QUIDEL

D3® Enterovirus 71 DFA REAGENT

For the qualitative identification of Enterovirus 71 in cell cultures by immunofluorescence.

FOR *IN VITRO* DIAGNOSTIC USE

℞ ONLY

INTENDED USE

The Diagnostics Hybrids, Inc. D[3] Enterovirus 71 DFA Reagent is intended for use in the qualitative identification of Enterovirus 71 in cell cultures by immunofluorescence.

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), nonenveloped, 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]

Hand, foot, and mouth disease (HFMD) is a common viral illness of infants and children. The disease causes fever and blister-like eruptions in the mouth and/or a skin rash. Enteroviruses, including Enterovirus 71, have been associated with HFMD and with outbreaks of the disease.[1,4]

PRINCIPLE OF THE PROCEDURE

The D[3] Enterovirus 71 DFA Reagent uses specific Enterovirus 71 murine monoclonal antibodies (MAb) labeled with fluorescein isothiocyanate for rapid identification of Enterovirus 71 antigens in cell culture.

The cells to be tested, on a slide prepared from a conventional tube cell culture or a shell vial monolayer or multiwell plate, are fixed in acetone. The Enterovirus FITC-labeled MAb reagent is added to the cells. After incubating for 15 to 30 minutes at 35°C to 37°C, the stained cells are washed with PBS (phosphate buffered

saline; may be ordered from Quidel). To prepare the slide for examination, a drop of Mounting Fluid (may be ordered from Quidel) 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 multiwell 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 100x to 400X. Virus infected cells will be stained in a **cytoplasmic** pattern with bright apple-green fluorescence while uninfected cells will contain no apple-green fluorescence but will fluoresce red from the Evans Blue counter-stain.[5]

REAGENTS AND MATERIALS PROVIDED

The D³ IFA Enterovirus Identification Kit contains the following:

D³ Enterovirus 71 DFA Reagent 5 mL

One dropper bottle containing two FITC-labeled murine monoclonal antibodies directed against an Enterovirus 71 antigen. The buffered, stabilized, aqueous solution contains Evans Blue and 0.1% sodium azide as preservative.

MATERIALS REQUIRED BUT NOT PROVIDED

- Fluorescence microscope with the correct filter combination for FITC (excitation peak = 490 nm, emission peak = 520 nm); magnification 200X to 400X.
- 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 Quidel).
- Live control viruses for positive culture controls: Known strains of Enterovirus 71 for use in monitoring the cell culture and staining procedures. Such control virus strains can be obtained from Quidel.
- Enterovirus 71/Coxsackievirus A16 Antigen Control Slides (01-00200, 10-slides; available from Quidel). 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, Enterovirus 71 or Coxsackievirus A16. The negative well contains non-infected cells. Each slide is intended to be stained only one time.
- 40X PBS Concentrate (01-090025, 25 mL; available from Quidel). 40X PBS concentrate consisting of 4% sodium azide (0.1% sodium azide after dilution to 1X using de-mineralized water)
- Mounting Fluid (01-002007b, 7 mL; available from Quidel). An aqueous, buffered, stabilized solution of glycerol and 0.1% sodium azide.
- E-Mix Refeed Medium or other standard refeed medium (available from Quidel).
- Cover slips (22 x 50mm) for antigen control slides and for specimen slides
- Universal Transport Medium (available from Quidel)
- Reagent-grade acetone (> 99% pure) chilled at 2°C to 8°C for fixation of prepared specimen slides, shell-vials and cultured cell preparations.

  NOTES:
  - Keep the reagent-grade acetone container tightly sealed to avoid hygroscopic absorption of water, which may cause a hazy, non-specific, background fluorescence.
  - A mixture of 80% acetone/20% demineralized water must be used for fixing cells in plastic multi-well plates. Store at ambient temperature (20°C to 25°C).
- Sterile graduated pipettes: 10 mL, 5 mL, 1 mL
- Sterile Pasteur pipettes or other transfer pipettes

  Caution: One should not use solvents such as acetone with polystyrene pipettes.
- Fine-tipped forceps

