D^3 DFA Herpes Simplex Virus Identification Kit

REF: 01-080000

For *in vitro* Diagnostic Use

Please contact Diagnostic HYBRIDS Technical Service for technical assistance regarding this procedure.

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US Patent Nos. 6,168,915; 6,280,928; 6,306,582; 6,376,172; 6,406,842; 6,495,316; and patents pending

Symbols Lexicon/Glossary

IVD	In Vitro Diagnostic Medical Device	LOT	Batch code/lot number
	Temperature limit	REF	Catalog number
	Consult instructions for use		Use by YYYY-MON-DD
	Do not reuse		Manufacturer
iP	Patent Numbers		
CONT NaN_3	Contains sodium azide	EC REP	Authorized representative in the European Community
NaN_3 4%	Contains 4% sodium azide when undiluted	CE	CE mark of conformity (Conformité Européen)

I. INTENDED USE

The Diagnostic Hybrids, Inc. D^3 DFA Herpes Simplex Virus Identification Kit is intended for use in the qualitative detection of human herpes simplex virus (HSV) in cell cultures by immunofluorescence using fluoresceinated monoclonal antibodies (MAbs). Negative results do not preclude an infection and should not be used as the sole basis for diagnosis, treatment or other management decisions.

Performance using direct specimen testing has not been evaluated.

II. SUMMARY AND EXPLANATION OF THE TEST

HSV infections in humans can cause lesions at a variety of sites, e.g., oral-facial, genital, eye, and cutaneous sites.

When an appropriately sensitive cell line is infected with HSV, a characteristic deterioration of cells, termed cytopathic effect (CPE), can be observed. Tube culture, a classic format for virus amplification, can take several days before CPE is evident. In the case of those specimens with low titers of virus, 7 days of culture may be required by the standard tube culture method before CPE can be observed[1,2,3,4,5].

The rate of isolation may be enhanced and the time required for HSV identification may be decreased by centrifugation of specimens in shell-vials or multi-well plates containing appropriately sensitive cell lines (centrifuge enhanced technique) [6,7,8].

Even so, CPE may be difficult to interpret due to, for instance, deterioration of cells, which can result from toxic components present in the clinical specimen making microscopic examination of the infected cells problematic. Because of this, immunofluorescence confirmation of cell culture is regarded as the standard for confirmation of a HSV positive result.

III. PRINCIPLE OF THE PROCEDURE

The Diagnostic Hybrids, Inc. D^3 DFA Herpes Simplex Virus Identification Kit uses a blend of HSV antigen-specific murine MAbs that are directly labeled with fluorescein for the rapid detection of HSV in cell culture.

The infected cells are fixed in acetone on a slide prepared from a tube culture or cell monolayer from either a shell-vial or multi-well plate. The HSV DFA Reagent is added to the cells to detect the presence of HSV specific viral antigens. After incubating for 15 to 30 minutes at 35º to 37ºC, the stained cells are washed with the diluted Phosphate Buffered Saline (1X PBS) and, using the supplied Mounting Fluid, prepared for examination. The slides or wells are examined using a fluorescence microscope equipped with the correct filter combination for fluorescein isothiocyanate (FITC) at a magnification of 200-400X. By staining with the HSV DFA Reagent, which contains Evans Blue as a counter-stain, virus infected cells will show bright apple-green fluorescence that will be distinguished from the counter-stained non-infected cells exhibiting red fluorescence.[9]

IV. REAGENTS

A. Kit Components

1. **HSV DFA Reagent**, 5-mL. One dropper bottle containing a blend of fluorescein labeled murine monoclonal antibodies, two directed against HSV-1 specific glycoproteins and two directed against HSV-2 specific glycoproteins. The buffered, stabilized, aqueous solution contains Evans Blue as a counter-stain and 0.1% sodium azide as preservative.
2. **Mounting Fluid**, 7-mL. One dropper bottle containing an aqueous, buffered, stabilized solution of glycerol and 0.1% sodium azide.
3. **HSV-1/HSV-2 Antigen Control Slides**, 5 slides. Individually packaged control slides containing wells with cell culture derived positive and negative control cells. Each HSV positive well is identified. The negative well contains non-infected cells. Each slide is intended to be stained only one time.
4. **40X PBS Concentrate**, 25-mL. One bottle of 40X PBS concentrate consisting of 4% sodium azide (0.1% sodium azide after dilution to 1X using de-mineralized water).

B. Warnings and Precautions

For *in vitro* diagnostic use.

1. No known test method can offer complete assurance that infectious agents are absent; therefore, all human blood derivatives, reagents and human specimens should be handled as if capable of transmitting infectious disease. It is recommended that reagents and human specimens should be handled in accordance with the OSHA Standard on Bloodborne Pathogens. [10]
   a. Cell culture isolation may have some potential to be hazardous. Personnel working with these cultures must be properly trained in safe handling techniques[11] and have experience with cell culture before attempting this procedure.
   b. All procedures must be conducted in accordance with the CDC 5th edition Biosafety in Microbiological and Biomedical Laboratories, 2007, and CLSI Approved Guideline M29-A, Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue.
2. All specimens and materials used to process them should be considered potentially infectious and handled in a manner which prevents infection of laboratory personnel.
   a. Biosafety Level 2 or other appropriate biosafety practices should be used when handling these materials.
   b. Decontamination of specimens and cultures is most effectively accomplished using a solution of sodium hypochlorite (1:10 final dilution of household bleach).
   c. Although Antigen Control Slides have been shown to be non-infectious, the same precautions taken in handling and disposing of other infectious materials should be employed in their use.
3. Never pipette reagents or clinical samples by mouth; avoid all contact of clinical samples with broken skin.
4. Avoid splashing and the generation of aerosols with clinical samples.
5. Use aseptic technique and sterile equipment and materials for all cell culture procedures.
6. Acetone, a reagent that is required for the test but not provided in the kit, is a flammable, volatile organic solvent. Use it in a well-ventilated area and keep away from flames and other sources of ignition.
7. Sodium azide is included in the 40X PBS Concentrate at 4%, and in the other solutions in this kit at 0.1%. A MSDS for sodium azide or for Diagnostic Hybrids, Inc. (DHI) reagents containing sodium azide is available by contacting DHI Technical Services.

