



**For the qualitative identification of Chlamydiae lipopolysaccharide (LPS) in inoculated cell cultures by immunofluorescence using fluoresceinated monoclonal antibodies (MAbs).**

## FOR *IN VITRO* DIAGNOSTIC USE

**R<sub>x</sub> ONLY**



### INTENDED USE

The Diagnostic Hybrids, Inc. D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit is intended for the qualitative identification of Chlamydiae lipopolysaccharide (LPS) in inoculated cell cultures by immunofluorescence using fluoresceinated monoclonal antibodies (MAbs).

**Performance has not been established with direct patient specimens.**

## SUMMARY AND EXPLANATION OF THE TEST

The Chlamydiae are prokaryotic parasites of eukaryotic cells. These organisms contain enzymes and are able to synthesize proteins; however, they lack a mechanism to generate their own ATP (adenosine-triphosphate) and must rely upon the host cell for survival.

The phylum Chlamydiae contains many species. *Chlamydia trachomatis* is primarily a human pathogen and is associated with endemic trachoma, inclusion conjunctivitis, lymphogranuloma venereum, nongonococcal urethritis epididymitis (male), cervicitis, salpingitis (female) and proctitis in either sex. *Chlamydophila psittaci* is an avian Chlamydia which is transmitted to humans by infected birds resulting in pneumonia. *Chlamydophila pneumoniae* can cause pneumonia similar to *Mycoplasma pneumoniae* and it has been implicated in upper respiratory infections (i.e., cough and sore throat).

Chlamydiae have a unique growth cycle exhibiting two life forms: the elementary body and the reticulocyte body. The elementary body, smaller in size, invades a susceptible cell through a phagocytic process. Once inside the cell, the elementary body surrounds itself with a protective vacuole and differentiates into an actively metabolizing reticulocyte body. The reticulocyte body divides through binary fission in the cell from approximately the 8th hour to 18 to 24 hours. During this time the Chlamydiae show their enhanced metabolic activity. At approximately 18 to 24 hours the reticulocyte bodies begin to differentiate into the smaller elementary bodies. The inclusions then consist of both reticulocyte bodies and (predominantly) elementary bodies. This entire process takes place in the vacuole. Between 48 to 72 hours, the vacuole will rupture, releasing the infectious elementary bodies and the growth cycle will continue.

Cell culture isolation of Chlamydiae utilizes cell types such as McCoy, a mouse fibroblast cell line, or BGMK (Buffalo Green Monkey Kidney), an African Green Monkey cell line, in shell vial or multi-well plate formats. The cells are overlaid with a Chlamydia isolation medium containing cycloheximide to enhance Chlamydial growth prior to inoculation of the clinical specimen. The cultures are incubated for 48 to 72 hours to allow for the

formation of the vacuole described above. The presence of this elementary body containing vacuole can be determined by many methods, i.e. staining using iodine, Giemsa, or fluorescein-conjugated monoclonal antibodies. The use of iodine and Giemsa has been shown to be less sensitive than the use of fluorescein-conjugated monoclonal antibodies.<sup>1</sup>

## PRINCIPLE OF THE PROCEDURE

The D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit uses a blend of two specific murine MAbs that are directly labeled with fluorescein isothiocyanate (FITC) for the qualitative detection of Chlamydiae in inoculated cell cultures.

Cell culture isolation of Chlamydiae utilizes either McCoy cells, a mouse fibroblast cell line, or Buffalo Green Monkey Kidney (BGMK) cells in shell vial or multi-well plate formats. The cells are overlaid with a Chlamydia isolation medium containing cycloheximide to enhance Chlamydial growth prior to inoculation of the clinical specimen. The inoculated cells are centrifuged for 60 minutes and incubated at 35°C to 37°C for 48 to 72 hours. The cells are fixed in acetone and the Chlamydiae DFA Reagent is added to the cells to stain any Chlamydiae specific antigens that may be present following culture. After incubating for 15 to 30 minutes at 35°C to 37°C, the stained cells are washed with the supplied Phosphate Buffered Saline (PBS). A drop of the supplied Mounting Fluid is added to the multi-wells or to a clean microscope slide for mounting a coverslip. The cells are examined at 100 to 400X magnification using a microscope equipped with the correct filter combination for fluorescein. Chlamydiae infected cells will have bright, apple-green fluorescent inclusions while non-infected cells will fluoresce red due to the Evans Blue counter-stain.

