D^3 IFA Enterovirus Identification Kit

REF: 01-050000

For *in vitro* Diagnostic Use

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

USA Toll-free: (866) 344-3477
Direct: +1(740) 589-3300
Fax: +1(740) 592-9820
Email: technical_services@dhiusa.com

US Patent Nos. 6,821,741 and 7,186,504 B2

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 IFA Enterovirus Identification Kit is intended for use in the qualitative identification of enteroviruses in cell cultures by immunofluorescence.

II. SUMMARY AND EXPLANATION OF THE TEST

Enteroviruses (genus Enterovirus, family *Picornaviridae*) are among the most common viruses infecting humans worldwide. Enteroviruses are small (approximately 30 nm), non-enveloped, single-stranded RNA viruses with an icosahedral capsid composed of 60 subunits consisting of four structural proteins (VP1 to VP4). Enteroviruses are associated with diverse clinical syndromes ranging from minor febrile illness to severe, potentially fatal conditions (e.g., aseptic meningitis, encephalitis, paralysis, myocarditis, and neonatal enteroviral sepsis) and could be linked with the development of some chronic diseases (e.g., type 1 diabetes and dilated cardiomyopathy)[1,2]. Each year, an estimated 10 to 15 million symptomatic enterovirus infections occur in the United States[3].

Human enteroviruses have traditionally been classified into echoviruses, coxsackieviruses group A and B, and polioviruses. This traditional taxonomy was based on the associated disease in humans and animal model systems, sometimes resulting in overlaps between groups and difficulties with classification. As a result, beginning in the 1960s, newly discovered enteroviruses received a numeric designation (e.g., enterovirus 71) instead of being assigned to one of the traditional groups[1,4].

Current taxonomy [4] takes into account molecular and biologic characteristics and divides human enteroviruses into four species (human enterovirus [HEV] A, B, C, and D) but keeps traditional names for individual serotypes. Because molecular techniques of enterovirus typing are becoming increasingly available, new enteroviruses continue to be identified, and enteroviruses 79 thru 101 have been recently described (CDC, unpublished data, 2005).

Echoviruses 22 and 23 have been reclassified as a new genus (*Parechovirus*) in *Picornaviridae* and are termed human parechoviruses 1 and 2, respectively [4,5]. Although they belong to genetically and biologically distinct genera, human parechoviruses and human enteroviruses share many epidemiologic and clinical characteristics[4].

III. PRINCIPLE OF THE PROCEDURE

The Diagnostic Hybrids, Inc. D^3 IFA Enterovirus Identification Kit uses a blend of enterovirus VP1 antigen-specific murine monoclonal antibodies (MAbs) that when combined with a fluorescein isothiocyanate labeled anti-mouse antibody allow rapid identification of enteroviral antigens in cell culture.

The cells to be tested, on a slide prepared from a conventional tube cell culture or a shell-vial monolayer, are fixed in acetone. The Enterovirus MAb Reagent is added to the cells. After incubating for 30-minutes at 35° to 37ºC, the stained cells are washed with the diluted Phosphate Buffered Saline (1X PBS). Anti-mouse Conjugate, which is labeled with fluorescein isothiocyanate (FITC), is added to the cells. After incubating for 30-minutes at 35 to 37ºC, the stained cells are washed again with fresh *1X PBS*. To prepare the slide for examination, a drop of the supplied Mounting Fluid is added to the stained cells and a coverslip is placed on the slide. To prepare the centrifuge enhanced cell cultures for examination, a drop of Mounting Fluid is placed on a clean microscope slide. The coverslip is removed from the shell-vial and placed on a drop of Mounting Fluid. For multi-well plates the Mounting Fluid is added to each well to cover the monolayers. The slides or wells are examined using a fluorescent microscope equipped with the correct filter combination for FITC at a magnification of 200 to 400X. Virus infected cells will be stained with bright apple-green fluorescence while non-infected cells will contain no apple-green fluorescence but will fluoresce a dull red from the Evans Blue counter-stain.[6]

