D^3 DFA Varicella-zoster Virus Identification Kit

REF: 01-020000

For *in vitro* Diagnostic Use

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

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US Patent Nos. 6,168,915; 6,376,172; 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
	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 Varicella-zoster Virus Identification Kit is intended for use in the qualitative detection of Varicella-zoster virus (VZV) 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 decision.

Performance testing has not been done on direct patient specimen testing.

II. SUMMARY AND EXPLANATION OF THE TEST

VZV is a latent DNA virus in the family *Herpesviridae.* The virus is associated with two disease states: chickenpox (primary infections) and shingles (reactivation of latent virus).

When an appropriately sensitive cell line is infected with VZV, 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- to 14-days of tube culture may be required before CPE can be observed.

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 necessary for confirmation of a VZV positive result.

III. PRINCIPLE OF THE PROCEDURE

The Diagnostic Hybrids, Inc. D^3 DFA Varicella-zoster Virus Identification Kit uses a blend of VZV antigen-specific murine MAbs that are directly labeled with fluorescein for the rapid identification of VZV 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 VZV DFA Reagent is added to the cells to detect the presence of VZV 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 200X-400X. By staining with the VZV 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 dull red fluorescence.

IV. REAGENTS

A. Kit Components

1. **VZV DFA Reagent**, 5-mL. One dropper bottle containing a blend of two fluorescein labeled murine monoclonal antibodies directed against a recombinant glycoprotein E (gE) from the Ellen strain of VZV. 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. **VZV Antigen Control Slides**, 5-slides. Five (5) individually packaged control slides containing wells with cell culture derived positive and negative control cells. Each VZV 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 demineralized 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.[1]
   a. Cell culture isolation may have some potential to be hazardous. Personnel working with these cultures must be properly trained in safe handling techniques[2] 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.

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.

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a. Reagents containing sodium azide should be considered poisons. If products containing sodium azide are swallowed, seek medical advice immediately and show product container, label, or MSDS to medical personnel. [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. Evaluate reagents containing sodium azide for proper use and disposal. When mixed with acids, aqueous solutions of sodium azide may liberate toxic gas. 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 VZV DFA Reagent is supplied at working strength. Any dilution to the DFA Reagent will decrease sensitivity.
10. Reagent should be used prior to its expiration date.
11. Each VZV Antigen Control Slide should be used only once. Do not re-use a Control Slide.
12. Microbial contamination of the VZV DFA Reagent may cause a decrease in sensitivity.
13. Store 1X PBS in a clean container to prevent contamination.
14. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
15. Do not expose the VZV 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 demineralized 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. VZV DFA Reagent	Store at 2° to 8°C in the dark
2. Mounting Fluid	
3. VZV 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

Reagent 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 VZV 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 VZV 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[3, 4]

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[5,6], 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. Several factors of specimen collection may affect the successful isolation of VZV. 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.[3] 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. VZV DFA Reagent
2. Mounting Fluid
3. 40X PBS Concentrate
4. VZV Antigen Control Slides

