard protocol not DBS. PBS: phosphate buffered saline; FITC: Fluorescein isothiocyanate; RT: room temperature; Ab: antibody; min: minutes

Additional equipment required	
Fluorescent microscope [‡]	Epifluorescent attachment/incident light microscope: (Used: ZEISS STANDARD 16) Light source: <i>mercury vapour lamp 50-200 W</i> Excitation filter: <i>KP 500 (Used: 450-490nm)</i> Splitting mirror: <i>TK 510</i> Barrier filter: <i>K 510 or K 530 (Used: 520)</i> Suppression filter: <i>BG-38</i>
Dilution tubes	12mm X 75 mm with screw caps recommended
Micropipettes	1-20 µl and 100-1000 µl
Slide holder	For immersion of slides during washing steps
Rotary/shaking platform	For slide washing steps
Incubator	For incubations at 37°C
Humidity chamber	A small box with PBS-wetted layer of absorbent paper; the kit container can also be used for this purpose
Distilled water	For dilution of PBS
Volumetric flask	For measuring distilled water
Timer	For timing 10/30 min during washes and incubations

‡: Characteristics of the microscope used in the evaluation (agreed with manufacturer) are given in bold

Appendix 2: Diagnosis of HIV infection

Diagnosis of HIV: Algorithm employed at the HPA Centre for Infections. Routine specimens are tested using two 4th Generation assays (combining anti-HIV and p24 antibody detection), plus GACPAT tests to distinguish between HIV-1 and HIV-2^{1,2}. Where the results from these tests are not straightforward (weak or discordant), or where clinical or epidemiological information suggests it is appropriate, supplementary assays may be employed. For example, suspected dual infections with HIV-1 and HIV-2 are tested using ImmunoComb II BiSpot HIV-1&2 EIA and may include an HIV Blot 2.2 Western Blot (Genelabs). Suspected recent infections may also be tested using HIV p24 Ag, HIV-1 RNA PCR and/or IgG/M/A anti-HIV EIA assays, as required. No specific information is available for specimens from commercial panels.

1. Parry JV, Mortimer PP. An immunoglobulin G antibody capture particle-adherence test (GACPAT) for antibody to HIV-1 and HTLV-I that allows economical large-scale screening. *AIDS* (1989) **3**: 173-176.
2. Parry JV, Connell JA, Garcia AB, Avillez F, Mortimer PP. GACPAT HIV 1+2: a simple, inexpensive assay to screen for, and discriminate between, anti-HIV 1 and anti-HIV 2. *Journal of Medical Virology* (1995) **45**: 10-16.

Appendix 3: Supplementary laboratory protocol provided by Manufacturer

Minor grammatical changes have been made.



ASSAY PROCEDURE HIV-1:

Preliminary work:

Preparation of PBS buffer working solution: 30ml PBS + 970ml distilled water
Preparation of humidity chamber

Step 1:

Dilute controls and all specimens 1:30 (e.g. 5µl + 145µl), mix the samples by vortex and spin the samples at 12000 rpm for 3 minutes

No further dilution if you test dry blood spots!

Step 2:

Add 15µl of controls and specimens to both uninfected cell well and infected cell well

Position 1: positive control serum

Position 2: negative control serum

Step 3:

Incubate at 37°C for 30 minutes (± 10 minutes)

Step 4:

Rinse the slides with PBS working solution and carry out the first washing step (10 minutes ± 5 minutes) with PBS working solution

Prepare the FITC conjugate working solution (dilution 1:10: e.g. 15µl + 135µl) during the washing step (DO NOT SHAKE)

Step 5:

Dry the slides (by tapping), but be careful, don't let them dry up

Add 15µl of the FITC conjugate working solution (start with the uninfected cell wells)

Step 6:

Incubate at 37°C for 30 minutes (± 10 minutes)

Step 7:

Rinse the slides with PBS working solution and carry out the second washing step (10 minutes ± 5 minutes) with PBS working solution

Prepare the Counterstaining working solution (dilution 1:30 or **1:10**: e.g. 10µl + 290µl or 30µl + 270µl) during the washing step

Step 8:

Dry the slides (by tapping), but be careful, don't let them dry up

Add 10µl of the Counterstaining working solution (start with the uninfected cell wells)

Step 9:

Incubate at room temperature for 5 minutes

Step 10:

Rinse the slides with PBS working solution

Place one small drop of Mounting Media on each well and carefully fix the glass coverslip

(hold the coverslip with one finger strongly and wipe off the excessive fluid with absorbent paper
(DO NOT MOVE THE SLIDE))

Step 11:

Turn on the microscope 10 to 15 minutes before you evaluate the samples

(CHECK THE CORRECT ADJUSTMENT OF THE MICROSCOPE)

Evaluate the controls first (start with the infected cell well and go on to the uninfected one) and then evaluate the samples in comparison to the controls

