Fluorognost HIV1 IFA DBS (Dry Blood Spot) PROCEDURE

1 PURPOSE

1.1 This document describes the detailed technical procedure for testing dried blood spot specimens for antibodies to HIV-1 using the Fluorognost HIV-1 Immunofluorescence Assay.

2 SCOPE

2.1 This procedure applies to all specimens, which are EIA repeatedly reactive during screening for HIV-1 antibodies.

3 RESPONSIBILITY

3.1 It is the responsibility of the technician performing the Fluorognost HIV-1 Immunofluorescence Assay on specimens of "clients" to adhere to the requirements of this document.

3.2 It is the responsibility of the Laboratory Manager(s) to ensure that all technicians perform this confirmatory testing on all HIV-1 repeatedly reactive specimens according to the requirements of this document.

4 DEFINITIONS

4.1 None.

5 REFERENCES AND RELATED DOCUNMNTATION

5.1 *Waldheim Pharmazeutika Fluorognost HIV-1 IFA insert, May, 1992*.

5.2 *Vironostika HIV-1 Microelisa System EIA Procedure, 91-21-0492*.

5.3 *Procedure for HIV-1 Repeat Testing and Confirmation, 91-21-0504.*

6 MATERIALS AND EQUIPMENT

6.1 Refer to procedural section entitled "Additional Materials Required" (9.3.2).

7 HEALTH AND SAFETY

7.1 See "CAUTION", "PRECAUTION" and "WARNING" statements embedded throughout the procedural text.

8 DOCUMENTATION REQUIREMENTS

8.1 IFA QC Log, Attachment A.

8.2 IFA Incubation Log, Attachment B.

8.3 IFA Results Log, Attachment C.

8.4 Fluorescent Microscope Bulb Time Tracking Log, Attachment D

9 PROCEDURE

9.1 Principle

9.1.1 Intended Use

The Fluorognost HIV-1 Immunofluorescence Assay (IFA) is an in vitro, qualitative assay for the detection of antibodies to Human Immunodeficiency Virus Type 1 (HIV-1) in human serum or plasma or dried blood spot. It is intended to be used as an additional, more specific test for antibodies to HIV-1 in human serum or plasma specimens found to be repeatedly reactive by screening procedures such as the Enzyme-Linked Immunosorbent Assay (EIA). Properly trained personnel can also use Fluorognost HIV-1 IFA as a screening test in hospital laboratories, medical clinics, physician's offices and emergency care situations and in blood banks or other settings where enzyme immunoassays are not practical or available.

9.1.2 Summary and Explanation of the Test

Human Immunodeficiency Virus Type 1 (HIV-1) is the major etiologic agent of Acquired Immunodeficiency Syndrome (AIDS). Although there are two types of AIDS-causing viruses, namely HIV-1 and HIV-2, infections with HIV-2 outside of West Africa are uncommon. The Fluorognost HIV-1 IFA is designed to identify the presence of HIV-1 virus specific antibodies. The assay uses immortalized human T-cells, which express HIV-1 antigens on their surface. The cells are fixed to the surface of an IFA glass slide. Fixed, uninfected T-cells are provided as a control. When a serum or plasma sample with HIV-1 antibodies comes in contact with the HIV-1 antigens on the slide, antigen/antibody binding takes place. Bound HIV-1 specific antibodies are detected with anti-human immunoglobulin conjugated to fluorescein isothiocyanate, which binds human antibodies and fluoresces when exposed to UV light. The interpretation of the degree and pattern of fluorescence of the infected cells of the IFA slide compared to the uninfected cells determines the HIV-1 status of the sample. Persons demonstrating antibodies to I-HIV-1 should be referred for medical evaluation, which may include testing by other techniques. A clinical diagnosis of AIDS can be made only if a person meets the case definition of AIDS established by the Centers for Disease Control.

Some individuals infected with HIV-2 may have antibodies that will recognize and bind to viral antigens present on the Fluorognost HIV-1 IFA slides. However, the degree and pattern of fluorescence may vary, even after seroconversion. The Fluorognost HIV-1 IFA is not intended to identify antibodies to HIV-2 or to distinguish HIV-2 from HIV-1.

9.1.3 Principle of the Test

The Fluorognost HIV-1 IFA is a test for HIV-1 antibodies based on specific antibody binding to HIV-1 (HTLV-IIIB isolate) antigens expressed on the surface of immortalized human T-cells fixed to a glass slide. T-cells not expressing HIV-1 antigens serve as controls for each test. Slides are processed to inactivate any virus

associated with infected cells, including complete air dying, refrigerated acetone treatment to remove the lipid components of the virus, and dehydration through evaporation of the acetone solvent. This inactivation process fixes both the HIV-1 infected and uninfected cells to their respective cell wells on the glass IFA slide.

To perform the assay, a serum, plasma or dried blood spot specimen is diluted and placed in the infected and uninfected cell well of the IFA slide and incubated. If HIV-1 antibodies are present in the sample, it will bind to HIV-1 viral antigens present on the infected cell surface. Unbound material is removed by aspiration and washing. Antibody to human immunoglobulin conjugated with fluorescein isothiocyanate (FITC) is added to the HIV-1 infected and uninfected cell wells of the IFA slide, and again incubated. Unbound material is removed by aspiration and washing. A glass coverslip is mounted to the IFA slide and the slide is viewed under a microscope with UV light.