IV. REAGENTS

A. Kit Components

1. **Enterovirus MAb Reagent**, 5-mL. One dropper bottle containing a blend of murine monoclonal antibodies directed against enteroviral antigens. The buffered, stabilized, aqueous solution contains 0.1% sodium azide as preservative.
2. **Anti-mouse Conjugate**, 5-mL. One dropper bottle containing FITC labeled anti-mouse antibodies. The buffered, stabilized, aqueous solution contains Evans Blue as a counter-stain and 0.1% sodium azide as preservative.
3. **Enterovirus Antigen Control Slides**, 5-slides. Five (5) individually packaged control slides containing wells with cell culture derived positive and negative control cells. Each positive well is identified as to the infected virus present, i.e., Echovirus, Coxsackie A, etc. 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).
5. **Mounting Fluid**, 7-mL. One dropper bottle of an aqueous, buffered, stabilized solution of glycerol and 0.1% sodium azide.

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 are handled in accordance with the OSHA Standard on Bloodborne Pathogens.
   a. Cell culture isolation may have some potential to be hazardous. Personnel working with cell cultures must be properly trained in safe handling techniques[7,8,9] 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. 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.
4. 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 Diagnostic HYBRIDS 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.

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. 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.

5. Evans Blue counter-stain is a potential carcinogen. If skin contact occurs, flush with water immediately.
6. The Enterovirus MAb Reagent and Anti-mouse Conjugate are supplied at working strength. Any dilution of these reagents will decrease sensitivity.
7. Reagents should be used prior to their expiration date.
8. Each Enterovirus Antigen Control Slide should be used only once. Do not re-use a Control Slide.
9. Microbial contamination of reagents may cause a decrease in sensitivity.
10. Store 1X PBS in a clean container to prevent contamination.
11. Never pipette reagents or clinical samples by mouth; avoid all contact of clinical samples with broken skin.
12. Avoid splashing and the generation of aerosols with clinical samples.
13. Use aseptic technique and sterile equipment and materials for all cell culture procedures.
14. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
15. Do not expose the Enterovirus MAb Reagent and Anti-mouse Conjugate 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	
Enterovirus MAb Reagent	Store at 2° to 8°C in the dark
Anti-mouse Conjugate	
Mounting Fluid	
Enterovirus Antigen Control Slides	Store at 2° to 8°C
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
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 Enterovirus MAb Reagent and Anti-mouse Conjugate 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 enterovirus 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.

For specimen collection and processing recommendations, refer to CLSI Approved Viral Culture Guidelines.[10]

A. Specimen Collection[11, 12]

Specimens accepted for enteroviral culture include: throat swabs or washes, cerebral spinal fluid (CSF), ocular tissue, vesicular or ulcerative lesion, and stool. Specimens should be received in viral transport medium. Specimens not received in viral transport medium should be transferred to a tube of transport medium immediately upon receipt.

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.[13] The specimens 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. Enterovirus MAb Reagent
2. Anti-mouse Conjugate
3. Enterovirus Antigen Control Slides
4. 40X PBS Concentrate
5. Mounting Fluid

B. Materials Required but not Provided

1. Fluorescence microscope with the correct filter combination for FITC (excitation peak = 490 nm, emission peak = 520 nm); magnification 200X to 400X.
2. Cell culture for enterovirus isolation. Suggested cell lines[14] include BGMK, A549, human diploid fibroblast, RD cells, Super E-Mix™ MixedCells™, and primary Rhesus monkey kidney cells (all are available from DHI).
3. Live control viruses for positive culture controls: Known strains of enterovirus 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. E-Mix™ Refeed Medium or other standard refeed medium (available from DHI).
7. Reagent-grade acetone (>99% pure) chilled at 2° to 8°C for fixation of prepared specimen slides, shell-vials and cultured cell preparations.
   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 (20º to 25ºC).
8. Sterile graduated pipettes: 10-mL, 5-mL, and 1-mL.
9. Sterile 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. Sterile 0.45-µm syringe filter.
13. Sterile 3-mL syringe.