- Wash bottle, 200 mL
- Sterile 0.45 µm syringe filter
- Sterile 3 mL syringe
- Bent-tip teasing needle (useful 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 bench top or with a pair of forceps taking care to avoid injury.
- Sodium hypochlorite solution (1:10 final dilution of household bleach)
- Humidified chamber (e.g., covered Petri dish with a damp paper towel placed in the bottom)
- Glass microscope slides
- Acetone-cleaned multi-well glass microscope slides
- 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.
- Sterile nylon flocked swabs or polyester swabs, which are non-inhibitory to viruses and cell culture
- Incubator, 35°C to 37°C (5% CO_2 or non-CO_2, depending on the cell culture format used)
- Centrifuge with free-swinging bucket rotor
- Demineralized water for dilution of 40X PBS Concentrate and for dilution of the reagent-grade acetone for use in polystyrene multi-well plates
- Aspirator Set-up: Vacuum aspirator with disinfectant trap containing sufficient household bleach (5% sodium hypochlorite) that the concentration is not decreased by more than 10-fold as it is diluted with discarded fluids.
- Wash Container: Beaker, wash bottle or Coplin jar for washing slides
- Fixing Container: Coplin jar, slide dish or polyethylene holder for slides for use in fixing the cells on the slides
- 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

WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use.
- 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.
  - Cell culture isolation may have some potential to be hazardous. Personnel working with cell cultures must be properly trained in safe handling techniques[6,7,8] and have experience with cell culture before attempting this procedure.
  - 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.
- All specimens and materials used to process them should be considered potentially infectious and handled in a manner which prevents infection of laboratory personnel.
  - Biosafety Level 2 or other appropriate biosafety practices should be used when handling these materials.
  - Decontamination of specimens and cultures is most effectively accomplished using a solution of sodium hypochlorite (1:10 final dilution of household bleach).
  - 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.

- 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.
- Sodium azide is used as a preservative (0.1%) in the D^3 Enterovirus 71 DFA Reagent.
  - Aqueous solutions of sodium azide, when mixed with acids, may liberate toxic gas.
  - Avoid disposal of these solutions down sanitary or industrial plumbing systems. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.
  - Avoid release to the environment.
- Evans Blue counter-stain is a potential carcinogen. If skin contact occurs, flush with water immediately.
- The D^3 Enterovirus 71 DFA Reagent is supplied at working strength. Any dilution of this reagent will decrease sensitivity.
- Reagents should be used prior to their expiration date.
- Each Enterovirus 71/Coxsackievirus A16 Antigen Control Slide (01-00201) should be used only once. Do not re-use a Control Slide.
- Microbial contamination of reagents may cause a decrease in sensitivity.
- Store 1X PBS in a clean container to prevent contamination.
- Never pipette reagents or clinical samples by mouth; avoid all contact of clinical samples with broken skin.
- Avoid splashing and the generation of aerosols with clinical samples.
- Use aseptic technique and sterile equipment and materials for all cell culture procedures.
- Reusable glassware must be washed and thoroughly rinsed free of all detergents.
- Do not expose the D^3 Enterovirus 71 DFA Reagent to bright light during staining or storage.
- Use of other reagents than those specified with the components of this kit may lead to erroneous results.
- Testing should be performed in an area with adequate ventilation.
- Dispose of containers and unused contents in accordance with Federal, State and Local regulatory requirements.
- Wear suitable protective clothing, gloves, and eye/face protection when handling the contents of this kit.
- Wash hands thoroughly after handling.
- For additional information on hazard symbols, safety, handling and disposal of the components within this kit, please refer to the Safety Data Sheet (SDS) located at quidel.com.

Storage

Table 1. Reagent Storage Conditions

D^3 Enterovirus 71 DFA Reagent	Store at 2°C to 8°C in the dark.

Stability

Reagent 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 D^3 Enterovirus 71 DFA Reagent should be kept to a minimum.

Discard 1X PBS if it becomes cloudy.

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.[9]

Collection[10,11]

Specimens acceptable for Enterovirus 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.

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°C to 8°C. This temperature can be attained by using cold packs, wet ice, foam refrigerant, or other coolants.[12] The specimens should be processed and tested as soon as possible and then stored at 2°C to 8°C.

Specimens should be stored at 2°C 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.

Specimen Preparation

There are no special requirements for processing of specimens for Enterovirus 71 culture.[13] Specimens should be processed according to established laboratory procedure.