If antibodies to HIV-1 are present in the serum, plasma, or dried blood spot specimen, a characteristic pattern of fluorescence will become visible under ultraviolet light. The interpretation of this fluorescence is evaluated by comparing and differentiating the pattern and intensity of fluorescence in the uninfected and infected cell well for each sample.

9.2 Specimen Storage

Studies indicate that completely dried blood spot specimens may be stored frozen (<-5°C), refrigerated (2-8°C), at controlled room temperature (17-23°C), or elevated temperatures (37°C) for up to three months as long as they are not exposed to elevated humidity. Although specimens stored at 37°C for up to three months did not exhibit a detectable loss in reactivity, routine storage at elevated temperatures is not recommended. If specimens are stored for longer than three months or at any conditions other than the ones listed above, the user must validate the stability of the specimens under those storage conditions. In experiments in which dried blood spot specimens were stored at elevated temperature and humidity (37°C, 60% relative humidity), a marked decrease in reactivity was noted after two weeks of exposure to these conditions. If specimens are to be stored in a humid environment, the user should include a desiccant and validate the stability of the specimens.

9.3 Reagents

9.3.1 Reagent Description

The Fluorognost HIV-1 IFA is available as a 10-, 25-, 50- and 100-Test Kit.Component	100-Test Kit	50-Test Kit	25-Test Kit	10-Test Kit
1. **IFA Slides** have inactived HIV-1 infected and uninfected control cell wells capable of detecting the presence or absence of HIV-1 antibodies	20 IFA slides, each containing parallel rows of 5 HIV-1 infected wells numbered (1i-5i) and 5 uninfected wells (1-5)	10 IFA slides, each containing parallel rows of 5 HIV-1 infected wells numbered (1i-5i) and 5 uninfected wells (1-5)	5 IFA slides, each containing parallel rows of 5 HIV-1 infected wells numbered (1i-5i) and 5 uninfected wells (1-5)	2 IFA slides, each containing parallel rows of 5 HIV-1 infected wells numbered (1i-5i) and 5 uninfected wells (1-5)
2. **Concentrated Diluent** consisting of 9.55 grams of sterile phosphate buffered sodium chloride in a sealed glass vial to make 1 liter of phosphate buffered saline (PBS) pH7.3.	One sealed vial containing 9.55 grams of sterile phosphate buffered saline for 1 liter of PBS.	One sealed vial containing 9.55 grams of sterile phosphate buffered saline for 1 liter PBS.	One sealed vial containing 9.55 grams of sterile phosphate buffered saline for 1 liter PBS.	One sealed vial containing 9.55 grams of sterile phosphate buffered saline for 1 liter PBS.
3. **Concentrated FITC-Conjugate** consisting of 300 µl of a concentrated solution of fluorescein isothiocynate conjugated with goat Anti-Human IgG, in a plastic vial with green color-coding. To be diluted with PBS for a working dilution.	One plastic vial containing 300 µl of Concentrated FITC Conjugate with green color-coding on the cap.	One plastic vial containing 300 µl of Concentrated FITC Conjugate with green color-coding on the cap.	One plastic vial containing 300 µl of Concentrated FITC Conjugate with green color-coding on the cap.	One plastic vial containing 300 µl of Concentrated FITC Conjugate with green color-coding on the cap.
4. **Mounting Media** consisting of a 3.0 ml solution of ready to use PBS buffered glycerol in a plastic dispenser bottle with cap.	One plastic dispenser bottle containing 3.0 ml of PBS buffered glycerol.	One plastic dispenser bottle containing 3.0 ml of PBS buffered glycerol.	One plastic dispenser bottle containing 3.0 ml of PBS buffered glycerol.	One plastic dispenser bottle containing 3.0 ml of PBS buffered glycerol.
5. **Negative Serum Control** consisting of 20 µl of heat	One plastic vial containing 20 µl of	One plastic vial containing 20 µl of Negative Serum	One plastic vial containing 20 µl of Negative Serum	One plastic vial containing 20 µl of Negative Serum

inactivated, human serum, negative for antibody to HIV-1. Non-reactive for Hepatitis B surface antigen and antibody to Hepatitis C virus. Preserved with 0.1% sodium azide in a plastic screw cap vial. To be diluted with PBS Diluent.	Negative Serum Control.	Control.	Control.	Control.
6. **Positive Serum Control** consisting of 20 µl of heat inactivated, human serum, positive for antibody to HIV-1. Non-reactive for Hepatitis B surface antigen and antibody to Hepatitis C virus. Preserved with 0.1% sodium azide in a plastic vial with **a RED Screw Cap**. To be diluted with PBS Diluent.	One plastic via with a **RED CAP** containing 20 µl of Positive Serum Control.	One plastic via with a **RED CAP** containing 20 µl of Positive Serum Control.	One plastic via with a **RED CAP** containing 20 µl of Positive Serum Control.	One plastic via with a **RED CAP** containing 20 µl of Positive Serum Control.
7. **Glass Coverslip** package consisting of 5 Coverslips. One Coverslip is used for each IFA slide.	Four packages of Coverslips, each containing 5 Coverslips (total of 20).	Two packages of Coverslips, each containing 5 Coverslips.	One packages of Coverslips, each containing 5 Coverslips.	One packages of Coverslips, each containing 5 Coverslips.