## REAGENTS AND MATERIALS PROVIDED

The D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit contains the following:

### Chlamydiae DFA Reagent\*

**5 mL**

One dropper bottle containing fluorescein labeled murine monoclonal antibodies directed against Chlamydiae LPS. The buffered, stabilized, aqueous solution contains Evans Blue as a counter-stain and 0.1% sodium azide as preservative

### Chlamydia Antigen Control Slides

**10 slides**

Five individually packaged control slides with wells containing cell culture-derived *Chlamydia trachomatis* positive cells and five individually packaged control slides with wells containing cell culture-derived negative cells. Each slide is intended to be stained only one time.

### 40X PBS Concentrate

**25 mL**

One bottle containing a 40X concentrate consisting of 4% sodium azide (0.1% sodium azide after dilution to 1X using de-mineralized water) in PBS.

### Mounting Fluid

**7 mL**

One dropper bottle containing an aqueous, buffer-stabilized solution of glycerol, and 0.1% sodium azide.

\***Note:** A reagent drop will vary from 40 to 60- $\mu$ L depending on the pressure and temperature of the solution, therefore, the number of examinations/tests per kit by procedure type is shown below.

Procedure Type	Number of Examinations per Chlamydiae DFA Reagent, 5 mL
<b>Shell-vials, tests:</b>	<b>20 to 30</b>
	<i>Assumptions: 4-drops, based on ~0.200 mL completely covering each shell-vial.</i>
<b>Multi-well Plates, tests:</b>	<b>24-well plate: 20 to 30</b>
	<i>Assumptions: 4-drops, based on ~0.200 mL completely covering each 24-well.</i>
	<b>48-well plate: 30 to 40</b>

Procedure Type	Number of Examinations per Chlamydiae DFA Reagent, 5 mL
	<i>Assumptions: 3-drops, based on ~0.150 mL completely covering each 48-well.</i>
<b>Cell Spot, tests:</b> <i>(from glass round bottom tube scraping)</i>	<b>100</b> <i>Assumptions: 1-drop, based on an average drop size of ~0.050 mL, covering 1 well (5 mm) of a 2-well slide.</i>

## MATERIALS REQUIRED BUT NOT PROVIDED

- Fluorescence microscope with the correct filter combination for FITC (excitation peak = 490 nm, emission peak = 520 nm)
- Cell culture for Chlamydiae isolation for example; McCoy or BGMK cell lines (*Both available from Quidel*)
- Cover slips (22 x 50mm) for Antigen Control Slides and for specimen slides
- Universal Transport Medium (*Available from Quidel*)
- Chlamydia Isolation Medium (Eagle's Minimum Essential Medium with 10% fetal bovine serum, 25mM HEPES, 2.2 grams/liter sodium bicarbonate, cycloheximide, and antibiotics) (*Available from Quidel*)
- Reagent grade acetone (>99% pure) chilled at 2°C to 8°C for fixation of shell vials

### NOTES:

- ▶ Keep the reagent grade acetone container tightly sealed to avoid hygroscopic absorption of water, which may cause a hazy, nonspecific, background fluorescence.
- ▶ A mixture of 80% acetone/20% de-mineralized water is 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, and 1 mL
- Sterile Pasteur pipettes or other "transfer"-type pipettes
- Fine-tipped forceps
- Wash bottle, 200 mL
- 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 bench top or with a pair of forceps taking care to avoid injury.
- Sodium hypochlorite solution, 0.5% (1:10 dilution of household bleach)
- Humid chamber (e.g., covered Petri dish with a damp paper towel placed in the bottom)
- Glass microscope slides
- Sterile nylon flock swab or polyester swab, non-inhibitory to Chlamydiae and tissue culture
- Incubator, 35°C to 37°C (CO<sub>2</sub> or non-CO<sub>2</sub>, depending on the cell culture format used)
- Centrifuge with free swinging bucket rotor
- De-mineralized water for dilution of PBS and for dilution of the reagent grade acetone for use in polystyrene multi-well plates
- Chlamydiae stock cultures for use in monitoring the cell culture and staining procedures (*Available from Quidel*)
- Aspirator Set-up: Vacuum aspirator with disinfectant trap containing sufficient household bleach (5%) that the concentration is not decreased by more than 100 fold as it is diluted with discarded fluids
- Wash Container: Beaker, wash bottle or Coplin jar for washing slides
- Inverted Light Microscope: Used for examining the monolayers of cells prior to inoculation and examination for toxicity (100X to 400X magnification capability)

## WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use only.
- For professional use only.
- Tissue culture cells may have some potential to be hazardous. Personnel working with these cultures must be properly trained in safe handling techniques<sup>2,3,4</sup> and have experience with tissue culture before attempting this procedure.