PROCEDURE

Preliminary Comments and Precautions

- Adhere to the recommended volumes and times in the following procedure to ensure that accurate results are obtained.
- 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.
- 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.
- Place the closed, humidified chamber for holding slides during staining in the incubator for equilibration to 35°C to 37°C prior to staining. By doing this, the test slides and reagents will come to temperature quickly, yielding more rapid, intense staining.
- Bring the D^3 Enterovirus 71 DFA Reagent to ambient temperature (20°C to 25°C) prior to use, and immediately return to refrigerator after use for storage at 2°C to 8°C.

Regarding Cell Culture Testing

- 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.
- 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.

- 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.
- Do not allow the monolayers to dry before fixing; this can lead to high background staining and decreased sensitivity.
- Do not allow the D^3 Enterovirus 71 DFA Reagent to dry on the monolayers; this can lead to high background.

Regarding Immunofluorescence Microscopy

- 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.
- Three aspects of the fluorescence microscope must be functioning properly and optimally in order to achieve maximum brightness of fluorescence:
  - The activation light source has a finite life and as it ages, its output decreases, resulting in lower fluorescence intensity from the DFA Reagent.
  - The light source is focused by a number of lenses and mirror(s). For maximum intensity, these must be properly aligned.
  - The filters used in the light path must be appropriate for the particular fluor, in this case, fluorescein.
- Fluorescent artifacts may be observed in the cell monolayers:
  - 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 DFA Reagent, resulting in highly intense fluorescence.
  - 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.
  - 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.
  - Inadequate washing can lead to general low grade fluorescence due to residual DFA Reagent remaining on the monolayer of cells.
- Protect stained slides and monolayers from light as much as possible during testing.
  - Bleaching or fading of the fluorescence of stained cells may occur on exposure to light, particularly light of high intensity.
  - This bleaching can occur when a stained cell is viewed in a fluorescence microscope for an extended period of time.

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 0.5 mL of each prepared specimen (see *Specimen Preparation*) to each of the cell culture types that are being used for Enterovirus 71 culture.
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 35°C to 37°C to allow virus adsorption to occur.
4. After adsorption, add 2 mL of appropriate refeed medium.
5. Incubate the tubes at 35°C to 37°C 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°C 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 71 Reagent to completely cover the dried, fixed cells on the slide.
13. Place the slides in a covered chamber at 35°C to 37°C 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 PBS. For many slides, a slide carrier that holds 10 to 20 slides can be placed in its container of 1X PBS. For effective rinsing, dip the slide(s) up and down several times.
15. Discard the used PBS and repeat the washing step using new 1X PBS.
16. 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
17. Gently blot the excess liquid.
18. Add a small drop of Mounting Fluid to each cell-containing well and cover the wells with a coverslip.
19. Examine the stained, mounted cells using a fluorescence microscope with magnifications between 200X to 400X (See *Regarding Immunofluorescence Microscopy*.)
20. Refer to *INTERPRETATION OF RESULTS*.

Cell Culture Testing – Shell Vial

1. Calculate the number of vials needed based on the staining protocol to be used (this staining protocol requires 2 vials):
   - One vial is required for each day the culture will be screened with the D^3 Enterovirus 71 DFA Reagent (i.e., staining at 48 to 72 hours, and then at 5 to 7 days, requires 2 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°C to 25°C.
6. Place stoppered shell-vials in an incubator at 35°C to 37°C.
7. When a monolayer is ready to be stained using the Enterovirus 71 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°C 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 D^3 Enterovirus 71 DFA Reagent to the fixed monolayers of patient and control samples, and rock to **ensure complete coverage** of the monolayer by the DFA Reagent.
15. Place stoppered shell-vials in a 35°C to 37°C incubator for 30 minutes.
16. Aspirate the D^3 Enterovirus 71 DFA Reagent from the monolayers.
17. Add 1 mL of the 1X PBS.
18. Remove the 1X PBS by aspiration; repeat the wash step, and again remove by aspiration.
19. Add 1 mL of 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 *Regarding Immunofluorescence Microscopy*.)
23. Refer to *INTERPRETATION OF RESULTS*.