9.3.2 Additional Materials Required

- Dilution Tubes - Dilution tubes, 12mm x 75mm (3"), with screw cap or stopper are recommended.
- Micropipettes - Micropipettes with a volume range of 1-20 ìl are recommended.
- Squeeze Bottle – This accessory is helpful for rinsing the IFA slides with PBS Diluent, for washing steps.
- Slide Holder - This accessory is helpful for immersion of the IFA slides into the PBS Diluent and for separation between the slides.
- Rotator or Shaker.
- Incubator - an incubator at 37°C is required.
- Humidity Chamber - a 6-7" Petri dish with cover can be used with a wetted layer of cloth or filter paper serving as the water reservoir. The kit container can also be used. Allow the humidity chamber to equilibrate for 30 minutes at 37°C.
- Distilled Water - Distilled water is required for preparation of the PBS Diluent.

- Graduated Cylinder- A graduated cylinder with a volume of at least 1.0 liter is required for the preparation/storage of the PBS Diluent.
- Fluorescent Microscope - A fluorescent microscope with an epifluorescent attachment using incident light fluorescence is required with the following hardware:
    - Light Source: Mercury Vapor Lamp 50-200W
    - Excitation Filter: KP 500
    - Splitting Mirror: TK510
    - Barrier Filter: K510 or K530
    - Suppression Filter: BG-38
- Vacuum Pump - A vacuum pump or other aspiration device is needed to remove liquid from the IFA slide wells. A chlorine bleach trap should be attached to the vacuum line.
- Timer - A timer capable of measuring 10 minutes and 30 minutes is recommended.

9.3.3 Storage

9.3.3.1 Store the Fluorognost HIV-1 IFA kit and individual reagents at 2-8°C.

9.3.4 Indications of Instability or Deterioration of Reagents

9.3.4.1 Changes in the physical appearance of the reagents supplied may indicate instability or deterioration of these materials. DO NOT use any reagent, which is cloudy and/or reveals microbial growth.

9.3.5 CAUTIONS

9.3.5.1 HANDLE PATIENT SAMPLES, IFA SLIDES AND FLUOROGNOST IFA SERUM CONTROLS AS IF CAPABLE OF TRANSMITTING AN INFECTIOUS AGENT.

9.3.5.2 HIV-1 infected T-cells have been fixed onto the infected cell wells of the IFA slide and virally inactivated by acetone and dehydration. The NEGATIVE AND POSITIVE SERUM CONTROLS have been virally inactivated by heat treatment. In addition, serum used to produce the CONTROLS was shown to be non-reactive for Hepatitis B surface antigen and antibodies to Hepatitis C virus. However, no known test method can offer assurance that products derived from human blood will not transmit infectious agents. Therefore, these components must be handled as if they are capable of transmitting infectious agents.

9.3.5.3 DO NOT pipette by mouth. Avoid splashing or forming aerosols with samples.

9.3.5.4 Wear disposable gloves through the test procedure. Dispose of gloves as biohazardous waste. Thoroughly wash hands after handling test reagents.

9.3.5.5 DO NOT smoke, eat or drink in areas where specimens or kit reagents are handled.

9.3.5.6 Wipe spills promptly with a 10% dilution of liquid household bleach and allow area to stand for at least 5 minutes for disinfection.

9.3.5.7 Dispose of all specimens and materials used in the Fluorognost HIV-1 IFA procedure as biohazardous waste. The recommended method of solid waste disposal is autoclaving for a minimum of 1 hour at 121°C. Disposable materials may be incinerated. Contaminated liquid materials should be mixed with a 10% dilution-of household bleach. Allow the mixture to stand for at least-5 minutes for disinfection prior to disposal.

- WARNING: DO NOT AUTOCLAVE LIQUIDS CONTAINING BLEACH.

9.3.5.8 The Positive and Negative Serum controls contain sodium azide as a preservative. Azide may react with lead or copper plumbing to form potentially explosive metal azides; use caution [as follows]. When disposing of these materials through a sink or other common plumbing system, always flush with generous amounts of water to prevent metal azide buildup. For further information, consult the manual.

- Safety Management No. CDC-22, "Decontamination of Laboratory Sink Drains to Remove Azide Salts " (Centers for Disease Control, Atlanta, GA, April 3 0, 1976).

9.3.5.9 Clamp all hoses firmly to the vacuum pump and traps to prevent separation during aspiration.

9.3.6 PRECAUTIONS

9.3.6.1 DO NOT INTERCHANGE REAGENTS BETWEEN KIT LOTS.

9.3.6.2 DO NOT use kit beyond its expiration date. The date is printed on kit boxes.

9.3.6.3 Bring all kit reagents and test specimens to room temperature (17-23°C) before use (approximately 30 minutes).

9.3.6.4 Keep all opened reagents refrigerated (2-8°C) when not in use.

9.3.6.5 Use aseptic technique when opening and withdrawing aliquots from the reagent vials. Avoid microbial contamination and cross-contamination of reagents. DO NOT use reagents, which are cloudy and reveal microbial growth.

9.3.6.6 Avoid the use of test specimens which are contaminated by microbial growth, or which have been frozen and thawed more than twice. See “Specimen Collection and Storage” section for details.

9.3.6.7 It is recommended that a separate pipette tip be used for each specimen and each reagent to avoid cross-contamination. In particular, trace contamination of the Negative Control, or any specimen not containing antibodies to HIV-1, will give rise to erroneous false positive results when compared with the Positive Control. Contamination of the FITC Conjugate by any human serum will cause partial or complete neutralization of this Conjugate and may give rise to erroneous false negative results.