T: Sodium azide at very low levels causes damage to health.

R28	Very toxic if swallowed.
R32	Contact with acids liberates very toxic gas.
S28	After contact with skin, wash immediately with plenty of water.
S45	In case of accident, or if you feel unwell, seek medical advice immediately.
S60	This material and its container must be disposed of as hazardous waste.

N: Sodium azide may present an immediate or delayed danger to one or more components of the environment.

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30175 Hannover, Germany

CE

REF PI-223en **v2010APR09**

R51/53 Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

S61 Avoid release to the environment. Refer to special instructions/safety data sheets.

a. Reagents containing sodium azide should be considered poisons. If products containing sodium azide are swallowed, seek medical advice immediately and show product container or label. [Refer to NIOSH, National Institute for Occupational Safety and Health; CAS# 2628-22-8; EC# 247-852-1; and also to GHS, The Globally Harmonized System of Classification and Labeling of Chemicals.]

b. Aqueous solutions of sodium azide, when mixed with acids, may liberate toxic gas.

c. Any reagents containing sodium azide should be evaluated for proper disposal. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. If products containing sodium azide are discarded into a drain, flush with a large volume of water to prevent azide build-up. Check with regulatory agencies to determine at what concentration sodium azide may cause a product to be regulated as hazardous waste.

8. Evans Blue counter-stain is a potential carcinogen. If skin contact occurs, flush with water immediately.
9. The HSV DFA Reagent is supplied at working strength. Any dilution will decrease sensitivity.
10. Reagents should be used prior to their expiration date.
11. Each HSV Antigen Control Slide should be used only once. Do not re-use a Control Slide.
12. Microbial contamination of the HSV DFA Reagent may cause a decrease in sensitivity.
13. Store 1X PBS in a clean container to prevent contamination.
14. Reusable glassware must be cleaned and thoroughly rinsed free of all detergents.
15. Do not expose the HSV DFA Reagent to bright light during staining or storage.
16. Use of other reagents than those specified with the components of this kit may lead to erroneous results.

C. Preparation of 1X PBS

1. After storage at 2° to 8°C, some salts in the 40X PBS Concentrate may have crystallized. Warm the solution to ambient temperature (20° to 25°C) to re-dissolve the crystals, then mix.
2. Add contents of the fully dissolved 25-mL 40X PBS Concentrate to 975-mL of de-mineralized water.
3. Label the 1X PBS with a sixty (60) day expiration date after reconstitution and store at ambient temperature.

D. Storage Instructions

TABLE 1: Reagent Storage Conditions	
1. HSV DFA Reagent	Store at 2° to 8°C in the dark.
2. Mounting Fluid	
3. HSV-1/HSV-2 Antigen Control Slides	Store at 2° to 8°C.
4. 40X PBS Concentrate NOTE: The Concentrate may crystallize when stored at 2° to 8°C. The crystals will dissolve when the Concentrate is warmed to ambient temperature.	Store liquid at 2° to 8°C prior to dilution.
5. 1X PBS	Store at ambient temperature (20° to 25°C).

E. Stability

Reagents and components will retain their full potency through the expiration date shown on the label of each bottle when stored at recommended temperatures. Light exposure of the HSV DFA Reagent should be kept to a minimum.

Discard 1X PBS if it becomes cloudy.

V. SPECIMEN COLLECTION AND PREPARATION

Proper collection and handling of the patient specimen are the most important factors in successful HSV detection. Specimen collection, specimen processing, and cell culture of viruses should be attempted only by personnel that have been trained in such procedures. Care should be taken during all specimen collection and handling to avoid generation of aerosols.

A. Specimen Collection[12, 13]

The possibility of virus isolation is increased when specimens are collected from the suspected site of infection as soon as possible after onset of the disease state. When possible, the specimen of choice is vesicular fluid removed from a fresh lesion by aspiration with a 26 or 27 gauge needle attached to a tuberculin syringe. For ulcerated lesions, use a sterile nylon flocked[14,15], rayon or polyester fiber-tipped swab to remove and discard pus without disrupting the lesion base, and then use a fresh sterile collection swab dipped in sterile physiological saline to vigorously swab the lesion base to obtain cells. Crusted lesions should have the crust removed and discarded by lifting the crust from the lesion with a sterile needle. A sterile nylon flocked, rayon or polyester fiber-tipped swab moistened in sterile physiological saline is then used to vigorously swab the base of the lesion.

All specimens should be immediately placed into viral transport medium to stabilize virus and inhibit microbial growth[16]. Several factors of specimen collection may affect the successful isolation of HSV. When swabs are used for specimen collection, sterile nylon flocked, rayon or polyester fiber-tipped swabs should be used. **Do not use calcium alginate and cotton swabs because they have been shown to inhibit virus replication.**

B. Specimen Transport and Storage

All potentially infectious agents should be transported according to International Air Transport Association (IATA), International Civil Aviation Organization, (ICAO), Titles 42 and 49 of the US Code of Federal Regulations, or other regulatory requirements, as may be applicable.

Specimens should be transported to the laboratory at 2° to 8°C. This temperature can be attained by using cold packs, wet ice, foam refrigerant, or other coolants[18]. The specimen should be processed and tested as soon as possible and then stored at 2° to 8°C.

Specimens should be stored at 2° to 8°C for no longer than 2 days before being tested. If longer storage is required, the specimens should be frozen at –70°C or lower.

Freezing and thawing of specimens should be avoided since this will result in a loss of viability of viruses, leading to decreased sensitivity of the test.