- Follow Universal Precautions when handling the contents of this kit and patient samples.
- All procedures must be conducted in accordance with the CDC 5<sup>th</sup> 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.
- Sodium azide is included in the 40x PBS Concentrate at 4%, and in the other solutions in this kit at 0.1%.
  - ▶ Sodium azide is considered poisonous. If the 40X PBS Concentrate is swallowed, seek medical advice immediately.
  - ▶ Aqueous solutions of sodium azide, when mixed with acids, may liberate toxic gas.
  - ▶ Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.
  - ▶ Avoid disposal of these solutions down sanitary or industrial plumbing systems.
  - ▶ Avoid release to the environment.
- 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.
- Evans Blue counter-stain is a potential carcinogen. If eye or skin contact occurs, immediately flush with copious amounts of water.
- Chlamydiae Antigen Control Slides are microscope slides onto which either cultured *non*-infected cells or *Chlamydiae trachomatis*-infected cells have been grown then fixed (killed) with acetone; a drying agent is included in the foil envelope to preserve antigen integrity; there is no residual acetone present.
- 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.
- Each Chlamydiae Antigen Control Slide should be used only once. Do not re-use a Control Slide.
- Wear suitable protective clothing, gloves, and eye/face protection when handling the contents of this kit.
- The Chlamydiae DFA Reagent is supplied at working strength. Any dilution will decrease sensitivity.
- Do not expose Chlamydiae DFA Reagent to bright light during staining or storage.
- Microbial contamination of Chlamydiae DFA Reagent may cause a decrease in sensitivity.
- Reagents should be used prior to their expiration date.
- Store 1X PBS in a clean container to prevent contamination.
- All specimens and materials used to process them should be considered potentially infectious and handled in a manner which prevents infection of laboratory personnel. Decontamination is most effectively accomplished using a 0.5% solution of sodium hypochlorite (1:10 dilution of household bleach). 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 tissue culture procedures.
- Reusable glassware must be washed and thoroughly rinsed free of all detergents.
- 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.
- 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](http://quidel.com).

### Preparation of 1X PBS Solution

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

## Storage

**Table 1. Reagent Storage Conditions**

<b>Chlamydiae DFA Reagent</b>	Store at 2°C to 8°C in the dark.
<b>Mounting Fluid</b>	
<b>Chlamydia Antigen Control Slides</b>	Store at 2°C to 8°C.
<b>40X PBS Concentrate</b> NOTE: The Concentrate may crystallize when stored at 2°C to 8°C. The crystals will dissolve when the Concentrate is warmed to ambient temperature.	Store liquid at 2°C to 8°C prior to dilution.
<b>1X PBS</b>	Store at ambient temperature (20°C to 25°C).

## 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 Chlamydiae DFA Reagent should be kept to a minimum.

Discard 1X PBS Solution if it becomes cloudy.

## SPECIMEN COLLECTION AND PREPARATION

Proper collection and handling of the patient specimen are the most important factors in successful Chlamydiae isolation. Specimen collection, processing, and cell culture isolation should be attempted only by laboratories trained in such procedures. Care should be taken during all specimen collection and handling to avoid generation of aerosols.

### Specimen Collection<sup>5</sup>

#### Cervical

- Wipe the cervix prior to collection to remove WBC and mucous debris.
- Insert a sterile, large-tipped polyester swab into the endocervix, rotate and remove. Discard this swab.
- Insert a sterile, polyester swab into the cervical os to collect cells from the transitional zone. Rotate the swab vigorously in firm contact with cervix surface to facilitate the collection of columnar epithelial cells.
- Withdraw swab without contacting vaginal surfaces.

NOTE: Recovery rates from females can be improved if the urethra is also sampled.