14. 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 (e.g., mycology teasing needle) against a benchtop or with a pair of forceps taking care to avoid injury.
15. Sodium hypochlorite solution (1:10 final dilution of household bleach).
16. Humidified chamber (e.g., covered Petri dish with a damp paper towel placed in the bottom).
17. Glass microscope slides.
18. Acetone-cleaned multi-well glass microscope slides.
19. Blotters for multi-well glass microscope slides: Two-well absorbent blotters, used to blot excess liquid from the mask to prevent spread of liquid or stained cells from one well to the other.
20. Sterile nylon flocked swabs or polyester swabs, which are non-inhibitory to virues and cell culture.
21. Incubator, 35° to 37°C (5% CO_2 or non-CO_2, depending on the cell culture format used).
22. Centrifuge with free-swinging bucket rotor.
23. 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 (Section VI.B.6. Note 2).
24. Aspirator Set-up: Vacuum aspirator with disinfectant trap containing sufficient household bleach (5%) that the concentration is not decreased by more than 10-fold as it is diluted with discarded fluids.
25. Wash Container: Beaker, wash bottle or Coplin jar for washing slides.
26. Fixing Container: Coplin jar, slide dish or polyethylene holder for slides for use in fixing the cells on the slides.
27. Inverted Light Microscope: Used for examining the monolayers prior to inoculation and examination for toxicity, confluency and for cytopathic effects (CPE). It should have between 40X to 100X magnification capability.

C. Preliminary Comments, 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 in the incubator for equilibration to 35° to 37°C prior to staining. By doing this, the test slides and reagents will come to temperature quickly, yielding more rapid, intense staining.
5. Bring the Enterovirus MAb Reagent and Anti-mouse Conjugate 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 Enterovirus MAb Reagent or Anti-mouse Conjugate 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 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 Anti-mouse Conjugate.
    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. Morphologically, staining artifacts do not have the appearance of a complete cell and typically do not appear to be on the plane of the monolayer. Cell debris, lint, etc. can non-specifically adsorb the Anti-mouse Conjugate, resulting in highly intense fluorescence.
    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 Anti-mouse Conjugate 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 Anti-mouse Conjugate 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 Anti-mouse Conjugate 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 is viewed in a fluorescence microscope for an extended period of time.

D. Specimen Preparation

There are no special requirements for processing of specimens for enteroviral culture[15]. Specimens should be processed according to established laboratory procedure.

E. Cell Culture Testing – Tube Culture

1. Examine the monolayers for proper morphology prior to inoculation.
2. Aspirate maintenance medium from the monolayers and add 0.2- to 0.4-mL of each prepared specimen (Section VI.D. 'Specimen Preparation') to each of the cell lines used for enterovirus cultures.
3. Place the tubes at an angle sufficient for the monolayers to be covered by the inoculum. Place tubes in an incubator for 1 hour at 35º to 37ºC to allow virus adsorption to occur.
4. After adsorption, add 2-mL of appropriate refeed medium.
5. Incubate the tubes at 35º to 37ºC in a roller drum at 1 to 3 rpm or in a stationary rack at an angle sufficient for the monolayers to be covered by the inoculum and medium. Examine the monolayers daily for evidence of toxicity or viral CPE.
6. When the monolayers are ready to be stained, remove the medium by aspiration and gently rinse the monolayer two times with 1-mL 1X PBS.
7. Add 0.5-mL of 1X PBS to the tube and prepare a suspension of the cells by scraping the monolayer using a pipette and breaking the cell aggregates up by pipetting up and down several times.
8. Prepare cell spots using about 25-µL of the suspension onto an acetone cleaned slide. Repeat this step for each specimen.
9. Air dry the wells completely.
10. Fix the cells to the slides using fresh, chilled 100% acetone. Let 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 slides from the fixative and allow to air dry.
12. Add one drop of the Enterovirus MAb Reagent to completely cover the dried, fixed cells on the slide.
13. Place the slides in a covered chamber at 35º to 37ºC for 30-minutes.
14. 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.
15. Discard the used PBS and repeat the washing step using new 1X PBS.
16. Gently blot the excess liquid.
17. Add one drop of the Anti-mouse Conjugate to completely cover the dried, fixed cells on the slide.
18. Place the slides in a covered chamber at 35º to 37ºC for 30-minutes.
19. 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.
20. Discard the used PBS and repeat the washing step using new 1X PBS.