VI. PROCEDURE

A. Materials Provided

1. HSV DFA Reagent.
2. Mounting Fluid
3. HSV-1/HSV-2 Antigen Control Slides
4. 40X PBS Concentrate

B. Materials Required But Not Provided

1. Fluorescence microscope with the correct filter combination for FITC (excitation peak = 490 nm, emission peak = 520nm); magnification 200X to 400X.
2. Cell culture for HSV isolation. Suggested cell lines include H&V-Mix™ MixedCells™, human newborn foreskin, MRC-5, Vero, and A549[17]. All available from DHI. Examples of HSV isolation methods include:
   a. Tube cultures containing monolayers of either a commercially prepared or user propagated cell line.
   b. Shell-vials, with glass coverslips, containing monolayers of either a commercially prepared or user propagated cell line.
   c. Multi-well plates (either 24-, or 48-well size), containing monolayers of either a commercially prepared or user propagated cell line.
3. Live control viruses for positive culture controls: Known strains of HSV concentration for use in monitoring the cell culture and staining procedures. Such control virus strains can be obtained from DHI.
4. Coverslips (22 x 50mm) for Antigen Control Slides and for specimen slides.
5. Universal Transport Medium. Available from DHI.
6. Tissue culture refeed medium (Eagle's Minimum Essential Medium with 2% fetal bovine serum, 25mM HEPES and antibiotics). Available from DHI.
7. Reagent grade acetone (>99% pure) chilled at 2° to 8°C for fixation on slides and in shell-vials.
   NOTE 1: Keep the reagent grade acetone container tightly sealed to avoid hygroscopic absorption of water, which may cause a hazy, non-specific, background fluorescence.
   NOTE 2: A mixture of 80% acetone 20% de-mineralized water is used for fixing cells in plastic multi-well plates. Store at ambient temperature (20° to 25°C).
8. Sterile graduated pipettes: 10-mL, 5-mL, and 1-mL.
9. Sterile Glass Pasteur pipettes or other transfer pipettes.
   Caution: One should not use solvents such as acetone with polyethylene transfer pipettes.
10. Fine-tipped forceps.
11. 200-mL wash bottle.
12. Bent-tip teasing needle (for removal of coverslip from a shell-vial); fashion the teasing needle by bending the tip of a syringe needle or similar object (i.e., mycology teasing needle) against a benchtop or with a pair of forceps, taking care to avoid injury.
13. Sodium hypochlorite solution (1:10 final dilution of household bleach).
14. Humidified chamber (e.g., covered Petri dish with a damp paper towel placed in the bottom).
15. Glass microscope slides.
16. Sterile nylon flocked swabs or polyester swabs, non-inhibitory to viruses and cell culture.
17. Incubator, 35° to 37°C (5% CO_2 or non-CO_2, depending on the cell culture format used).
18. Centrifuge with free-swinging bucket rotor.
19. De-mineralized water for dilution of 40X PBS Concentrate (Section IV.C.) and for dilution of the reagent grade acetone for use in polystyrene multi-well plates (See Section VI.B. Note 2).
20. Aspirator Set-up: Vacuum aspirator, with disinfectant trap, containing sufficient household bleach (5%) such that the concentration is not decreased by more than 100 fold during aspiration procedures.
21. Wash Container: Beaker, wash bottle or Coplin jar for washing slides.
22. Fixing Container: Coplin jar, slide dish or polyethylene holder for slides for use in fixing the cells on the slides.

23. Inverted Light Microscope: Used for examining monolayers prior to inoculation and examination for toxicity, confluency and for CPE. It should have between 40X to 100X magnification capability.

C. Preliminary Comments and Precautions

1. Adhere to the recommended volumes and times in the following procedure to ensure that accurate results are obtained.
2. For specimen swabs received in transport medium with glass beads, vortex vigorously for about 15 seconds to dissociate adhered cells. For swabs not received in transport medium, transfer them to a tube of transport medium containing glass beads and vortex vigorously for about 15 seconds to dissociate adhered cells.
3. When staining with fluorescent reagents and examining cells microscopically for fluorescence, it is very important to include controls, both positive and negative, to monitor the procedure and performance of the reagents. It is recommended that such controls be run with each batch of patient specimens.
4. The closed, humidified chamber for holding the slides during incubation should be kept in the incubator so it is at incubator temperature when the slides are placed in it. By doing this, the cells and antibody solution will come up to temperature more rapidly.
5. It is recommended that the HSV Reagent be brought to ambient temperature (20° to 25°C) prior to use and immediately returned to 2° to 8° C after use.

CELL CULTURE TESTING:

6. Good Laboratory Practice dictates that positive and negative virus controls be run with each new batch of cells to confirm their performance in culturing specific viruses.
7. It is good practice to retain the medium removed from the positive monolayers until after staining results have been obtained. If there is any question concerning the specimen results, the medium can be passed to another monolayer for repeat testing.
8. If staining cell monolayers in polystyrene multi-well plates, the acetone fixative must be diluted with de-mineralized water to 80% by adding 20-mL of de-mineralized water to 80-mL of acetone (Refer to Section VI.B.7. Note 2)..
9. Do not allow the monolayers to dry before fixing; this can lead to high background staining and decreased sensitivity.
10. Do not allow the DFA Reagent to dry on the monolayers; this can lead to high background.

IMMUNOFLUORESCENCE MICROSCOPY:

11. Examine the positive and negative controls before examining the test specimens. If one of these fails to perform as expected, review the steps and conditions under which the test was performed to determine the root cause(s). Do not report results for patient samples until controls perform as expected.
12. Three aspects of the fluorescence microscope that must be functioning properly and optimally in order to achieve maximum brightness of fluorescence:
    a. The activation light source has a finite life and as it ages, its output decreases, resulting in lower fluorescence intensity from the DFA Reagent.
    b. The light source is focused by a number of lenses and mirror(s). For maximum intensity, these must be properly aligned.
    c. The filters used in the light path must be appropriate for the particular fluor, in this case, fluorescein.
13. There are several fluorescent artifacts that may be observed in the cell monolayers being examined:
    a. Cell debris, lint, etc. can nonspecifically adsorb the DFA Reagent, resulting in highly intense fluorescence. These can be identified by their morphology, i.e., they don't have the appearance of a complete cell and typically do not appear to be on the same plane as the monolayer like the other cells.
    b. A low grade, yellow-green fluorescence may sometimes be seen, particularly in areas that have piled cells or are near holes in the cell monolayer. In both cases, the diffusion of the entrapped DFA Reagent is retarded during the wash step, resulting in the non-specific fluorescence.
    c. Intense fluorescence around the periphery of slide wells is indicative of drying of the DFA Reagent during incubation, suggesting that it was incubated too long or the humidity was not controlled.
    d. Inadequate rinsing can lead to general low grade fluorescence due to the residual DFA Reagent remaining on the monolayer of cells.
14. Bleaching or fading of the fluorescence of the stained cells may occur on exposure to light, particularly light of high intensity.
    a. This bleaching can occur when a stained cell is viewed in a field for an extended period of time.
    b. Stained slides and multi-well plates should also be protected as much as possible during testing.