#### Urethral

- Insert a sterile, fine-tipped polyester swab 2 to 4 cm into the male urethra or 1 cm into the female urethra and hold in place for 5 seconds.
- Rotate the swab several times to obtain columnar epithelial cells and withdraw.

NOTE: Patient should not have urinated within one hour of collection.

#### Eye

- Gently swab the lower conjunctiva with a sterile, fine-tipped polyester swab, collecting patient mucous membrane cells.

#### Nasopharynx and Throat

- Gently insert a sterile nasopharyngeal fine-wire polyester swab into one or both anterior nares to the posterior pharynx; rotate to collect mucous membrane cells and withdraw.

- Swab the posterior pharynx vigorously with a large-tipped, sterile polyester swab.  
NOTE: Nasal aspirates collected by intubation are a superior source of the agent in infants with pneumonia.

### **Rectal Mucosa**

- To collect cells from the mucosal surface, insert a sterile polyester swab 1-cm past the anal sphincter, rotate in firm contact with the mucosal surface and withdraw.

Several factors of specimen collection may affect the successful isolation of Chlamydiae. When swabs are used for specimen collection, cotton or polyester swabs should be used, as calcium alginate swabs have been shown to inhibit replication.<sup>4</sup> Immerse swab immediately in appropriate transport medium. This will serve to stabilize Chlamydiae, if present, and will inhibit undesirable bacterial and fungal overgrowth. Place swab in transport tube, break off shaft, and tightly secure cap.

### **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 U.S. Code of Federal Regulations, or other regulatory requirements, as may be applicable.

Specimens should be transported on wet ice to the laboratory and 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 48 hours before being tested. If longer storage is required, the specimens should be frozen at -70°C or lower.<sup>4</sup>

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

## **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.
- The closed, humidified container for holding the slides during incubation should be kept in the incubator so it is at incubator temperature when the slides are placed in it. By doing this, the cells and antibody solution will come up to temperature rapidly, yielding the expected stain intensity.
- It is recommended that the MAbs reagents be brought to ambient temperature prior to use and immediately returned to 2°C to 8°C after use.

### **Regarding Cell Culture Testing**

- Good Laboratory Practice dictates that positive and negative Chlamydia controls be run with each new batch of cells to confirm their performance in culturing Chlamydiae.

- It is good practice to retain the medium removed from the positive monolayers until after staining results have been obtained. If there is any question concerning the specimen results, the medium can be passed to another monolayer and incubated for the appropriate time period for repeat testing.
- If using cell cultures in polystyrene, multi-well plates, the acetone fixative must be diluted with water to 80% by adding 20 mL of 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 antibody reagents to dry on the monolayers; this can lead to high background.

### Regarding Immunofluorescence Microscopy

- It is good practice to 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 cause(s). Do not report results until controls perform as expected.
- There are three aspects of the fluorescence microscope that 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 DFAs.
  - ▶ 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.
- There are several fluorescent artifacts that may be observed in the cell monolayers being examined:
  - ▶ Cell debris, lint, etc. can nonspecifically adsorb DFAs, resulting in highly intense fluorescence. These can be identified by their morphology, i.e., they don't have the appearance of a complete cell and typically do not appear to be a part of the monolayer like the other cells.
  - ▶ 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 DFAs is retarded during the wash step, resulting in the nonspecific 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 controlled.
  - ▶ Inadequate washing can lead to general, low grade fluorescence due to residual DFAs remaining on the monolayer of cells.
- Bleaching or fading of the fluorescence of the stained cells may occur on exposure to light, particularly light of high intensity. This bleaching can occur when the microscope slide is viewed in a field for an extended period of time. Slides should be protected as much as possible during the assay.

### Specimen Preparation

- Swabs containing specimen material should be handled with sterile forceps. The swab should be rotated in 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 0.5% sodium hypochlorite solution (1:10 dilution of household bleach). Decontaminate the forceps in between specimen disposal.
- 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 Chlamydiae into the medium.
- 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 Chlamydiae in a specimen, each laboratory should establish the efficacy of its specimen preparation procedure.