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):
   - One well is required for each day the culture will be screened with the D^3 Enterovirus 71 DFA Reagent (i.e., staining at 48 to 72 hours, and then at 5 to 7 days, requires 2 wells).
   - 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°C to 25°C.
6. Place the covered multi-well plates in a 35°C to 37°C incubator with a humidified, 5% CO_2 atmosphere.
7. When a monolayer is ready to be stained using the Enterovirus 71 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°C 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 D^3 Enterovirus 71 DFA Reagent to the fixed monolayers of patient and control samples; to each well of a 48-well plate, add 3 drops of the D^3 Enterovirus 71 DFA Reagent to the fixed monolayers of patient and control samples. Rock to **ensure complete coverage** of the monolayer by the DFA Reagent.
15. Place the covered multi-well plate in a 35°C to 37°C, humidified incubator for 30 minutes.
16. Aspirate the D^3 Enterovirus 71 DFA Reagent from the monolayers.
17. Add 1 mL of the 1X PBS and mix to wash.
18. Remove the 1X PBS by aspiration, repeat the wash step, and again remove by aspiration.
19. Add 1 mL of demineralized water.
20. Remove the demineralized water by aspiration.
21. Add 2 to 3 drops of Mounting Fluid to each monolayer, then cover the plate.
22. Examine the stained monolayers using a fluorescence microscope with magnifications between 200X to 400X. (See *Regarding Immunofluorescence Microscopy*.)
23. Refer to *INTERPRETATION OF RESULTS*.

QUALITY CONTROL

Reagents

- A fresh Enterovirus 71 / Coxsackievirus A16 Antigen Control Slide should be stained each time the staining procedure is performed to ensure proper test performance.
- 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.
- The negative well will show only negative cells staining a dull red.
- 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.

Cell Culture

- Positive and negative enterovirus controls should be run with each new batch of cells to confirm their performance in culturing enteroviruses.
- To ensure viral sensitivity, enterovirus-inoculated control monolayers should be included each time a new lot of cell culture is used.
- 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.
- If control cultures fail to perform correctly, results are considered invalid.

INTERPRETATION OF RESULTS

Examination of Samples and Controls

- Examine controls first to ensure proper test performance before examining patient specimens.
- A positive reaction for enterovirus is one in which bright apple-green fluorescence is observed in the infected cells.
- Non-infected cells will fluoresce dull red due to the Evans Blue counter-stain included in the D^3 Enterovirus 71 DFA Reagent.
- Examine the entire cell spot or monolayer of cells before reporting final negative results. Do not report results for patient samples unless controls perform as expected.

Artifacts of Staining

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

Results from Culture Isolation/Confirmation

- The bright apple-green fluorescence staining pattern is **cytoplasmic**.
- 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.” If enterovirus-specific fluorescence is observed, report as “Enterovirus 71 isolated by cell culture.”

LIMITATIONS OF THE PROCEDURE

- Inappropriate specimen collection, storage, and transport may lead to false negative culture results.[15]
- Incubation times or temperatures other than those cited in the test instructions may give erroneous results.

- 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.
- Since the MAbs have been prepared using defined strains, they may not detect all antigenic variants or new strains of the Enterovirus 71, should they arise. MAbs may fail to detect strains of viruses which have undergone minor amino acid changes in the target epitope region.
- A negative result does not rule out the presence of virus.
- Performance of the kit can only be assured when components used in the assay are those supplied by Quidel.
- Prolonged storage of the D^3 Enterovirus 71 DFA Reagent under bright light will decrease the staining intensity.
- Light background staining may occur with specimens contaminated with *Staphylococcus aureus* strains containing large amounts of protein A. Protein A will nonspecifically 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.
- Any *nuclear* non-specific fluorescence present in cultured cells or on antigen control slides should be interpreted as negative for Enterovirus 71.

SPECIFIC PERFORMANCE CHARACTERISTICS

Enterovirus Reactivity

D^3 Enterovirus 71 DFA Reagent was tested for cross-reactivity against a wide variety of cells and microorganisms. No cross-reactivity was observed for 83 virus strains (cultured and processed for staining) or for 18 host culture cell types. Nineteen (19) microorganisms, including 18 bacteria, and 1 yeast were tested for cross-reactivity. 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 (see *LIMITATIONS OF PROCEDURE*) while all other bacterial cultures were negative. (Table 2 below lists the organisms which were tested for cross-reactivity with the D^3 Enterovirus 71 DFA Reagent.)