D. Specimen Preparation

1. Swabs containing specimen material should be handled with sterile forceps. The swab should be rotated in viral transport medium and then pressed against the inside of the tube to allow excess fluid to drain back into the transport medium. Discard the swab into an appropriate disinfectant such as sodium hypochlorite solution (1:10 final dilution of household bleach). Decontaminate the forceps in between specimen disposal.
2. Disrupt cellular material in the transport medium by vortexing with sterile glass beads for 30 to 60 seconds, sonication at 10kc/sec for 30 to 60 seconds, or by other methods determined by the laboratory to be effective in disrupting cellular debris. This will enhance the release of cell-associated virus into the medium.
3. To remove bacterial, fungal, and cellular debris, centrifuge the transport medium at 700xg for 10 minutes. Supernatant is then used as the inoculum. Heavily contaminated specimens, noted by a cloudy yellow coloration, may be further clarified by filtration through a sterile 0.45 micron membrane filter. The filtrate is then used as the inoculum. Since such procedures may reduce the number of viruses in a specimen, each laboratory should establish the efficacy of its specimen preparation procedure.

E. Cell Culture Testing - Tube Culture

1. One of the two laboratories that conducted studies for clearance of this assay using tubes [presented in Section X ('Specific Performance Characteristics')], used one tube per specimen and the other used two; both laboratories terminated the cultures within 7 days.
    - It is generally recommended that specimens be inoculated into a minimum of two vessels (tubes, shell-vials, or wells; or a combination of these) containing the same or different cell types that are permissive for the suspected or requested virus(es). However – providing the specimen is appropriately collected and taken during the early infectious process (indicated by a viable lesion) – a single vessel may be sufficient for HSV cultures from genital specimens, which often contain high viral titers[18].
2. Examine the monolayers for proper morphology prior to inoculation.
3. Using a sterile pipette, remove medium from the cell culture container and re-feed with at least 2-mL of fresh pre-warmed (25º to 37ºC) refeed medium. Aseptic technique is essential at all times during inoculation and cell culture handling.
4. Using a sterile 1-mL graduated pipette, inoculate 0.2 to 0.4-mL of the clinical specimen into each tube. It is recommended that all clinical specimens be inoculated in duplicate for backup.
5. Incubate the tubes at 35º to 37ºC in a roller drum at 1 to 3 rpm.
6. Examine the monolayers daily for evidence of viral CPE (including toxicity, microbial contamination, cell death, pH extremes and non-specific cellular degeneration)[20], for at least 5 to 7 days and every other day thereafter for 14-days.
7. Rinse the cells 2 to 3 times with 1-mL volumes of 1X PBS.
8. Discard each rinse into a biohazard container.
9. Add 0.5 to 1-mL of 1X PBS to each tube.
10. Scrape cells from the tube surface and re-suspend in the 1X PBS using a sterile pipette.
11. Prepare cell spots using about 25-µL of the suspension onto an acetone-cleaned slide. Repeat this step for each specimen.
12. Air dry the wells completely.
13. Fix the cells to the slides using fresh, chilled 100% acetone. Let stand for 5 to 10 minutes, at 20º to 25ºC.
    Caution: Acetone is volatile and flammable; keep away from open flames.
14. Remove the slides from the fixative and allow to air dry.
15. Add one drop of the HSV DFA Reagent to completely cover the dried, fixed cells spots on each of the slides.
16. Also, add one drop of the HSV DFA Reagent to each of the wells of a fresh HSV Antigen Control Slide. An Antigen Control Slide should be stained only once, as it contains individual wells of viral-infected cells and non-infected cells.
17. Place the slides in a covered chamber at 35º to 37ºC for 15 to 30 minutes.
18. Rinse the stained cells using the 1X PBS. For only a few slides, this can be done using a beaker of the 1X PBS. For many slides, a slide carrier that holds 10 to 20 slides can be placed in its container with 1X PBS. For effective rinsing, dip the slide(s) up and down a minimum of four times.
19. Discard the used 1X PBS and repeat the rinse step using fresh 1X PBS.
20. Rinse the stained cells using de-mineralized water. For only a few slides, this can be done using a beaker of the de-mineralized water. For many slides, a slide carrier that holds 10 to 20 slides can be placed in its container with de-mineralized water. For effective rinsing, dip the slide(s) up and down a minimum of four times.
21. Blot the excess liquid, add a small drop of Mounting Fluid to each cell-containing well and cover the wells with a coverslip.
22. Examine the stained, mounted cells using a fluorescence microscope with magnifications between 200X to 400X (See Section VI.C. 11-14, 'Immunofluorescence Microscopy').
23. Refer to Section VII., 'Interpretation of Results'.

F. Cell Culture Testing - Shell-vial

1. The laboratory that conducted studies for clearance of this assay using shell-vials [presented in Section X ('Specific Performance Characteristics')], used one shell-vial per test, and terminated cultures within 24-hours of incubation.
    a. It is generally recommended that specimens be inoculated into a minimum of two vessels (tubes, shell-vials, or wells; or a combination of these) containing the same or different cell types that are permissive for the suspected or requested virus(es). However – providing the specimen is appropriately collected and taken during the early infectious process – a single vessel may be sufficient for HSV cultures from genital specimens, which often contain high viral titers[19].
    b. Shell-vial cultures are typically terminated after one to four days, depending on the particular agent. Overnight incubation may be adequate for rapidly growing viruses such as HSV[19].
2. Examine the monolayers for proper morphology prior to inoculation.
3. Aspirate medium from the monolayers and add 1-mL of appropriate refeed medium to each shell-vial.
4. Add 0.2 to 0.4-mL of prepared specimen to each shell-vial.
5. Centrifuge the shell-vials at 700xg for 1-hour at 20° to 25°C.
6. Place stoppered shell-vials in an incubator at 35º to 37ºC.