9.3.6.8 DO NOT interchange vial or bottle caps and stoppers; this will lead to cross-contamination of reagents. For ease of use, the Positive Serum Control is color coded with RED labeling and has a RED screw cap. The Negative Serum Control has blue labeling and a clear screw cap. Always designate separate reservoirs for specific reagents and specimens and/or their dilutions.

9.3.6.9 Handle the Fluorognost HIV-1 IFA slides by the rim or by the frosted end of the slide only. Never touch the IFA slide cell wells where HIV-1 infected and uninfected cells have been attached. Handle the sealed individual slide sachets without applying pressure to the labeled side of the slide.

9.3.6.10 Use distilled water to dilute reagents in order to avoid substances which may interfere with the assay.

9.3.6.11 Adherence to the procedures outlined within the product insert in the kit or in the Instructions for Laboratory Training and Qualification Brochure is necessary for the successful use of this product. When properly conducted, the Fluorognost HIV-1 IFA will accurately demonstrate the presence or absence of antibodies to HIV-1. While this is a criterion for infection by the HIV-1 virus, it must not be considered as equivalent to a diagnosis of the Acquired Immunodeficiency Syndrome (AIDS). Such a diagnosis can only be made by a physician according to accepted medical criteria.

9.3.6.12 Wash hands thoroughly after handling test reagents and on completion of the assays.

9.4 Procedure

9.4.1 Critical Steps and Cautions

9.4.1.1 DO NOT shake the FITC-Conjugate. Gently mix the working dilution of conjugate.

9.4.1.2 Cross-contamination of the FITC-Conjugate by human serum will cause partial or complete neutralization of the conjugate and may give rise to false negative assay results.

9.4.1.3 Separate pipette tips should be used for each specimen and each reagent to avoid cross-contamination. Trace contamination of the Negative Serum Control or any specimen not containing antibodies to HIV-1 will give rise to erroneous false positive results.

9.4.1.4 Use only serum or plasma or DBS test specimens with the Fluorognost HIV-1 IFA.

9.4.1.5 DO NOT contact the IFA slide cell layer with the pipette tip.

9.4.1.6 Switch the UV microscope mercury vapor lamp on at least 15 minutes before using.

9.4.1.7 7 UV light will cause FITC-Conjugate fluorescence to fade with time. Be careful not to expose each microscopic field to UV light for over 15-30 seconds.

9.4.1.8 It is strongly recommended that the IFA slides be evaluated immediately after the mounting procedure is completed.

9.4.1.9 If the image appears to be dim, check to make sure that the beam barrier has been fully withdrawn and your eyes are adjusted to darkness. If image brightness is still too low, use a lens with a higher aperture.

9.4.1.10 Make sure that the UV microscope, filters, objectives and light sources are operating properly and that the microscope attachments are adjusted and aligned correctly.

9.4.2 Assay Setup

9.4.2.1 All procedural steps should be followed precisely as written. Failure to do so may result in aberrant test results.

9.4.2.2 Bring all reagents to room temperature (15-23°C) before use (approximately 30 minutes

9.4.2.3 Record the lot number and expiration date of the kit on the IFA QC Log. Test specimen codes and results should also be recorded on the log.

9.4.2.4 Construct a humidity chamber as described in the section "Additional Materials Required."

9.4.2.5 Turn on the mercury vapor lamp of the fluorescence microscope at least 15 minutes before the first observation. The UV microscope should be fitted with the hardware and accessories described in the section of the product insert titled "Additional Materials Required. Not Supplied."

9.4.2.6 Connect a vacuum pump and chlorine bleach trap or other aspiration device to remove liquid waste from the IFA slides.

9.4.3 Preparation of Reagents

9.4.3.1 Phosphate-Buffered Saline Diluent: Completely dissolve contents of the Concentrated PBS Diluent Container in 1.0 liter of distilled water. Mix well.

CAUTION: The PBS Diluent does not contain a preservative. To prolong the integrity of the PBS Diluent, prepare PBS Diluent with sterile water. After each use, sterile filter the Diluent into a new sterile container before storing at 2-8°C. Discard if there are visible signs of microbial contamination. Do not reuse the reconstituted Diluent after 8 months.

9.4.3.2 FITC-Conjugate

9.4.3.2.1 For each slide, dilute 30 ìl of FITC-Conjugate (green screw cap) with 270 ìl of PBS diluent. Gently mix. DO NOT SHAKE.

9.4.3.2.2 Avoid exposing the FITC-Conjugate to direct sunlight extremely bright illumination.

9.4.3.2.3 Store at 2-8°C for subsequent tests.

9.4.3.3 Serum Controls

9.4.3.3.1 For each batch of IFA testing, dilute 5 ìl of Positive Serum Control (RED screw cap) with 145 ìl of PBS diluent to form a working dilution. Gently mix by inverting the closed vial several times.

9.4.3.3.2 For each batch of IFA testing, dilute 5 ìl of Negative Serum Control with 145 ìl of PBS diluent to form a working dilution. Gently mix by inverting the closed vial several times.

9.4.3.3.3 Store at 2-8ºC.

9.4.4 Dilution of Serum Test Specimens

9.4.4.1 Test Specimen Working Dilution For Serum: Add 300 ìl of PBS diluent to 10 ìl of test specimen in a tube with a conical bottom and cap to prepare working dilution. Use on the day of dilution.