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 VZV isolation. Suggested cell lines include H&V-Mix™ MixedCells™, human newborn foreskin, MRC-5, CV-1 and A549.[7] All are available from DHI.
3. Coverslips (22 x 50mm) for Antigen Control Slides and for specimen slides.
4. Universal Transport Medium. Available from DHI.
5. Tissue culture refeed medium (Eagle's Minimum Essential Medium with 2% fetal bovine serum, 25mM HEPES and antibiotics). Available from DHI.
6. Reagent-grade acetone (>99% pure) chilled at 2° to 8°C for fixation of 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% demineralized water is used for fixing cells in plastic multi-well plates. Store at ambient temperature (20° to 25°C).
7. Sterile graduated pipettes: 10-mL, 5-mL, and 1-mL.
8. Sterile Pasteur pipettes or other transfer pipettes
   Caution: One should not use solvents such as acetone with polyethylene transfer pipettes.
9. Fine-tipped forceps.
10. Sterile 0.45-µm syringe filter.
11. Sterile 3-mL syringe.
12. 200-mL wash bottle.
13. 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.
14. Sodium hypochlorite solution (1:10 final dilution of household bleach).
15. Humidified chamber (e.g., covered Petri dish with a damp paper towel placed in the bottom).
16. Glass microscope slides.
17. Sterile nylon flocked swabs or polyester swabs, which are non-inhibitory to viruses and cell culture.
18. Incubator, 35° to 37°C (5% CO_2 or non-CO_2, depending on the cell culture format used).
19. Centrifuge with free-swinging bucket rotor.
20. Demineralized 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 (Item VI.B.6. Note 2).
21. Live control viruses for positive culture controls: Known strains of VZV for use in monitoring the cell culture and staining procedures. Such control virus strains can be obtained from DHI.
22. Aspirator Set-up: Vacuum aspirator with disinfectant trap containing sufficient household bleach (5%) such that the concentration is not decreased by more than 10-fold as it is diluted with discarded fluids.
23. Wash Container: Beaker, wash bottle or Coplin jar for washing slides.
24. Fixing Container: Coplin jar, slide dish or polyethylene holder for slides for use in fixing the cells on the slides.
25. Inverted Light Microscope: Used for examining monolayers of cells 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 transfer 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. Place the closed, humidified chamber for holding slides during staining into the incubator for equilibration to 35°C to 37°C prior to staining. By doing this, the test slides and reagent will come to temperature quickly, yielding more rapid, intense staining.
5. Bring the DFA Reagent to ambient temperature (20° to 25°C) prior to use, and immediately return to refrigerator after use for storage at 2° to 8°C.

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 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 and incubated for the appropriate time period for repeat testing.
8. When using cell cultures in polystyrene multi-well plates, dilute the acetone fixative to 80% by adding 20-mL of demineralized water to 80-mL of acetone (Section VI.B.6.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. Fluorescent artifacts may be observed in the cell monolayers:
    a. Cell debris, lint, etc. can non-specifically 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 are not seen on the same plane of the monolayer as the other cells would be.
    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 well controlled.
    d. Inadequate washing can lead to general low grade fluorescence due to residual DFA Reagent remaining on the monolayer of cells.
14. Protect stained slides and monolayers from light as much as possible during testing.
    a. Bleaching or fading of the fluorescence of stained cells may occur on exposure to light, particularly light of high intensity.
    b. This bleaching can occur when a stained cell in viewed in a fluorescence microscope for an extended period of time.

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 a sodium hypochlorite solution (1:10 dilution of household bleach). Decontaminate the forceps after 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. The laboratory that conducted studies for clearance of this assay using tube culture [presented in Section X ('Specific performance characteristics')] used one tube per specimen and terminated cultures within 14-days.
    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).[8]
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. Examine the monolayers daily for evidence of toxicity or viral CPE.
6. Examine the monolayers daily for at least 5- to 7-days and every other day thereafter for 14-days. Assess cultures for evidence of viral replication (CPE) and also identification of problems such as toxicity, microbial contamination, cell death, pH extremes and non-specific cellular degeneration[9].
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 1.0-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 VZV DFA Reagent to completely cover the dried, fixed cells on each of the slides.
16. Also, add one drop of the VZV DFA Reagent to each of the wells of a fresh VZV Antigen Control Slide. Each Antigen Control Slide can be stained only once.
17. Place the slides in a covered humidified 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 washing step using fresh 1X PBS.
20. Rinse the stained cells using demineralized water. For only a few slides, this can be done using a beaker of the demineralized water. For many slides, a slide carrier that holds 10- to 20- slides can be placed in its container with demineralized water. For effective rinsing, dip the slide(s) up and down a minimum of four times.
21. Blot the excess liquid.
22. Add a small drop of Mounting Fluid to each cell-containing well and cover the wells with a coverslip.
23. Examine the stained, mounted cells using a fluorescence microscope with magnifications between 200X to 400X (See Section VI.C. 11-14, 'Regarding Immunofluorescence Microscopy').
24. Refer to Section VII. 'Interpretation of Results'7.