21. 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.
22. Gently blot the excess liquid.
23. Add a small drop of Mounting Fluid to each cell-containing well and cover the wells with a coverslip.
24. Examine the stained, mounted cells using a fluorescence microscope with magnifications between 200X to 400X (Section VI.C.11-14, 'Immunofluorescence Microscopy').
25. Refer to Section VII. 'Interpretation of Results'.

F. Cell Culture Testing – Shell-vial

1. Calculate the number of shell-vials needed based on the staining protocol to be used (this staining protocol requires 2 shell-vials):
   a. One shell-vial is required for each day the culture will be screened with the D^3 IFA Enterovirus Identification Kit (i.e., staining at 48- to 72-hours, and then at 5- to 7-days, requires 2 shell-vials).
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 Enterovirus MAb 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 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 Enterovirus MAb Reagent to the fixed monolayers of patient and control samples, and rock to ensure complete coverage of the monolayer by the MAb Reagent.
15. Place stoppered shell-vials in a 35º to 37ºC incubator for 30-minutes.
16. Aspirate the Enterovirus MAb 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 4-drops of the Anti-mouse Conjugate to the fixed monolayers of patient and control samples, and rock to ensure complete coverage of the monolayer by the Conjugate.
20. Place stoppered shell-vials in a 35º to 37ºC incubator for 30-minutes.
21. Aspirate the Anti-mouse Conjugate from the monolayers.
22. Add 1-mL of the 1X PBS.
23. Remove the 1X PBS by aspiration; repeat the wash step, and again remove by aspiration.
24. Add 1-mL of de-mineralized water.
25. Remove the de-mineralized water by aspiration.
26. 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.
27. Examine the stained monolayers using a fluorescence microscope with magnifications between 200X to 400X (Section VI.C.11-14, 'Immunofluorescence Microscopy').
28. Refer to Section VII. 'Interpretation of Results'.

G. Cell Culture Testing – Multi-well Plate

1. Calculate the number of wells needed for the staining protocol to be used (this staining protocol requires 2-wells):
   a. One well is required for each day the culture will be screened with the D^3 IFA Enterovirus Identification Kit (i.e., staining at 48- to 72-hours, and again at 5- to 7-days, requires 2 wells).
   b. It is recommended that each replicate well be on a different multi-well plate. This allows each plate to be processed on the appropriate day.
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 wells 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 35º to 37ºC incubator with a humidified, 5% CO_2 atmosphere.
7. When a monolayer is ready to be stained using the Enterovirus MAb 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% aqueous 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 completely.
14. To each well of a 24-well plate, add 4-drops of the Enterovirus MAb Reagent to the fixed monolayers of patient and control samples; to each well of a 48-well plate, add 3-drops of the Enterovirus MAb Reagent to the fixed monolayers of patient and control samples. Rock to ensure complete coverage of the monolayer by the MAb Reagent.
15. Place the covered multi-well plate in a 35º to 37ºC, humidified incubator for 30-minutes.
16. Aspirate the Enterovirus MAb Reagent from the monolayers.
17. Add 1-mL of the 1X PBS and mix to wash.
18. Remove 1X PBS by aspiration; repeat the wash step, and again remove by aspiration.
19. To each well of a 24-well plate, add 4-drops of the Anti-mouse Conjugate to the fixed monolayers of patient and control samples; to each well of a 48-well plate, add 3-drops of the Anti-mouse Conjugate to the fixed monolayers of patient and control samples. Rock to ensure complete coverage of the monolayer by the Conjugate.
20. Place the covered multi-well plate in a 35º to 37ºC, humidified incubator for 30-minutes.
21. Aspirate the Anti-mouse Conjugate from the monolayers.
22. Add 1-mL of the 1X PBS and mix to wash.
23. Remove the 1X PBS by aspiration, repeat the wash step, and again remove by aspiration.
24. Add 1-mL of de-mineralized water.
25. Remove the de-mineralized water by aspiration.
26. Add 2- to 3-drops of Mounting Fluid to each monolayer, then cover the plate.
27. Examine the stained monolayers using a fluorescence microscope with magnifications between 200X to 400X (Section VI.C.11-14, 'Immunofluorescence Microscopy').
28. Refer to Section VII. 'Interpretation of Results'.