9.4.4.2 Elution Procedure For Use Of Dried-Blood Spot Samples (controls or specimens)

9.4.4.2.1 Use a clean paper punch to prepare 1/4" (.05 sq. inch) disks from a uniformly spotted section of a completely dried DBS specimen. See Procedure For Storage And Retrieval Of Post-Test HIV-1 Specimens, Section 9.2. Start punching at the center of the spot, and store the remaining DBS in bag with a desiccant. One punched disk is sufficient for several Fluorognost assays.

9.4.4.2.2 Place each disk in a small (1-2 ml) disposable tube.

9.4.4.2.3 Add 250 ìl of working dilution prepared from Fluorognost HIV-1. Concentrated Diluent (or PBS w/o Ca and Mg, pH 7.3) to each tube. Take care that the buffer covers the disk completely.

9.4.4.2.4 Elute at 2-8°C overnight, or for 2 hours with vigorous agitation (e.g., on a rotator) at room temperature. Record times on IFA Incubation Log, Attachment B.

9.4.4.2.5 After elution, use a pipette tip to remove 15 ìl (0.015 ml) from the reddish eluate and to transfer this volume directly to an infected cell well of a Fluorognost HIV-1 slide. Repeat this to transfer an identical volume to the corresponding uninfected cell well. Avoid disturbing the pellet. The vessel with the disk and the remaining eluate can be stored for one day at 2-8°C. DO NOT freeze the eluate. Eluates from other methods can also be used provided the dilution factor is 1:400 or less.

9.4.5 Assay Procedure

9.4.5.1 Remove the number of Fluorognost HIV-1 IFA slides required for the planned testing from the kit container and label each slide with current date, tech initials and slide number. Although each IFA slide can process five specimens, each run must be validated by the use of one Positive and one Negative Serum Control. Hence, the kits of 10, 25, 50 and 100 tests can process maximum 8, 23, 48 and 98 test specimens respectively.

9.4.5.2 Each IFA slide contains two parallel rows of cell wells. The HIV-1 infected cell wells (Ii-5i) are surrounded by RED Teflon coating and the uninfected control cell wells (1-5) are surrounded by a BLUE Teflon coating. A dilute test specimen is placed in the corresponding HIV-1 infected and uninfected cell wells.

9.4.5.3 Place 15 ìl of the diluted positive serum control in the cell well labeled "1" (uninfected cells in BLUE colored lower row of the IFA slide). Place 15 ìl of the same control in the cell well labeled "1i" (infected cells in RED colored upper row of the IFA slide).

9.4.5.4 Place 15 ìl of the diluted Negative Serum Control in the cell well labeled "2" (uninfected cells in BLUE colored lower row of the IFA slide). Place 15 ìl of diluted negative serum control in cell well labeled "2i" (infected cell well in RED colored upper row of the IFA slide. If Serum Controls are placed in a different arrangement, be sure to place the Negative Serum control between any test specimen and the Positive Control. Correspondingly place 15 ìl of DBS positive control eluate in well "3" then "3i" and 15 ìl of DBS negative control eluate in well "4" then "4i".

9.4.5.5 Add 15 µl of the first diluted test specimen to the cell labeled “5” (uninfected cells in BLUE colored lower row of the IFA slide). Add 15 µl to the cell well labeled “5i” (infected cell well in the RED colored upper row of the IFA slide). Additional test specimens should be placed in corresponding sequential wells.

CAUTION: CAREFULLY EXAMINE THE SLIDE TO BE SURE THAT SAMPLE WAS ADDED TO EACH WELL. DO NOT CONTACT THE CELL LAYER WITH THE PIPETTE TIP AND DO NOT ALLOW THE CELL WELLS TO DRY.

9.4.5.6 Following specimen application, transfer the IFA slide to the humidity chamber and incubate at 37 ± 2°C for 30 minutes. Record times on the IFA Incubation Log (Attachment B).

CAUTION: DO NOT TILT OR SHAKE THE SLIDE DURING TRANSFER TO AND FROM THE HUMIDITY CHAMBER OR INCUBATOR AS CROSS-CONTAMINATION CAN OCCUR WHICH MAY LEAD TO FALSE POSITIVE RESULTS.

9.4.5.7 Following incubation, carefully remove the slide from the humidity chamber and replace humidity chamber in incubator. Thoroughly aspirate each of the cell

wells of the IFA slide on a level surface. Be careful not to scratch the cell well surfaces.

9.4.5.8 First Wash - Briefly rinse the IFA slide with a squeeze bottle containing PBS diluent and immediately place the IFA slide into the slide holder containing a fresh volume of PBS diluent. Make sure that all cell wells on the IFA slide are submerged. Then place the slide holder on a rocking or rotary platform for 10 minutes at room temperature. Record Times on the IFA Incubation Log, (Attachment B).

9.4.5.9 Following the first washing step, remove the slide from the slide holder and carefully tap off the liquid from each cell well and/or blot the surrounding area, making sure that the entire surface of the IFA slide is free of excess fluid.

9.4.5.10 Place 15µl of the diluted FITC-Conjugate in each IFA slide well position containing a test specimen or Serum Control.

9.4.5.11 Transfer the IFA slide to the humidity chamber and incubate at 37 ± 2° C for 30 minutes. Record Times on the IFA Incubation Log, (Attachment B).

NOTE: THERE IS NO FURTHER RISK OF CROSS-CONTAMINATION.

9.4.5.12 Following incubation, remove the slide from the humidity chamber and aspirate the liquid from each of the cell wells.