F. Cell Culture Testing - Shell-vial

1. The laboratory that conducted studies for clearance of this assay using shell-vial culture [presented in Section X ('Specific Performance Characteristics')], used one shell-vial per specimen per test, and terminated cultures at a minimum of 72-hours.
    NOTE: Shell-vial cultures are typically terminated after 1- to 7-days, depending on the particular agent. Frequently two time points are utilized, e.g. day two and days four to five for VZV. Selection of time points for termination of cultures and immunostaining of monolayers for the detection of viral antigens is best based on the laboratory's experience and needs.[8]
2. Examine the monolayers for proper morphology prior to inoculation.
3. Aspirate maintenance 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 using the VZV DFA Reagent, 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 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. Discard the acetone into a biohazard container.
12. Add 0.5-mL of 1X PBS to wet the monolayer.
13. Swirl and then aspirate.
14. Add 4 drops of the VZV DFA Reagent to the fixed monolayers of patient and control samples, and 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 VZV 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 0.5- to 1.0-mL of demineralized water.
20. Remove the demineralized water by aspiration.

21. Lift the coverslip from the bottom of the shell-vial using a bent-tip needle on a syringe barrel. Grasping it with the fine-tipped forceps, transfer it, monolayer-side down, to a small drop of Mounting Fluid on a standard microscope slide.
22. Examine the stained monolayers using a fluorescence microscope with magnifications between 200X to 400X. (See Section VI. C. 11-14, 'Regarding Immunofluorescence Microscopy').
23. Refer to Section VII. 'Interpretation of Results'.

G. Cell Culture Testing – Multi-well Plate

1. The laboratories that conducted studies for clearance of this assay using multi-well plates [presented in Section X ('Specific performance characteristics'), used one well per specimen, and terminated cultures at a minimum of 72-hours.
   NOTE: Multi-well plate cultures are typically terminated after 1- to 4-days, depending on the particular agent. Frequently two time points are utilized, e.g. day two and days four to five for VZV. Selection of time points for termination of cultures and immunostaining of monolayers for the detection of viral antigens is best based on the laboratory's experience and needs.[8]
2. Examine the monolayers for proper morphology prior to inoculation.
3. Aspirate maintenance 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.
4. Add 0.2- to 0.4-mL of prepared specimen to the appropriate well of a multi-well plate.
5. Centrifuge the multi-well plates at 700xg for 1-hour at 20° to 25°C.
6. Place the covered multi-well plates in a 35° to 37°C incubator with a humidified, 5% CO_2 atmosphere.
7. When a monolayer is ready to be stained using the VZV DFA Reagent, remove the medium by aspiration 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 80% acetone and let stand 5- to 10-minutes at 20° to 25°C.
    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.
11. Remove the fixative by aspiration.
12. Add 0.5-mL of the 1X PBS to wet the monolayer.
13. Swirl and then aspirate.
14. To each well of a 24-well plate, add 4 drops of the VZV DFA Reagent to the fixed monolayers of patient and control samples; to each well of a 48-well plate, add 3 drops of the VZV DFA Reagent to the fixed monolayers of patient and control samples. Rock to ensure complete coverage of the monolayer by the Reagent.
15. Place the covered multi-well plate in a 35º to 37ºC, humidified incubator for 15 to 30-minutes.
16. Aspirate the VZV 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 0.5- to 1.0-mL of demineralized water.
20. Remove the demineralized water by aspiration.
21. Add 3 drops of Mounting Fluid to each monolayer, and cover the plate.
22. Examine the stained monolayers using a fluorescence microscope with magnifications between 200X to 400X. (See Section VI.C. 11-14, 'Regarding Immunofluorescence Microscopy').
23. Refer to Section VII. 'Interpretation of Results'.

H. Quality Control

1. **Reagents**
   a. A fresh VZV Antigen Control Slide should be stained each time the staining procedure is performed to ensure proper test performance.
   b. The positive well will show infected areas of bright apple-green fluorescence where negative cells will fluoresce a dull red due to the included Evans Blue counter-stain.
   c. The negative well will show only negative cells staining a dull red.
   d. Positive and negative controls must demonstrate appropriate fluorescencefor specimen results to be considered valid.

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, a VZV-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 observed 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 VZV is one in which bright 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 Reagents.
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 cytoplasmic.
2. Examine the entire cell spot or monolayer of cells for VZV-specific flourescent cells. If no fluorescent cells are found, report: "No Varicella-zoster virus isolated by cell culture."
3. If fluorescent cells are found, report results as, "Varicella-zoster virus isolated by cell culture".