H. Quality Control

1. Reagents
   a. A fresh Enterovirus Antigen Control Slide should be stained each time the staining procedure is performed to ensure proper test performance.
   b. The positive wells will show multiple infected cells of bright apple-green fluorescence with negative cells staining 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 fluorescence 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 enterovirus controls should be run with each new batch of cells to confirm their performance in culturing enteroviruses.
   b. To ensure viral sensitivity, enterovirus-inoculated control monolayers should be included each time a new lot of cell culture is used.
   c. A non-inoculated monolayer from each lot should be stained to serve as a negative control. 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 enterovirus is one in which bright apple-green fluorescence is observed in the infected cells.

3. Non-infected cells will fluoresce dull red due to the Evans Blue counter-stain included in the Anti-mouse Conjugate.
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 enterovirus-specific fluorescent cells. If no fluorescent cells are found, report: "No enterovirus isolated by cell culture."
3. If enterovirus-specific fluorescence is observed, report as "Enterovirus isolated by cell culture."

VIII. LIMITATIONS OF PROCEDURE

1. Inappropriate specimen collection, storage, and transport may lead to false negative culture results[16].
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. Since the MAbs have been prepared using defined virus strains, they may not detect all antigenic variants or new strains of the enteroviruses, should they arise. MAbs may fail to detect strains of viruses which have undergone minor amino acid changes in the target epitope region.
5. A negative result on a direct or cultured specimen does not rule out the presence of virus.
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 Anti-mouse Conjugate under bright light will decrease the staining intensity.
8. Limited cross-reactivity was seen with Adenovirus types 11 and 16.
9. Light background staining may occur with specimens contaminated with *Staphylococcus aureus* 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.
10. Any **nuclear** non-specific fluorescence present in cultured cells or on antigen control slides should be interpreted as negative for enterovirus.

IX. SPECIFIC PERFORMANCE CHARACTERISTICS

A. Enterovirus reactivity

A total of 224 clinical enterovirus isolates were re-cultured and tested with the D^3 IFA Enterovirus Identification Kit at two locations (Site 1: 168, Site 2: 56). The identity of each isolate was determined by neutralization. The D^3 IFA Enterovirus Identification Kit correctly identified all enterovirus isolates when compared to the neutralization results. The data is summarized in Table 2.