9.4.5.13 Second Wash - Briefly rinse the IFA slide with a squeeze bottle containing PBS diluent and immediately place the IFA slide into the slide holder containing a fresh volume of PBS diluent. Make sure that all cell wells on the IFA slide are submerged. Then place the slide holder on a rocking or rotary platform for 10 minutes at room temperature. Record Times on the IFA Incubation Log, (Attachment B).

CAUTION: DO NOT ALLOW THE IFA SLIDE TO DRY

9.4.5.14 Following the second washing step, remove the slide from the slide holder and carefully tap off all excess liquid off of the IFA slide and/or blot the surrounding area.

9.4.5.15 Place one drop of Mounting Media in each well to insure that each cell well is covered. Align and cover each IFA slide with a coverslip (one coverslip per slide). Gently press down on the coverslip making sure there are no air bubbles. For correct microscopic evaluation of the IFA cell wells the Mounting Media MUST completely fill the space between each IFA slide cell well position and the coverslip.

9.4.5.16 At this point, the IFA slide is ready for evaluation and interpretation of the assay results. It is strongly recommended that the IFA slide(s) be evaluated immediately after the mounting procedure is complete.

CAUTION: THE IFA SLIDES MUST BE CAREFULLY PROTECTED FROM BRIGHT LIGHT TO AVOID A GRADUAL BLEACHING OF THE FLUORESCEIN.

9.5 Quality Control

The Fluorognost HIV-1 IFA Positive and Negative Serum Controls included in the kit and the dried-blood spot controls must be included with each run, regardless of the number of specimens tested. To validate the IFA run, first examine the Negative Serum Control, inspecting both the uninfected and HIV-1 infected cells in that order. Then examine the Positive Serum Control inspecting the uninfected and infected cells, as above. The following is a description of the expected appearance of the Serum Controls.

9.5.1 Negative Serum Control

9.5.1.1 The contours of the individual cells in the Negative Control Serum in both the uninfected BLUE cell well "2" and the HIV-1 infected RED cell well "2i" should be barely visible and cytoplasm of the cells must not exhibit any specific fluorescent staining greater than the surrounding cellular matrix. The appearance of the uninfected and infected cells of the Negative Control should be virtually indistinguishable.

9.5.2 Positive Serum Control

9.5.2.1 The uninfected cells located in the BLUE cell well "1" must not exhibit any specific cytoplasmic staining greater than the surrounding cellular matrix and should have an appearance similar to that seen with the Negative Serum controls located in the BLUE cell well "2" and the RED cell well "2i." In contrast, the HIV-1 infected cells of the Positive Control located in RED cell well "1i" should exhibit an intense apple green staining of the cytoplasm which can be easily differentiated from the uninfected cells.

9.5.2.2 As an additional internal control, the HIV-1 infected cell well is a "mixed cell environment" containing 40-60% infected PALL T-cells mixed with uninfected PALL T-cells. As a result, the uninfected cells within cell well of the Positive Control will not exhibit specific fluorescent staining greater than the surrounding cellular matrix.

9.5.3 Dried-Blood Spot Controls

9.5.3.1 The dried-blood spot controls should exhibit the staining according to the respective controls, negative and positive. The serum controls and the DBS controls must exhibit expected staining in order for run to be qualified. Refer to Attachment A for recording of DBS controls and serum controls.

9.5.4 Indications of Instability or Deterioration of the IFA Slide

9.5.4.1 The Negative Serum Control must not exhibit any specific fluorescent staining in either the HIV-1 infected or the uninfected cells. The Positive Serum Control must demonstrate a typical positive cytoplasmic fluorescence pattern in the HIV-1 infected cells. The uninfected cell well of the Positive Serum Control must

NOT exhibit any specific fluorescent staining and should have an appearance similar to that seen with the Negative Serum Control. If any IFA result is not in accordance with the aforementioned criteria, deterioration of the Fluorognost HIV-1 IFA slides, the Positive or Negative Serum Control, or the FITC-Conjugate may be suspected. Any kit showing deviation from the criteria, or showing an atypical fluorescence pattern with the Positive and/or Negative Serum Control should NOT be used

9.5.5 Result Validity.

9.5.5.1 A test run is valid if both sets of controls, the dried blood spot controls and kit serum controls, are qualified. If the test run is valid, all results are validated.

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9.5.5.2 If the positive and negative kit controls are qualified, but one or both dried blood spot controls are not, an investigation should be launched whether the run was not performed in accordance with the requirements stated in this and referenced procedures. This includes correct performance of the test procedure, proper reagent quality (within-date, no evidence of contamination) and equipment that meets specs.

9.5.5.3 If it is established that all requirements for the run were met, except for dried blood controls, reactive results are validated, but non-reactive results are not. In this case all non-reactive results should be requeued for another run.

9.5.5.4 All test results should be invalidated if by investigation it is determined that the run was not performed in accordance with the requirements of this procedure.

9.6 Interpretation of Validated Results

9.6.1 The presence or absence of antibodies to HIV-1 in a test specimen is determined by the subjective comparison and differentiation of the intensity and the pattern of fluorescence between the uninfected control cells and the HIV-1 infected cells. The IFA test result is then interpreted as NEGATIVE, POSITIVE, OR INDETERMINATE. Record results on the IFA Results Sheet, (Attachment C).