7. When a monolayer is ready to be stained, remove the medium and add 1-mL of 1X PBS.
8. Swirl to mix and then aspirate.
9. Repeat this wash with another 1-mL of 1X PBS and then aspirate.
10. Add 1-mL of fresh, chilled 100% acetone and allow to stand for 5 to 10 minutes at 20° to 25°C.
    Caution: Acetone is volatile and flammable; keep away from open flames.
11. Remove the fixative by aspiration.
12. Add 0.5-mL of 1X PBS to wet the monolayer.
13. Swirl and then aspirate completely.
14. Add 4 drops of the HSV DFA Reagent to the fixed monolayers of patient and control samples. Rock to ensure complete coverage of the monolayer by the Reagent.
15. Place stoppered shell-vials in a 35º to 37ºC incubator for 15 to 30 minutes.
16. Aspirate the HSV DFA Reagent from the monolayers.
17. Add 1-mL of the 1X PBS.
18. Remove the 1X PBS by aspiration, repeat the wash step and again remove by aspiration.
19. Add 1-mL of de-mineralized water.
20. Remove the de-mineralized water by aspiration.
21. Lift the coverslip from the bottom of the shell-vial, grasping it with the fine tipped forceps; then transfer it, monolayer-side down, to a small drop of Mounting Fluid on a labeled microscope slide.
22. Examine the stained monolayers using a fluorescence microscope with magnifications between 200X to 400X. (See Section VI. C. 11-14, 'Immunofluorescence Microscopy')
23. Refer to Section VII., 'Interpretation of Results'.

G. Cell Culture Testing – Multi-well Plate

1. The laboratory that conducted studies for clearance of this assay using multi-well plates [presented in Section X ('Specific Performance Characteristics')], used one well per genital specimen, and terminated cultures within 24 hours of incubation. Studies were conducted using the 48-well size of multi-well plate; use of smaller diameter wells was not evaluated.
2. It is generally recommended that specimens be inoculated into a minimum of two vessels (tubes, shell-vials, or wells; or a combination of these) containing the same or different cell types that are permissive for the suspected or requested virus(es). However – providing the specimen is appropriately collected and taken during the early infectious process – a single vessel may be sufficient for HSV cultures from genital specimens, which usually contain high viral titers[19].
    a. Multi-well plate cultures are typically terminated after one to four days, depending on the particular agent. Overnight incubation may be adequate for rapidly growing viruses such as HSV[21].
3. Examine the monolayers for proper morphology prior to inoculation.
4. Aspirate medium from the monolayers and add 1-mL of appropriate refeed medium to each 24-well multi-well plate monolayer; add 0.8-mL to each 48-well plate monolayer.
5. Add 0.2 to 0.4-mL of prepared specimen to the appropriate well of a multi-well plate.
6. Centrifuge the multi-well plates at 700xg for 1hour at 20° to 25°C.
7. Place the covered multi-well plates in a 35° to 37°C incubator with a humidified, 5% CO_2 atmosphere.
8. When a monolayer is ready to be stained, remove the medium and add 1-mL of 1X PBS.
9. Swirl to mix and then aspirate.
10. Repeat this wash with another 1-mL of 1X PBS and then aspirate.
11. Add 1-mL of 80% aqueous acetone and let stand 5 to 10 minutes.
    NOTE: Do not allow the 80% acetone fixative to remain in the polystyrene wells longer than 10 minutes since it may craze and cloud the plastic, making it difficult to examine the monolayers.
    Caution: Acetone is volatile and flammable; keep away from open flames.
12. Remove the fixative by aspiration.
13. Add 0.5-mL of the 1X PBS to wet the monolayer.
14. Swirl and then aspirate completely.
15. Add 4 drops of the HSV DFA Reagent to the fixed monolayers of patient and control samples in each 24-well multi-well plate monolayer; add 3 drops of the HSV DFA Reagent to the fixed monolayers of patient and control samples in each 48-well plate monolayer. Rock to ensure complete coverage of the monolayer by the Reagent.
16. Place the covered multi-well plate in a 35º to 37ºC, humidified incubator for 15 to 30 minutes.
17. Aspirate the HSV DFA Reagent from the monolayers.
18. Add 1-mL of the 1X PBS.
19. Aspirate the 1X PBS, repeat the rinse step and aspirate.
20. Add 1.0 -mL of de-mineralized water.
21. Aspirate the de-mineralized water.
22. Add 3 drops of Mounting Fluid to each monolayer, and cover the plate.
23. Examine the stained monolayers using a fluorescence microscope with magnifications between 200X to 400X. (See Section VI.C. 11-14 'Immunofluorescence Microscopy'.)
24. Refer to Section VII. 'Interpretation of Results'.

H. Quality Control

1. Reagents
    a. A fresh HSV Antigen Control Slide should be stained each time the staining procedure is performed to ensure proper test performance.
    b. The positive well will show multiple infected areas of bright green fluorescence with negative cells fluorescing a dull red due to the included Evans Blue counter-stain.
    c. The negative well will show only fluorescence-negative cells staining a dull red.
    d. Positive and negative controls must demonstrate appropriate staining characteristics for specimen results to be considered valid. Antigen Control Slides may also aid in the interpretation of patient specimens.
2. Cell Culture
    a. Positive and negative virus controls should be run with each new batch of cells to confirm their performance in culturing specific viruses.
    b. To ensure viral sensitivity, an HSV-inoculated control monolayer should be included each time a new lot of cell culture is used.
    c. Also, a non-inoculated monolayer from each lot should be kept and re-fed every 3 to 7 days and monitored for normal cell growth; it may be used as a negative cell control when examining for CPE. All cell cultures should be stored at 35º to 37ºC. Adverse storage conditions or handling procedures will also be reflected in the negative control.
    d. If control cultures fail to perform correctly, results are considered invalid.

VII. INTERPRETATION OF RESULTS

A. Examination of Samples and Controls

1. Examine controls first to ensure proper test performance before examining patient specimens.
2. A positive reaction for HSV is one in which apple-green fluorescence observed in the infected cells.
3. Non-infected cells will stain dull red due to the Evans Blue counter-stain included in the DFA Reagent.
4. Examine the entire cell spot or monolayer of cells before reporting final negative results.
5. Do not report results for patient samples unless controls perform as expected.