Appendix 4: Details of specimens requiring repeat testing

Patient status	Specimen category	No. of category repeated	Final IFA result	Details	Details of repeat test profiles (final result) [†]	
HIV-1 pos	HIV-1 pos	3/102 (2.9%) (2.0% excluding possible human error)	2 x Pos 1 x Indet	High background, one with polar cap staining of infected and uninfected cell wells ^a One clearly positive on retest suggesting a possible user handling error ^b	-Nx2, lx4 (I) ^a -Nx2; Px4 (P) ^b -Px6 (P)	
	WB HIV-1 pos	10/35 (28.6%)	2 x Pos 4 x Indet 4 x Neg	8/10 specimens from suspected seroconverters, including one very cloudy specimen ^c (?poor quality) One reader changed reading upon second examination ^d	-Nx1, lx5 (I) -Nx3, lx2, Px1 (I) -Nx8 (N) ^c -Nx3(2), Px3(4) (P) ^d -Nx6 (N)	-Nx4, lx2 (N) -Nx1, Px5 (P) -Nx8 (N) -Nx4, lx4 (I) -Nx2, Px4 (P)
	WB HIV-1 indet	5/6 (83.3%) (100% including IFA neg specimen not retested)	5 x Neg	All specimens from suspected seroconverters A 6 th IFA neg specimen was not retested due to insufficient volume (Nx2)	-Nx6 (N) -Nx6 (N) -Nx6 (N)	-Nx4; lx1; Px1 (N) -Nx6 (N)
	WB HIV-1 neg	4/5 (75.0%) (100% including IFA neg specimen not retested)		2/3 specimens from suspected seroconverters; 1 from late-stage infection A 5 th IFA neg specimen was not retested due to insufficient volume (Nx2)	-Nx6 (N) -Nx6 (N) -Nx7, lx1 (N) -Nx4, Px2 (N)	
	HIV-1 pos - DBS	3/50 (6.0%)	1 x Pos 2 x Neg	2/3 specimens from suspected seroconverters	-Nx6 (N) -Nx6; lx1; Px1 (N)	-Nx1; Px3 (P)
HIV-1 neg	HIV-1 neg	5/100 (5.0%) (7%, including defective slides)	5 x Neg 2 x Indet	Mainly repeated due to high levels of background staining, some with polar cap staining in uninfected cell wells; 2 deficient cell well (test defective) ^e	-Nx3; lx3 (I) -Nx2 (well quality issue) (N) ^e -Nx2 (well quality issue) (N) ^e	-Nx7; lx1 (N) -Nx4 (N) -Nx5, lx3 (N) -Nx1; lx5 (I)
	WB HIV-1 indet	1/10 (10.0%)	1 x Neg	Polar cap staining in infected and uninfected wells (non-specific)	-Nx5; lx1 (N)	
	HIV-1 neg - DBS	3/50 (6.0%)	3 x Neg		-Nx3, lx1 (N) -Nx4; lx1; Px1 (N)	-Nx5; Px1 (N)
HIV-2 pos	HIV-2 pos (panel)	5/20 (25.0%)	2 x Pos 2 x Indet 1 x Neg	Non-specific staining observed	-Nx3; Px3 (I) -Nx3; lx2; Px1 (I) -Nx4; lx2 (N)	-Px6 (P) -lx1; Px5 (P)

† Each reading on each well is counted as a single result (every well read being by two readers); N= negative; I= indeterminate; P= positive;

Appendix 5: Details of specimens giving false negative/ indeterminate results

Patient status	Specimen category	IFA result	No. (category total)	Ab status	Seroconverter (suspected)	% of IFA failure attributable to seroconverters	Comments
HIV-1 positive	Antibody pos	Indet	1 (102)	1 x pos	0 (1)	100	2/4 false negative specimens from a single patient taken at different times
	WB HIV-1 pos	Neg	4 (35)	4 x pos	3 (1)	100	
		Indet	4 (35)	4 x pos	2 (1)	75	
	WB HIV-1 neg	Neg	5 (5)	5 x neg	1	25	4/5 specimens from the same patient taken at different times. This patient had a highly untypical anti-HIV evolution.
	WB HIV-1 indet	Neg	5 (6)	3 x neg, 2 x pos, 1 nk (prob neg)	5	100	
	DBS	Neg	2 (50)	1 x neg, 1 pos	2	100	
	TOTAL			21		16	76.2
HIV-1 negative	Antibody neg	Indet	2 (100)	2 x neg	0		

Ab: antibody; Indet: Indeterminate; Neg: negative; nk: not known
 From HIV-1 positive patients, 10/21 were HIV-antibody negative; 12 anti-HIV positive

A handwritten signature in black ink on a light gray background. The signature reads "Keith Perry" in a cursive, flowing script.

Signature of Principal Investigator, Dr Keith Perry, to confirm that this report is a fair representation of the work performed.