9.6.2 Evaluation and interpretation of a test should be carried out for each test specimen in the following sequence:

9.6.2.1 Evaluate the UNINFECTED CONTROL CELLS (BLUE) of the test specimen for fluorescent staining. There should not be any specific cytoplasmic staining greater than the surrounding cellular matrix and the cells should be similar in appearance to the uninfected and infected cells of the Negative Serum Control (BLUE cell well "2" and RED cell well "2i") and the uninfected cells of the Positive Serum Control (BLUE cell well "i").

9.6.2.2 Evaluate HIV-1 INFECTED CELLS (RED) of the test specimen for fluorescent staining. Use the interpretation criteria (9.2.2.1 and 9.2.2.2, below) to evaluate and interpret the IFA test result.

9.6.3 Negative Result Interpretation Criteria

9.6.3.1 A test specimen in interpreted as NEGATIVE when there is no specific fluorescent staining of the infected cells and there is no significant difference in the intensity of fluorescent staining and the pattern of fluorescence between the HIV-1 infected and uninfected cells. The test specimen is reported as negative.

9.6.3.2 Description of Negative IFA Result: In test specimens having a NEGATIVE IFA result interpretation, both the uninfected and infected cells of the test specimen will have an appearance similar to the uninfected and infected cells of the Negative Control and the uninfected cells of the Positive Serum Control.

9.6.4 Positive Result Interpretation Criteria

9.6.4.1 A test specimen is interpreted as POSITIVE when there is a specific cytoplasm staining pattern in the HIV- infected cells and there is a significant difference in the intensity of fluorescent staining and the pattern of fluorescence between the HIV-1 infected and uninfected cells. The test specimen is reported as positive.

9.6.4.2 Description of Positive IFA Result: Positive HIV-1 infected cells will exhibit a concentrated fluorescence in the cytoplasmic of the cell. Because the PALL T-cell nucleus occupies a large portion of the cell volume, the cytoplasm tends to be compressed into a dense acentric structure, which is localized at one end of the cell. A positive cytoplasmic pattern can vary in appearance from an acentric "half moon" to a "poplar cap," depending on the orientation of the T-cell on the slide well. A positive pattern can range from diffuse to finely reticulated and the intensity can vary from a very intense brilliant apple-green color to a less intense apple-green color. The infected cell well contains 40-60% infected cells, often termed a "mixed cell" environment. Therefore, a positive specimen will exhibit specific fluorescence in at most 40-60% of the cells. The remaining cells are uninfected and serve as a built-in internal control and should have an appearance similar to the Negative Serum Control. **CAUTION**: It is ALWAYS NECESSARY to evaluate both the uninfected cell well and the infected cell well before interpreting the final IFA result. Be sure to scan a range of 3-5 microscopic fields within each cell well before completing the evaluation.

9.6.5 Indeterminate Result Interpretation Criteria

9.6.5.1 A test specimen is interpreted as INDETERMINATE when:

- There is fluorescent staining present IN BOTH the HIV-1 infected and uninfected cells, or
- when it is NOT possible to clearly differentiate the intensity of fluorescent staining and the pattern of fluorescence between the HIV-1 infected and uninfected cells, or
- when duplicates are discordant. The test specimen is reported as INDETERMINATE and repeat testing of the original specimen should be carried out in duplicate. If an INDETERMINATE result persists, it may be necessary to obtain a fresh test specimen for follow-up testing.

9.6.5.2 The INDETERMINATE IFA interpretation does not imply that HIV-1 antibodies are, or are not, present in the test specimen. It simply means that the HIV-1 status of the serum or plasma or DBS specimen cannot be resolved through the

use of that specific Fluorognost HIV-1 IFA test run. INDETERMINATE ASSAY RESULTS MUST NOT BE CONSIDERED POSITIVE OR NEGATIVE. The correct evaluation, in such situations, must be based on subsequent repeat testing and/or immunoblot testing and clinical evaluation.

9.6.5.3 Description of Indeterminate IFA Result: In most cases, INDETERMINATE IFA results are due to the presence of non-specific staining. In specimens with non-specific staining reactions, the fluorescence intensity can vary from very weak to very intense and staining will be exhibited in BOTH the infected and uninfected cells. When the specimen reacts equally with both the infected and uninfected cells, the IFA interpretation result must be regarded as inconclusive and reported as INDETERMINATE. Non-specific staining can be categorized as cellular and/or extracellular and can occur as a result of a variety of conditions and from a number of sources. Cellular and extracellular staining can also occur in the same sample. The following is a brief description of typical non-specific staining reactions:

- ***Non-specific Cellular Staining***: Non-specific cellular staining can occur when antibodies from the test sera bind with non-HIV-1 protein in both the HIV-1 infected and uninfected control cells. For example, sera from patients with Systemic Lupus Erythematosus (SLE) can produce intense cell membrane staining without cytoplasmic staining. Sera that possess antinuclear antibodies (ANA) react with the nucleus but not the cytoplasm of the cells. Some sera stain the cytoplasm of the cells and often display an appearance of a “polar cap".

- ***Non-specific Extracellular Staining***: Non-specific extracellular staining can appear in a wide variety of patterns including an amorphous film, droplets, particulate matter, bacterial/fungal contamination and dead cells. This type of staining generally does not interfere with the interpretation of the IFA results long as the positive or negative result criteria are fulfilled.