VIII. LIMITATIONS OF PROCEDURE

1. Inappropriate specimen collection, storage, and transport may lead to false negative culture results[10].
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 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.
6. The VZV DFA Reagent cannot differentiate between the various VZV genotypes or the wild type or vaccine strain(s).
7. Performance of the kit can only be assured when components used in the assay are those supplied by Diagnostic Hybrids, Inc.
8. Prolonged storage of the VZV DFA Reagent under bright light will decrease the staining intensity.
9. Light background staining may occur from specimens contaminated with certain *Staphylococcus aureus* (e.g. Cowan) strains containing large amounts of protein A. Protein A will bind to 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. Results from cell cultures with bacterial contamination must, therefore, be interpreted with caution.

IX. EXPECTED VALUES

The clinical studies described in Section X ('Specific Performance Characteristics') used only specimens collected and cultured for the presence of VZV. Most of the specimen types used in the clinical studies were swabs taken from skin lesions (with two taken as respiratory specimens (NP) and one CSF). Specimens were taken from the following body sites (and presented as # positive/# specimens) are described in Table 2 below.

TABLE 2: Specimen Sources

Source	Total specimens	Unknown +/Total	Genital +/Total	Penis +/Total	Vaginal +/Total	Cervical +/Total	Rectal +/Total	perineum** +/Total	Eyelid +/Total	Face +/Total	Mouth* +/Total	Skin‡ +/Total	NP+/Total	CSF/Brain +/Total
Site 1	99	0/8	0/1	0/0	0/0	0/0	0/1	0/11	0/1	4/14	0/2	17/61	0/0	0/0
Site 2	35	0/0	0/0	1/2	0/0	0/0	0/0	1/3	0/0	0/2	0/0	9/27	0/1	0/0
Site 3	120	2/51	0/6	0/1	0/9	0/1	0/0	0/3	0/0	1/9	0/5	4/33	1/1	0/1

*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 the three Study Sites are tabulated below.

Of the specimens evaluated in these studies (which had been submitted to the laboratories as swabs taken from lesions for both HSV and VZV testing), a large

proportion were from patients between the ages of 18 and 40. Prevalence of VZV within the population tested was quite low (due in part to varicella vaccination programs).[11] The patient demographics are listed below (Table 3).

TABLE 3 Demographics by Age and Gender

Source	Site 1 Values are # pos / Total		Site 2 Values are # pos / Total			Site 3 Values are # pos / Total	
Age / Gender	F	M	F	M	Gender not reported	F	M
TOTALS	63	36	10	10	12	80	40
<2y	0/1	0/4	0	0/2	0	0	0/1
2y to 10y	0	0/1	0/1	0/1	0	1/3	0/2
10y to18y	1/6	1/3	1/1	1/1	0	0/4	0/3
18y to 40y	0/18	1/3	0/1	0/1	0	0/39	0/13
>40y	11/38	7/24	3/6	4/5	0	2/33	5/21
Age not reported	0/0	1/1	0/1	0	1/12	1/1	0

X. SPECIFIC PERFORMANCE CHARACTERISTICS

This study included two hundred and fifty-four (254) prospectively collected specimens submitted for VZV culture. Each specimen was evaluated by the D^3 DFA Varicella-zoster Virus Identification Kit (D^3 DFA VZV Kit) and compared to a currently-marketed VZV identification kit. A combination of fresh (61) and frozen (193) specimens were tested. Three fresh specimens from Site 2 were toxic to cell culture and were not evaluated by either test. One specimen from Site 3 was negative in the multi-well plate culture, but was positive in the tube culture 10-days post inoculation. These evaluations were conducted at two external laboratory sites and one in-house laboratory: (**1**) A reference laboratory in the Southeastern United States; (**2**) A reference laboratory in the Southwestern United States; and (**3**) Diagnostic Hybrids, Inc. in-house virology laboratory. The numbers of fresh and frozen specimens tested are summarized below (Table 4).

TABLE 4: Number of Fresh vs. Frozen Specimens

Site	Culture		Site Total
	Fresh	Frozen	
1	57	42	99
2	1	31	32
3	0	120	120

Percent Agreement between the D^3 DFA VZV Kit and comparator tests was calculated and tabulated in Table 5 for all the tested specimens is presented below (Table 4).