Stringent conditions for cross-reactivity testing were achieved by using a high concentration of primary monoclonal antibody and high titers of microorganisms. The D^3 Enterovirus 71 DFA Reagent was prepared at 2X the concentration that is provided in the kit. Each of the tested viruses was prepared as infected cell monolayers (1000-$TCID_{50}$ viruses were inoculated into permissive multi-well plate cultures and incubated for 24 hours, to yield a 2+ to 4+ cytopathic effect, processed and stained with 2X subject. Stained cells were examined at 200X magnification.), and processed and stained with the 2X D^3 Enterovirus 71 DFA Reagent according to the procedure detailed in this product insert.

The adenovirus and parainfluenza viruses were inoculated in to R-Mix™ cultures at 1000-$TCID_{50}$ for a 24-hour incubation time at 37°C. The infected cultures were acetone-fixed and stained with a 2X concentration of the subject device for 30 minutes at 37°C. Afterwards, the stained cultures are examined at 200X magnification for any fluorescent staining cells. The adenovirus infections were verified using the D^3 Ultra™ Respiratory Virus individual typing reagents. For parainfluenza 4a and 4b, an in-house RUO MAb was used as the positive control.

Bacteria strains were tested by propagating ATCC derived stocks on nutrient agar plates. These organisms were grown on an agar plates then streaked onto fresh plates for colony isolation. Individual colonies were selected and diluted in PBS to a desired turbidity of ~4 by MacFarland standards. Eight-well blank slides were spotted at CFUs (colony forming units) ranging from 2.3 $x10^5$ to 5.2 $x10^{10}$/well at 10 µL per well and allowed to

air dry (~30 minutes). These slides were fixed with 100% acetone for 10 minutes then air dried. The D^3 Enterovirus 71 DFA Reagent at 2X concentration was tested in duplicate on the prepared slides. After 1 hour at 37°C, the slides were rinsed with PBS then dotted with mounting fluid. The slides were examined at 400X using a fluorescence microscope for fluorescence. Table 2 summarizes the results.

Table 2. Cross-reactivity Testing

Organism	Strain or Type	D^3 Enterovirus 71 DFA Reagent at 2X concentration*	Concentrations of targets (viruses: $TCID_{50}$ inoculum level; bacteria: CFU)
Viruses			
Adenovirus	Type 1 VR-1	—	1000-$TCID_{50}$
	Type 3 VR-3	—	1000-$TCID_{50}$
	Type 5 VR-5	—	1000-$TCID_{50}$
	Type 6 VR-6	—	1000-$TCID_{50}$
	Type 7 VR-7	—	1000-$TCID_{50}$
	Type 8 VR-1366	—	1000-$TCID_{50}$
	Type 10 VR-1087	—	1000-$TCID_{50}$
	Type 11 Clinical Isolate	—	1000-$TCID_{50}$
	Type 13 VR-14	—	1000-$TCID_{50}$
	Type 14 VR-15	—	1000-$TCID_{50}$
	Type 17 Clinical Isolate	—	1000-$TCID_{50}$
	Type 18 VR-19	—	1000-$TCID_{50}$
	Type 31 VR-1109	—	1000-$TCID_{50}$
Influenza A	Clinical Isolate	—	Control Slide
	Aichi VR-547	—	1000-$TCID_{50}$
	Malaya VR-98	—	1000-$TCID_{50}$
	Hong Kong VR-544	—	1000-$TCID_{50}$
	Denver VR-546	—	1000-$TCID_{50}$
	Port Chalmers VR-810	—	1000-$TCID_{50}$
	Victoria VR-822	—	1000-$TCID_{50}$
	New Jersey VR-897	—	1000-$TCID_{50}$
	WS VR-1520	—	1000-$TCID_{50}$
	PR VR-95	—	1000-$TCID_{50}$
Influenza B	Clinical Isolate	—	Control Slide
	Hong Kong VR-791	—	1000-$TCID_{50}$
	Maryland VR-296	—	1000-$TCID_{50}$
	Mass VR-523	—	1000-$TCID_{50}$
	GL VR-102	—	1000-$TCID_{50}$
	Taiwan VR-295	—	1000-$TCID_{50}$
	JH-001 Clinical Isolate	—	1000-$TCID_{50}$
	Russia VR-790	—	1000-$TCID_{50}$
Respiratory Syncytial Virus (RSV)	Clinical isolate	—	Control Slide
	Long VR-26	—	1000-$TCID_{50}$
	Washington VR-1401	—	1000-$TCID_{50}$
	9320 VR955	—	1000-$TCID_{50}$
Parainfluenza 1	Clinical Isolate	—	Control Slide
	C-35 VR-94	—	1000-$TCID_{50}$