9.6.5.4 It is important to note that it is also possible for a test specimen to present specific HIV-1 staining in the presence of non-specific staining. A specimen for which the positive interpretation criteria are clearly fulfilled is reported as POSITIVE. For some specimens, the non-specific staining may mask the presence of specific HIV-1 staining and hence, those samples should be reported as INDETERMINATE. If the IFA reader does not have confidence in the IFA interpretation, the test result should be considered INDETERMINATE and follow-up testing with the original specimen should be carried out as outlined above. Caution must be exercised with sera containing microbial contamination since bacteria/fungus may reduce or eliminate antibody titer to HIV-1 and lead to a false-negative interpretation.

9.7 Limitations Of The Procedure.

9.7.1 The Fluorognost HIV-1 IFA protocol and the criteria for interpretation of the Fluorognost HIV-1 IFA test results must be performed in strict accordance with these instructions to obtain accurate results.

9.7.2 Insufficient data are available to support use of Fluorognost HIV-1 IFA with individual body fluid specimens other than serum or plasma or DBS. Similarly, use of the Fluorognost HIV-1 IFA with pooled or processed plasma and products made from such pools has not been evaluated, and testing of such specimens is not recommended.

9.7.3 While no specific group of specimens prone to false positive or false negative results can be identified, INDETERMINATE Fluorognost HIV-1 IFA results can be expected to occur with a slightly higher frequency in specimens from patients with certain autoimmune diseases such as Systemic Lupus Erythematosus, or in severe paraproteinaemias as seen in certain types of leukemias. On the other hand, viral infections such as hepatitis, cytomegalovirus, and Epstein-Barr virus have been demonstrated to be without significant influence on the results of Fluorognost HIV-1 IFA. Hyperlipemic or extremely icteric test specimens may cause evaluation problems resulting from the precipitation of specimen components onto the IFA slide. In addition, specimens heavily contaminated with bacteria may show atypical background fluorescence and may cause proteolytic degradation of the antibody to HIV-1. The testing of these samples is not recommended and every attempt should be made to obtain another specimen without those characteristics.

9.7.4 The intensity of the fluorescence observed in HIV-1 positive specimens does not bear a strict correlation to the antibody titer, or to the presence of antibody directed against particular HIV-1 antigens.

9.7.5 Although a POSITIVE IFA for antibodies to HIV-1 usually indicates infection with the virus, a diagnosis of Acquired Immunodeficiency Syndrome, AIDS, can only be established on clinical grounds, provided that an individual meets the case definition of AIDS established by the Centers for Disease Control.

9.7.6 An individual with a POSITIVE IFA for antibodies to HIV-1 should be referred for medical evaluation, which may include additional testing. The clinical implications of antibodies to HIV-1 in an asymptomatic detectable HIV-1 in their blood cells, and may develop Immunodeficiency with the passage of time.

9.7.7 Due to variations in test performance and the uncertainty associated with INDETERMINATE IFA results, it is recommended that all INDETERMINATE samples be retested using the original specimen. A fresh specimen may also be requested for follow-up testing. The correct evaluation in such situations must be based on subsequent repeat testing and Immunoblot and clinical evaluation.

9.7.8 Fluorognost HIV-1 IFA clinical studies have demonstrated that there is a high degree of assay reproducibility and that IFA reader disagreements are uncommon. However, in the event that more than one reader participates in the interpretation of the IFA and/or a final result cannot be confidently determined by the IFA reader(s), the assay must be repeated using the original specimen.

9.7.9 A NEGATIVE IFA does not exclude the possibility of infection with HIV-1. Antibody testing should not be used in lieu of blood donor exclusion and self-exclusion procedures.

10 ATTACHMENTS

10.1 Attachment A, IFA QC LOG

10.2 Attachment B, IFA INCUBATION LOG

10.3 Attachment C, IFA RESULTS LOG

10.4 Attachment D, Fluorescent Microscope Bulb Time Tracking Log

ATTACHMENT A

IFA QC LOG

	Serum Pos	Serum Neg	DBS Pos	DBS Neg	
Lot #					
Acceptable Range					
Date					**Tech ID**

Reviewed By: _____ Date:_____

ATTACHMENT B

IFA INCUBATION LOG

Elution Date: _____
Testing Date: _____

Elution **Overnight at 2-8ºC or 2 hour; shake at 17-23ºC (RT)**

Tube #	Time In /Temp	Time Out	Tech ID

Sample Incubation **37 ± 2ºC for 30 minutes**

Slide #	Time In/Temp	Time Out	Tech ID

First Wash **17 – 23ºC (RT) for 10 minutes**

Slide #	Time In/ Temp	Time Out	Tech ID

Conjugate **37 ± 2ºC for 30 minutes**

Slide #	Time In/Temp	Time Out	Tech ID

Second Wash **17 – 23ºC (RT) for 10 minutes**

Slide #	Time In/Temp	Time Out	Tech ID

Reviewed By: _____ **Date: _____**

ATTACHMENT C

IFA RESULTS LOG

Slide Number:				
Sample #	**Sample #**	**Sample #**	**Sample #**	**Sample #**
Result	**Result**	**Result**	**Result**	**Result**

Slide Number:				
Sample #	**Sample #**	**Sample #**	**Sample #**	**Sample #**
Result	**Result**	**Result**	**Result**	**Result**

Slide Number:				
Sample #	**Sample #**	**Sample #**	**Sample #**	**Sample #**
Result	**Result**	**Result**	**Result**	**Result**

Slide Number:				
Sample #	**Sample #**	**Sample #**	**Sample #**	**Sample #**
Result	**Result**	**Result**	**Result**	**Result**

ATTACHMENT D

Fluorescent Microscope Bulb Time Tracking Log

Date Bulb Replaced	Technician Initials