B. Artifacts of Staining

1. Dried edges of the monolayer or cell clumps may non-specifically fluoresce due to antibody trapping.
2. Dead, rounded cells may non-specifically fluoresce dull olive-green due to specimen toxicity or improper cell storage.
3. Properly controlled humidity during staining and adequate washing between steps helps to eliminate non-specific staining.

C. Results from Culture Isolation/Confirmation

1. The bright apple-green fluorescence staining pattern is finely granular in appearance, and associated with the cytoplasm and/or nucleus.
2. Examine the entire cell spot or monolayer of cells for Herpes simplex virus-specific fluorescent cells. If no fluorescent cells are found, report: "No Herpes simplex virus isolated by cell culture."
3. If fluorescent cells are found in the HSV stained monolayer showing an expected staining pattern, report the result as "Herpes simplex virus isolated by cell culture".

VIII. LIMITATIONS OF PROCEDURE

1. Inappropriate specimen collection, storage, and transport may lead to false negative culture results[20].
2. Incubation times or temperatures other than those cited in the test instructions may give erroneous results.
3. Detection of viruses will vary greatly depending upon the specimen quality and subsequent handling. A negative result does not exclude the possibility of virus infection. Results of the test should be interpreted in conjunction with information available from epidemiological studies, clinical evaluation of the patient and other diagnostic procedures.
4. The effects of antiviral therapy on the performance of this kit have not been established.
5. Since the MAbs used in this kit have been prepared using defined virus strains, they may not detect all antigenic variants or new strains of the viruses, should they arise. MAbs may fail to detect strains of viruses which have undergone minor amino acid changes in the target epitope region. MAbs in this kit have been shown to react with cells infected with the HSV strains HSV-1(f), HSV-1(MacIntyre), HSV-2(G), and HSV-2(MS).
    a. The kit's HSV DFA Reagent is a blend of HSV-1 and HSV-2 MAbs, therefore it is not type-specific and therefore cannot be used to differentiate HSV-1 and HSV-2.
    b. These MAbs are created from hybridomas using viral-infected cells as the immunogen. The particular viral antigens detected by the antibodies are undetermined.
6. Performance of the kit can only be assured when components used in the assay are those supplied by DHI.
7. Prolonged storage of the HSV DFA Reagent under bright light will decrease the staining intensity.
8. Light background staining may occur with specimens contaminated with *Staphylococcus aureus* strains containing large amounts of protein A. Protein A will specifically bind the Fc portions of conjugated antibodies. Such binding can be distinguished from viral antigen binding on the basis of morphology, i.e., *S. aureus*-bound fluorescence appears as small (~1 micron diameter), bright dots.

IX. EXPECTED VALUES

The clinical studies described in Section X ('Specific Performance Characteristics') used only specimens that were collected and cultured for the presence of *Herpes simplex* types 1 and 2. The majority of the specimen types (n=517) used in the clinical studies were swabs taken from skin lesions. The rest of the specimens were respiratory specimens (n=8) and CSF specimens (n=2). The specimen sources and positivity with the Comparison device are described below (Table 2).

TABLE 2: Specimen Sources

Study Site	Total specimens	Unknown +/Total	Genital +/Total	Penis +/Total	Vaginal +/Total	Labia +/Total	Cervical +/Total	Rectal +/Total	perineum** +/Total	Eye +/Total	Urethra +/Total	Eyelid +/Total	Face +/Total	Mouth* +/Total	Skin‡ +/Total	Bronchial/BAL Sputum/NP+/Total	CSF/Brain +/Total
1	107		18/37	1/1	12/14	6/8	1/1	0/1	4/9	0/0	1/1	1/1	2/4	9/14	3/10	5/6	0/0
2	129	5/11	2/7	2/7	19/42	8/11	5/12	2/2	0/2	1/2	0/0	0/0	1/2	2/15	3/10	0/5	0/1
3	150	7/19	1/3	2/8	16/42	3/6	1/2		3/4	0/3	0/1		2/3	3/12	9/44	1/2	0/1
4	141		40/141														

*mouth: mouth, lip, tongue, gum, throat
**perineum: groin, buttock, gluteal, coccyx, sacral, pubic, perianal
‡skin: skin lesion, skin, finger, wrist, chest, axilla, abdomen, thigh, blister

Demographics by age and gender for the specimens that were tested at 3 of the 4 Study Sites are tabulated below (specimen information from Study Site 4 was not available).

Of the specimens evaluated in these studies (which had been submitted to the laboratories for HSV testing), 224 of 384 were taken from patients between 18 and 40. The percent positivity for HSV isolated from culture from the 18 to 40 year age range was 47%, 32% from pediatric patients, and 35% from patients older than 40 years of age, The prevalence of HSV isolated from lesion specimens ranged anywhere from 28% to 59%, The patient demographics are listed below (Table 3).

TABLE 3: Demographics by Age and Gender

Site	Study Site 1 Values are # pos / Total		Study Site 2 Values are # pos / Total		Study Site 3 Values are # pos / Total		
Age / Gender	F	M	F	M	F	M	Gender not reported
TOTALS	84	23	103	26	114	34	2
<2y	0/3	2/2	0	0	1/2	0/1	0
2y to 10y	4/5	1/2	0/3	0/1	0/4	0/1	0
10y to18y	3/4	0	4/10	0/3	1/6	0/3	0
18y to 40y	31/48	7/11	32/67	4/14	26/66	6/18	0
>40y	11/24	4/8	8/23	2/8	9/33	4/11	0
Age not reported	0	0	0	0	0/3	0	1/2

X. SPECIFIC PERFORMANCE CHARACTERISTICS

This study included five hundred and thirty (530) prospectively collected specimens submitted for Herpes simplex culture. Each specimen was evaluated by the Diagnostic Hybrids, Inc. D^3 DFA Herpes Simplex Virus Identification Kit (D^3 DFA HSV ID Kit) and compared to currently-marketed HSV identification kits. A combination of fresh (250) and frozen (280) specimens were tested. Three specimens from Study Site 4 were not evaluated due to bacterial contamination of the monolayers, leaving 527 for analysis. These studies were conducted at three external laboratory sites located in the mid-west United States and one in-house virology laboratory. Each of the study sites used different HSV kits as Comparison devices. Therefore, each site is listed separately due to the likely variability of the antibodies used with each kit.