Organism	Strain or Type	D³ Enterovirus 71 DFA Reagent at 2X concentration*	Concentrations of targets (viruses: $TCID_{50}$ inoculum level; bacteria: CFU)
Parainfluenza 2	Clinical Isolate	—	Control Slide
	Greer VR-92	—	1000-$TCID_{50}$
Parainfluenza 3	Clinical Isolate	—	Control Slide
	C-234 VR-93	—	1000-$TCID_{50}$
Parainfluenza 4	M-25	—	1000-$TCID_{50}$
Parainfluenza 4b	CH-19503	—	1000-$TCID_{50}$
Metapneumovirus	A1 subtype Clinical Isolate	—	Control Slide
Measles	Rubeola	—	Control Slide
Mumps	N/A	—	Control Slide
CMV	AD169	—	Control Slide
Varicella-zoster	Webster	—	Control Slide
Coxsackievirus	A9	—	Control Slide
	A9 (ODH-36685 Clinical Isolate)	—	Control Slide
	A16 (Ya, Al, Ro, Sa, Wi, Ha, Ro2 Clinical Isolates)	—	1000-$TCID_{50}$
	A24	—	Control Slide
	B1, B2, B3, B4, B5, B6	—	Control Slide
	B2 (ODH-185 Clinical Isolate)	—	Control Slide
Enterovirus 70	Commercial Control	—	Control Slide
Enterovirus 71	Ir, Vi, Ji, Br, En, Fl, Gu, Clinical Isolates	+	1000-$TCID_{50}$
Enterovirus 71	Commercial Control	+	Control Slide
Enterovirus 68	Ca. Zu, Sm Clinical Isolates	—	1000-$TCID_{50}$
Echovirus	Types 4, 6, 9, 11, 30, 34	—	Control Slide
Echovirus 7	ODH-594684 Clinical Isolate	—	Control Slide
Bacteria			
Acinetobacter calcoaceticus		—	4.17e10 CFU
Bordetella bronchiseptica		—	3.10e5 CFU
Bordetella pertussis		—	3.30e10 CFU
Corynebacterium diphtheriae		—	2.30e5 CFU
Chlamydia trachomatis LGV-II		—	Control Slide
Escherichia coli		—	1.71e10 CFU
Gardnerella vaginalis		—	5.60e9 CFU
Haemophilis influenzae type A		—	7.50e9 CFU
Klebsiella pneumoniae		—	2.01e10 CFU
Moraxella cartarrhalis		—	1.50e10 CFU
Proteus mirabilis		—	2.67e10 CFU
Pseudomonas aeruginosa		—	5.20e10 CFU
Salmonella enteriditis		—	1.27e10 CFU
Salmonella typhimurium		—	1.65e10 CFU
Staphylococcus aureus†		+	3.20e5 CFU
Streptococcus agalactiae		—	3.99e10 CFU
Streptococcus pneumoniae		—	4.10e5 CFU
Streptococcus pyogenes		—	3.10e10 CFU
Yeast			
Candida glabrata		—	2.0e10 CFU

Organism	Strain or Type	D³ Enterovirus 71 DFA Reagent at 2X concentration*	Concentrations of targets (viruses: $TCID_{50}$ inoculum level; bacteria: CFU)
* Positive (+) or Negative (–) for Reactivity † Reactivity with *Staphylococcus aureus* is more than likely due to binding the protein A produced by *Staphylococcus aureus*. Staining of *Staphylococcus aureus* appeared as small points of fluorescence while all other cultures were negative. This has been noted in labeling in the section "Limitations of the Assay": The Protein A produced by the bacterium, *S. aureus*, will bind the Fc portion of some of the fluorescein-labeled monoclonal antibodies used in this kit. 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.			

Eighteen (18) host culture cell types were tested for cross-reactivity. Cell cultures were prepared in shell-vial format. Confluent monolayers or cell spots were stained with the 2X 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 (Table 3).

Table 3. Cell lines Tested for Cross-Reactivity with D³ Enterovirus 71 DFA Reagent

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

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GLOSSARY

REF Catalogue number	LOT Batch code
 Use by	 Manufacturer
 Temperature limitation	 Intended use
 Consult e-labeling instructions for use	 Do not reuse
IVD For *In Vitro* diagnostic use	CONT NaN_3 Contents/Contains