TABLE 2: Enterovirus isolates correctly identified by D^3 IFA Enterovirus Identification Kit compared to neutralization results

Enterovirus Type	# of Isolates	Enterovirus Type	# of Isolates
Coxsackie A9	20	Echovirus 9	17
Coxsackie A16	5	Echovirus 11	20
Coxsackie A24	1	Echovirus 13	1
Coxsackie B1	6	Echovirus 14	4
Coxsackie B2	20	Echovirus 15	1
Coxsackie B3	11	Echovirus 18	5
Coxsackie B4	9	Echovirus 21	1
Coxsackie B5	16	Echovirus 24	2
Coxsackie B6	1	Echovirus 25	2
Echovirus 3	3	Echovirus 30	18
Echovirus 4	8	Enterovirus 70	2
Echovirus 5	8	Enterovirus 71	5
Echovirus 6	10	Poliovirus type 1	4
Echovirus 7	12	Poliovirus type 2	5
		Poliovirus type 3	6

B. Enterovirus Specificity

Diagnostic Hybrids, Inc. D^3 IFA Enterovirus Identification Kit was tested for cross-reactivity against a wide variety of cells and microorganisms. No cross-reactivity was observed for 59 virus strains (cultured and processed for staining) or for 17 host culture cell types. Thirty-two (32) bacterial cultures 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 (Section VIII.9, 'Limitations of Procedure') while all other bacterial cultures were negative. (Tables 3, 4 and 5 below list the organisms which were tested for cross-reactivity with the Enterovirus MAb Reagent.)

- Forty-one (41) virus strains were tested for cross-reactivity. No cross reactivity was observed for the viruses listed below.

TABLE 3: Virus strains used in cross-reactivity study

Adenovirus	Type 1	RSV	Long
	Type 3		Wash
	Type 5		9320
	Type 6	Parainfluenza 1	C-35
	Type 7	Parainfluenza 2	Greer
	Type 14	Parainfluenza 3	C 243
	Type 18	HSV-1	1F
Influenza A	Aichi		CWOH 0026
	Mal		CWOH 0015
	Hong Kong		MacIntyre
	Denver	HSV-2	MS
	Port Chalmers		Strain G
	Victoria	CMV	Towne
	New Jersey		Davis
	PR		AD169
Influenza B	Hong Kong	Varicella	Webster
	Maryland		Ellen
	Mass	Mumps	Bion, (CDC V5-004)
	Taiwan	Rubeola	Bion
	GL	Epstein Barr	Bion
	Russia		

- Eighteen (18) host culture cells were tested for cross-reactivity; no cross-reactivity was observed for the following:

TABLE 4: Cell lines used in cross-reactivity study

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

- Thirty-two (32) bacterial cultures were stained and examined for cross-reactivity, including *Staphylococcus aureus*, a protein A producing bacterium ('Section VIII.9, Limitations of Procedure'). Staining of *S. aureus* appeared as small points of fluorescence while all other cultures were negative.

TABLE 5: Bacteria used in cross-reactivity study

Acholeplasma laidlawii	Mycoplasma hominis
Acinetobacter calcoaceticus	Mycoplasma orale
Bordetella bronchiseptica	Mycoplasma pneumoniae
Bordetella pertussis	Mycoplasma salivarium
Candida glabrata	Neisseria gonorrhoeae
Chlamydia psittaci	Peptostrepococcus anaerobius
Chlamydia trachomatis	Proteus mirabilis
Clostridium diphtheriae	Pseudomonas aeruginosa
Escherichia coli	Salmonella enteriditis
Gardnerella vaginalis	Salmonella typhimurium
Haemophillus influenzae type A	Staphylococcus aureus
Klebsiella pneumoniae	Streptococcus agalactiae
Legionella pneumophila	Streptococcus pneumoniae
Moraxella cartarrhalis	Streptococcus pyogenes
Mycobacterium avium	Trichomonas vaginalis
Mycobacterium intracellulare	Ureaplasma urealyticum

Stringent conditions for cross-reactivity testing were achieved by using a high concentration of primary monoclonal antibody and high titers of microorganisms. The primary Enterovirus MAb Reagent was prepared at 1.5X the concentration that is provided in the kit. The fluorescein-labeled goat anti-mouse secondary reagent (Anti-mouse Conjugate) was used as supplied in the kit. Each of the tested viruses was prepared as infected cell monolayers (250 infected cells inoculated into a shell-vial culture and incubated for 24- to 48-hours, to yield a 3+ to 4+ infection), and processed and stained with the 1.5X Enterovirus MAb Reagent according to the procedure detailed in this product insert. Bacterial strains were cultured, processed as suspensions, then spotted on microscope slides (yielding > 150 bacteria per 400X microscope field), then stained with the

1.5X Enterovirus MAb Reagent according to the procedure in this product insert. Cell cultures were stained as confluent monolayers.