The numbers of fresh and frozen specimens tested at each study site are summarized below (Table 4).

TABLE 4: Numbers of Specimens Evaluated per Site

Site	Culture Confirmation		Study Site Total
	Fresh	Frozen	
1	4	103	107
2	96	33	129
3	150	0	150
4	0	141	141

Percent Agreement between the D^3 DFA HSV ID Kit and Comparison devices was calculated and tabulated for all the tested specimens, and is presented below (Table 5).

TABLE 5: Specimen Total: D^3 DFA HSV ID Kit and Comparison Devices using Various Culture Methodologies

D^3 DFA HSV ID Kit \ Comparison Device	+	-
+	**200**	1
-	1	**325**

	Agreed	95% CI [a]
Positive Percent Agreement [b] (PPA) =	99.5%	97.3% to 100%
Negative Percent Agreement [c] (NPA) =	99.7%	98.3% to 100%

A. Study Site 1: A total of 107 specimens were cultured for HSV. Briefly, 200-µL from each specimen was inoculated into one tube culture each of MRC-5 and A-549. The inoculated cells were incubated at 35° to 37°C and examined daily for CPE for seven days. Tube cultures exhibiting CPE were scraped and cell spots made on two multi-well slides; one slide was processed according to the D^3 DFA HSV ID Kit product insert and the other slide was processed according to the Comparison device product insert procedure. Tube cultures exhibiting no CPE at 7-days were also scraped and cell spots made to confirm the absence of HSV. The cell spots were fixed with acetone in accordance with the respective product insert. All 107 specimens were tested for the presence of HSV. All calculations for confidence intervals were done according to the Exact Method.[Error! Bookmark not defined.] The results of this study site are summarized below (Table 6).

TABLE 6: Study Site 1: D^3 DFA HSV ID Kit and Comparison Device using Tube Culture

D^3 DFA HSV ID Kit \ Comparison Device	+	-
+	**63**	0
-	0	**44**

	Agreed	95% CI
PPA =	100%	94.2% to 100%
NPA =	100%	91.9% to 100%

B. Study Site 2: A total of 129 specimens were cultured for HSV. Briefly, 200-µL from each specimen was inoculated into duplicate MRC-5 shell-vials (one for each device being tested). The inoculated cultures were centrifuged at 700xg for 60-minutes, incubated at 35° to 37°C for 24-hours. One shell-vial from each set was then stained in accordance with the respective product insert procedures (D^3 DFA HSV ID Kit and Comparison device). All calculations for confidence intervals were done according to the Exact Method.[Error! Bookmark not defined.] The results of this study site are summarized below (Table 7).

TABLE 7: Study Site 2: D^3 DFA HSV ID Kit and Comparison Device using Shell-Vials

D^3 DFA HSV ID Kit \ Comparison Device	+	-
+	**50**	0
-	0	**79**

	Agreed	95% CI
PPA =	100%	92.8% to 100%
NPA =	100%	95.3% to 100%

C. Study Site 3: A total of 150 specimens were cultured for HSV. Briefly, 200-µL from each specimen was inoculated into single MRC-5 tubes. The inoculated tube cultures were incubated at 35° to 37°C and examined daily for CPE . Cell cultures exhibiting CPE were scraped and cell spots made on two multi-well slides; one slide was processed according to the D^3 DFA HSV product insert procedure and the other slide was processed according to the Comparison device product insert procedure. Cell cultures exhibiting no CPE at 7-days were also scraped and cell spots prepared to confirm the absence of HSV. All 150 specimens were tested for the presence of HSV. All calculations for confidence intervals were done according to the Exact Method.[Error! Bookmark not defined.] The results of this study site are summarized below (Table 8).

[a] "95% CI" refers to 95% Confidence Intervals, which were calculated according to Exact method (Clopper, C. and S. Pearson, Biometrika 26:404-413, 1934).

[b] "Positive Percent Agreement", or "PPA", values were calculated according to {(Total Number of Positive Results in Agreement by both Subject and Comparison Devices) divided by ((Total Number of Positive Results in Agreement by both Subject and Comparison Devices) plus (Number of Results Positive by Comparison Device but Negative by Subject))} multiplied by 100%.

[c] "Negative Percent Agreement", or "NPA", values were calculated according to {(Total Number of Negative Results in Agreement by both Subject and Comparison Devices) divided by ((Total Number of Negative Results in Agreement by both Subject and Comparison Devices) plus (Number of Results Negative by Comparison Device but Positive by Subject))} multiplied by 100%.

TABLE 8: Study Site 3: D³ DFA HSV ID Kit and Comparison Device using Tube Culture

D³ DFA HSV ID Kit	Comparison Device +	Comparison Device -
+	**47**	1
-	1	**101**

	Agreed	95% CI
PPA =	97.9%	88.9% to 99.9%
NPA =	99.1%	94.7% to100%

D. Study Site 4: A total of 144 specimens were cultured for HSV. Briefly, 100-µL from each specimen was inoculated into duplicate H&V-Mix™ multi-well plates[18] (48-well size). The inoculated cells were centrifuged at 700xg for 60-minutes, incubated at 35° to 37°C for 24-hours. One well from each set was then stained in accordance with the respective product insert procedures (D³ DFA HSV ID Kit and Comparison device). Three specimens were not evaluated due to bacterial contamination of the monolayers. The results of this study site are summarized below (Table 9).

TABLE 9: Study Site 4: D³ DFA HSV ID Kit and Comparison Device using Multi-Well Plates

D³ DFA HSV ID Kit	Comparison Device +	Comparison Device -
+	**40**	0
-	0	**101**

	Agreed	95% CI
PPA =	100%	91.1% to 100%
NPA =	100%	96.3% to 100%

E. Cross-reactivity Testing:
The Diagnostic Hybrids, Inc. D³ DFA Herpes Simplex Virus Identification Kit's DFA Reagent was tested for cross-reactivity against a wide variety of cells and microorganisms. No cross-reactivity was observed for 59 virus strains or for 17 host culture cell types. Twenty-seven (27) bacterial cultures, one yeast and one protozoan culture were stained and examined for cross-reactivity, including *Staphylococcus aureus,* a protein-A-producing bacterium. Staining of *S. aureus* appeared as small points of fluorescence (See Limitations of Procedure, Section VIII.). [See Tables below for cross-reactivity study results.]