TABLE 5: Percent Agreement of All Tests

D^3 DFA VZV Kit	Comparison Device +	Comparison Device –
+	**42**	1
–	0	**208**

Positive Percent Agreement [a] (PPA)	100%
95% CI [b] – PPA	91.6% to 100%
Negative Percent Agreement [c] (NPA)	99.5%
95% CI – NPA	97.3% to 99.9%

A. Study Site 1: A total of 99 specimens were cultured for VZV using multi-well plates. Briefly, two hundred microliters (200-µL) of each specimen were inoculated using one well per specimen. The inoculated cells were centrifuged at 700xg for 60-minutes, incubated at 35° to 37°C for up to 72-hours then stained in accordance with each respective product insert (DHI and Comparison device). All calculations for confidence intervals were done according to the Exact Method[b]. The results of this testing are summarized below (Table 6).

TABLE 6: Study Site 1 – D^3 DFA VZV Kit and Comparison Device in Multi-Well Plates.

D^3 DFA VZV Kit	Comparison Device +	Comparison Device –
+	**22**	0
–	0	**77**

	Agreed	95% CI
PPA =	100%	84.6% to 100%
NPA =	100%	95.2% to 100%

B. Study Site 2: A total of 35 specimens were cultured for VZV. Three fresh specimens from this Site were toxic to cell culture and were not evaluated. Briefly, 200-µL from the specimens was inoculated into duplicate shell-vials. The inoculated cells were incubated at 35° to 37°C for 72-hours then stained in accordance with the respective product insert (DHI and Comparison devices). All calculations for confidence intervals were done according to the Exact Method[b]. The results of this testing are summarized below (Table 7).

TABLE 7: Study Site 2 –D^3 DFA VZV Kit and Comparison Device in Shell-vials.

D^3 DFA VZV Kit	Comparison Device +	Comparison Device –
+	**11**	1
–	0	**20**

	Agreed	95% CI
PPA =	100%	71.5% to 100%
NPA =	95.1%	76.2% to 99.9%

C. Study Site 3: A total of 120 specimens were cultured for VZV. Briefly, two hundred microliters (200-µL) of each specimen was inoculated into one well per specimen of a multi-well plate and a single cell culture tube. The inoculated plates were centrifuged at 700xg for 60-minutes, incubated at 35° to 37°C for 72-hours then stained in accordance with each respective product insert (DHI and Comparison devices). The results of this testing are summarized in Table 8a. The inoculated tubes were read for CPE daily for 14-days. Tubes exhibiting CPE were scraped and cell spots made on multi-well slides according to the Comparison device's product insert procedure (the same procedure was used for both the DHI and the Comparison devices). Tubes exhibiting no CPE at 14-days were also scraped and cell spots made to confirm the absence of VZV. The cell spots were fixed with acetone in accordance with each respective product insert (DHI and Comparison device). All calculations for confidence intervals were done according to the Exact Method[b]. The results of this testing are summarized below (Table 8b).

TABLE 8a: Study Site 3 – D^3 DFA VZV Kit and Comparison Device in Multi-Well Plates

D^3 DFA VZV Kit	Comparison Device +	Comparison Device –
+	**8**	0
–	0	**112**

	Agreed	95% CI
PPA =	100%	63.1% to 100%
NPA =	100%	96.8% to 100%

TABLE 8b: Study Site 3 – D^3 DFA VZV Kit and Comparison Device in Tube Cultures.

D^3 DFA VZV Kit	Comparison Device +	Comparison Device –
+	**9**	0
–	0	**111**

	Agreed	95% CI
PPA =	100%	66.4% to 100%
NPA =	100%	96.7% to 100%

[a] "Positive Percent Agreement", or "PPA", values were calculated according to **{**[Total Number of Positive Results in Agreement by both DHI and Comparison Tests) divided by [(Total Number of Positive Results in Agreement by both DHI and Comparison Tests) plus (Number of Results Positive by the Comparison Test but Negative by the DHI test)]**}** multiplied by 100%.