X. BIBLIOGRAPHY

1 Pallansch MA, Roos RP. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Knippe DM, Howley PM, eds. Fields Virology. 4th ed. Philadelphia, PA: Lippincott Williams and Wilkins; 2001:723-775.

2 Khetsuriani N, Parashar UD. Enteric viral infections. In: Dale DC, Federman DD, eds. Scientific American medicine. New York, NY: WebMD, Inc.; 2003:1758-1766.

3 Strikas RA, Anderson L, Parker RA. Temporal and geographic patterns of isolates of nonpolio enteroviruses in the United States, 1970-1983. J Infect Dis 1986;153:346-351

4 Stanway G, Brown F, Christian P, et al. *Picornaviridae*. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, eds. Virus taxonomy---classification and nomenclature of viruses. 8th report of the International Committee on the Taxonomy of Viruses. Amsterdam, The Netherlands: Elsevier Academic Press; 2005:757-778.

5 Stanway G, Joki-Korpela P, Hyypia T. Human parechoviruses---biology and clinical significance. Rev Med Virol 2000;10:57-69.

6 Specter, S., Hodinka, R. L., and Young, S.A. 2000, *Clinical Virology Manual*, Washington D.C., ASM Press, 420-424.

7 *Biosafety in Microbiological and Biomedical Laboratories* (BMBL), 5th edition, 2007, CDC-NIH manual. [http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm]

8 *Biosafety Manual*, 3rd edition, 2004. World Health Organization [Manual may be available in additional languages; refer to WHO web page http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/]

9 *Laboratory Biosafety Guidelines*, 3rd edition, 2004. Published by authority of the Minister of Health, Population and Public Health Branch, Centre for Emergency Preparedness and Response [Guideline is available in French or English; refer to web page [http://www.phac-aspc.gc.ca/publicat/lbg-ldmbl-04/index.html]

10 Clinical and Laboratory Standards Institute. *Viral Culture; Approved Guidelines.* CLSI document M41-A [ISBN 1562386239]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA 2006.

11 Eisenberg, Henry D. 1992. Clinical Microbiology Procedures Handbook, published by American Society for Microbiology, Washington DC, p 8.2.3.

12 Clinical and Laboratory Standards Institute. *Viral Culture: Proposed Guideline.* CLSI document M41-P. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne Pennsylvania 19087-1898 USA, 2006. pp. 15 - 17

13 Clinical and Laboratory Standards Institute. *Viral Culture; Approved Guidelines.* CLSI document M41-A [ISBN 1562386239]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA 2006, Section 7.4.

14 IBID, pg. 28

15 IBID, pp. 22 - 25

16 Leland, Diane S. (1996). *Clinical Virology,* published by W.B. Saunders, Philadelphia, PA.

WARRANTY STATEMENT

These products are warranted to perform as described in their labeling and the Diagnostic Hybrids, Inc. literature when used in accordance with their instructions. THERE ARE NO WARRANTIES WHICH EXTEND BEYOND THIS EXPRESS WARRANTY AND DIAGNOSTIC HYBRIDS, INC. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR WARRANTY OF FITNESS FOR PARTICULAR PURPOSE. Diagnostic Hybrids, Inc. sole obligation and purchaser's exclusive remedy for breach of this warranty shall be, at the option of Diagnostic Hybrids, Inc. to repair or replace the products.