Stringent conditions for cross-reactivity testing were achieved by using a high concentration of the HSV DFA Reagent and relatively high titers of microorganisms. The DFA Reagent was prepared at 1.5X the concentration that is provided in the kit.

- Fifty-nine (59) virus strains were tested for cross-reactivity. Depending on the particular virus, 500 to 715 $TCID_{50}$ were inoculated into shell-vial culture and incubated for 24 to 48 hours, to yield a 1+ to 3+ infection, processed and stained with the 1.5X DFA Reagent according to the procedure as detailed in this product insert. No cross-reactivity was observed for the viruses listed below (Table 10).

TABLE 10: Virus Strains Tested for Cross-Reactivity with HSV DFA Reagent

Organism	Strain or Type	Inoculum ($TCID_{50}$)	Organism	Strain or Type	Inoculum ($TCID_{50}$)
Adenovirus	Type 1	715	Influenza B	Hong Kong	715
	Type 3	715		Maryland	715
	Type 5	715		Mass	715
	Type 6	715		Taiwan	715
	Type 7	715		GL	715
	Type 8	715		JH-001 isolate	715
	Type 10	715		Russia	715
	Type 13	715	RSV	Long	715
	Type 14	715		Wash	715
	Type 18	715		9320	715
	Type 31	715	Parainfluenza 1	C-35	715
	Type 40	715	Parainfluenza 2	Greer	715
	Type 41	715	Parainfluenza 3	C 243	715
Influenza A	Aichi	715	Parainfluenza 4a	M-25	715
	Mal	715	Parainfluenza 4b	CH19503	715
	Hong Kong	715	CMV	Towne	700
	Denver	715		Davis	700
	Port Chalmers	715		AD169	700
	Victoria	715	VZV	Webster	500
	New Jersey	715		Ellen	500
	PR	715	Epstein-Barr	Commercially available slides stained.[dd]	
	WS	715	Rubeola		
			Mumps		
Echovirus	Types 4, 6, 9, 11, 30, 34	Commercially available slides stained.[d]	HPV	Types 6, 11	Commercially available slides stained.[d]

[d] Test material is from commercially available prepared slides. Each positive well contains 10 to 50% reactive cells.

TABLE 10: Virus Strains Tested for Cross-Reactivity with HSV DFA Reagent

Organism	Strain or Type	Inoculum ($TCID_{50}$)	Organism	Strain or Type	Inoculum ($TCID_{50}$)
Coxsackie-virus	Types B1, B2, B3, B4, B5, B6	Commercially available slides stained.[d]			

- Seventeen (17) host culture cell types were tested for cross-reactivity. Cell cultures were prepared in shell-vial format. Confluent monolayers were stained with the 1.5X DFA Reagent according to the procedure as detailed in this product insert, then examined for cross-reactivity. No cross-reactivity was observed for cell culture types listed below (Table 11):

TABLE 11: Cell lines Tested for Cross-Reactivity with HSV DFA Reagent

A-549	NCI-H292
BGMK	pCMK
HEp-2	pRhMK
LLC-MK2	RhMK II
MDCK	pRK
MRC-5	RD
MRHF	R-Mix
Mv1Lu	Vero
	WI-38

- Twenty-nine (29) microorganisms, including 27 bacterial cultures, one yeast and one protozoan, were stained with the 1.5X DFA Reagent according to the procedure as detailed in this product insert, then examined for cross-reactivity. Except for *Staphylococcus aureus*, which was cross-reactive with the HSV DFA Reagent (see above), all microorganisms tested negative. Concentrations for each bacterial organism cultured by DHI for cross-reactivity testing were determined from suspensions of the bacteria in PBS by spectrophotometer according to McFarland standards of levels 1.0 and 2.0 (equaling approximately 3.0×10^6 and 6.0×10^6 CFU per mL). Slides were prepared with spots of 0.01-mL of the suspensions to give either 3.0×10^4 or 6.0×10^4 per spot. At the same time, 1-mL of each suspension was plated on an appropriate agar dish for colony confirmation. According to the confirmation agar cultures, initial concentrations of the bacterial organisms in the study ranged from 6.4×10^4 to 2.9×10^7 CFU. Results of testing are listed below (Table 12).

TABLE 12: Microorganisms Tested for Cross-Reactivity with HSV DFA Reagent

BACTERIA	CFU TESTED
Acinetobacter calcoaceticus	9.7×10^5
Bordetella bronchiseptica	1.7×10^5
Bordetella pertussis	4.6×10^6
Corynebacterium diphtheriae	2.5×10^6
Escherichia coli	2.6×10^5
Gardnerella vaginalis	5.0×10^5
Haemophilis influenzae type A	9.3×10^5
Klebsiella pneumoniae	6.4×10^6
Legionella pneumophila	6.5×10^4
Moraxella cartarrhalis	6.4×10^4
Neisseria gonorrhoeae	1.3×10^6
Proteus mirabilis	2.1×10^6
Pseudomonas aeruginosa	1.0×10^7
Salmonella enteriditis	2.5×10^6
Salmonella typhimurium	1.7×10^6
Staphylococcus aureus	1.0×10^7
Streptococcus agalactiae	9.6×10^6
Streptococcus pneumoniae	8.0×10^5
Streptococcus pyogenes	2.9×10^7
Acholeplasma laidlawi	~6×10^7
Mycoplasma hominis	~6×10^4
Mycoplasma orale	~6×10^4
Mycoplasma pneumoniae	~6×10^4
Mycoplasma salivarium	~6×10^7
Ureaplasma urealyticum	~6×10^4
Chlamydophila pneumoniae	Commercially available slides stained. [d]
Chlamydia trachomatis	Commercially available slides stained. [d]
YEAST	
Candida glabrata	8.7×10^6
PROTOZOAN	
Trichomonas vaginalis	[Commercially available slides]

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