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

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

D. Cross-Reactivity Testing

The Diagnostic Hybrids, Inc. D[3] DFA Varicella-zoster Virus Identification Kit VZV DFA Reagent was tested for cross-reactivity against a wide variety of cells and microorganisms. No cross-reactivity was observed for 55 virus strains (cultured and processed for staining) or for 20 host culture cell types. Twenty-seven (27) bacterial cultures and one (1) yeast 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 (Limitations of Procedure, Section VIII.) while all other bacterial cultures were negative. [See Tables below for cross-reactivity study results.]

Stringent conditions for cross-reactivity testing were achieved by using a high concentration of the VZV 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-five (55) virus strains were tested for cross-reactivity. Depending on the particular virus, 150 to 2100 TCID50 viruses were inoculated into a 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 detailed in the product insert. No cross-reactivity was observed for the viruses listed below (Table 10).

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

Organism	Strain or Type	Inoculum Concentration ($TCID_{50}$)	Organism	Strain or Type	Inoculum Concentration ($TCID_{50}$)
Adenovirus	Type 1	350	RSV	Long	350
Adenovirus	Type 5	350	RSV	Wash	350
Adenovirus	Type 6	350	RSV	9320	350
Adenovirus	Type 7	350	Parainfluenza 1	C-35	Commercially available slides stained. [d]
Adenovirus	Type 8	350	Parainfluenza 2	Greer	
Adenovirus	Type 10	350	Parainfluenza 3	C 243	
Adenovirus	Type 14	350	HSV-1	1F	150
Adenovirus	Type 18	350	HSV-1	CWOH 0026	150
Adenovirus	Type 31	350	HSV-1	CWOH 0015	150
Influenza A	Aichi	2,100	HSV-1	MacIntyre	150
Influenza A	Mal	2,100	HSV-2	MS	150
Influenza A	Hong Kong	2,100	HSV-2	Strain G	150
Influenza A	Denver	2,100	CMV	Towne	700
Influenza A	Port Chalmers	2,100	CMV	Davis	700
Influenza A	Victoria	2,100	CMV	AD169	700
Influenza A	PR	2,100	Echovirus	4	Commercially available slides stained. [d]
Influenza B	Hong Kong	350	Echovirus	6	
Influenza B	Maryland	350	Echovirus	9	
Influenza B	Mass	350	Echovirus	11	
Influenza B	Taiwan	350	Echovirus	30	
Influenza B	GL	350	Echovirus	34	
Influenza B	Russia	350	Coxsackievirus	B1	Commercially available slides stained. [d]
Poliovirus	Type 1	Commercially available slides stained. [d]	Coxsackievirus	B2	
Poliovirus	Type 2		Coxsackievirus	B3	
Poliovirus	Type 3		Coxsackievirus	B4	
Epstein-Barr	Commercially available slides stained. [d]		Coxsackievirus	B5	
Rubeola			Coxsackievirus	B6	
Mumps					

- Twenty (20) 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 the following cell lines as presented below (Table 11).

TABLE 11: Cell Lines Tested for Cross-Reactivity with VZV DFA Reagent

A549	Mv1Lu	RD
BGMK	HFF	RhMK II
HEp-2	McCoy	R-Mix
LLC-MK2	NCI-H292	Vero
MDCK	pCMK	WI-38
MRC-5	pRhMK	Vero 76
MRHF	pRK	

- Twenty-eight (28) microorganisms, including one (1) yeast culture and twenty-seven (27) bacterial cultures, 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 VZV 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 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: Bacteria and Yeast Tested for Cross-Reactivity with VZV 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	1.7×10^5
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	$\sim 6 \times 10^7$
Mycoplasma hominis	$\sim 6 \times 10^4$
Mycoplasma orale	$\sim 6 \times 10^4$
Mycoplasma pneumoniae	$\sim 6 \times 10^4$
Mycoplasma salivarium	$\sim 6 \times 10^7$
Ureaplasma urealyticum	$\sim 6 \times 10^4$
These were procured as prepared slides:	Proportion of cells reactive
Chlamydophila pneumoniae	10 to 50%
Chlamydia trachomatis	10 to 50%
YEAST	
Candida glabrata	8.7×10^6

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

XI. BIBLIOGRAPHY

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WARRANTY STATEMENT