### Cell Culture Testing – Shell Vial

1. The laboratories that conducted studies for clearance of this assay (presented in section *SPECIFIC PERFORMANCE CHARACTERISTICS*) inoculated duplicate shell vials and incubated for either 48 or 72 hours.
2. Determine the number of vials needed based on the number of specimens and controls to be used.
3. Examine the monolayers for proper morphology prior to inoculation.
4. Aspirate maintenance medium from the monolayers and add 1 mL of Chlamydia Isolation Medium to each shell vial.
5. Add 0.2 to 0.4 mL of prepared specimen to each shell vial.
6. Centrifuge the shell vials at 700xg for 1-hour at 20°C to 25°C.
7. Place stoppered shell vials in an incubator at 35°C to 37°C for 48 to 72 hours.
8. Remove the medium and add 1 mL of 1X PBS.
9. Swirl to mix and then aspirate.
10. Repeat this wash with another 1 mL of 1X PBS and then aspirate.
11. Add 1 mL of chilled 100% acetone and allow it to stand for 5 to 10 minutes at 20°C to 25°C.
12. Remove the fixative by aspiration.
13. Add 0.5 mL of 1X PBS to wet the monolayer.
14. Swirl and then aspirate completely.
15. Add 4 drops of the Chlamydiae DFA Reagent to the fixed monolayers of patient and control samples, and rock to ensure complete coverage of the monolayer by the Reagent.
16. Place stoppered shell vials in a 35°C to 37°C incubator for 15 to 30 minutes.
17. Aspirate the Chlamydiae DFA Reagent from the monolayers.
18. Add 1 mL of the 1X PBS.
19. Remove the 1X PBS by aspiration, repeat the wash step and again remove by aspiration.
20. Add 1 mL of de-mineralized water.
21. Remove the de-mineralized water by aspiration.
22. Lift the coverslip from the bottom of the shell vial using a bent-tip needle on a syringe barrel, and, grasping it with the fine tipped forceps, transfer it, monolayer-side down, to a small drop of Mounting Fluid on a standard microscope slide.
23. Examine the stained monolayers using a fluorescence microscope with magnifications between 100X and 400X. (See section *Regarding Immunofluorescence Microscopy*)
24. Refer to section *INTERPRETATION OF RESULTS*.

### Cell Culture Testing – Multi-well Plate

1. The laboratories that conducted studies for clearance of this assay (presented in the section *SPECIFIC PERFORMANCE CHARACTERISTICS*) inoculated duplicate wells and incubated for either 48 or 72 hours.
2. Determine the number of wells needed based on the number of specimens and controls to be used.
3. Examine the monolayers for proper morphology prior to inoculation.
4. Aspirate maintenance medium from the monolayers and add 1 mL of Chlamydia Isolation Medium to each 24-well multi-well plate monolayer; add 0.8 mL to each 48-well plate monolayer.
5. Add 0.2 to 0.4 mL of prepared specimen to the appropriate well of a multi-well plate.
6. Centrifuge the multi-well plates at 700xg for 1 hour at 20°C to 25°C.
7. Place the covered multi-well plates in a 35°C to 37°C incubator with a humidified, 5% CO<sub>2</sub> atmosphere for 48 to 72 hours.
8. Remove the medium and add 1 mL of 1X PBS.
9. Swirl to mix and then aspirate.
10. Repeat this wash with another 1 mL of 1X PBS and then aspirate.



11. Add 1 mL of 80% aqueous acetone and let stand 5 to 10 minutes 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.
12. Remove the fixative by aspiration.
13. Add 0.5 mL of the 1X PBS to wet the monolayer.
14. Swirl and then aspirate completely.
15. Add 4 drops of the Chlamydiae DFA Reagent to the fixed monolayers of patient and control samples in each 24-well multi-well plate; add 3 drops of the Chlamydiae DFA Reagent to the fixed monolayers of patient and control samples in each 48-well plate. Rock to **ensure complete coverage** of the monolayer by the Reagent.
16. Place the covered multi-well plate in a 35°C to 37°C, humidified incubator for 15 to 30 minutes.
17. Aspirate the Chlamydiae DFA Reagent from the monolayers.
18. Add 1 mL of the 1X PBS.
19. Remove the 1X PBS by aspiration, repeat the wash step and again remove by aspiration.
20. Add 1 mL of de-mineralized water.
21. Remove the de-mineralized water by aspiration.
22. Add 2 to 3 drops of Mounting Fluid to each monolayer, cover the plate.
23. Examine the stained monolayers using a fluorescence microscope with magnifications between 100X and 400X. (See *Regarding Immunofluorescence Microscopy*)
24. Refer to *INTERPRETATION OF RESULTS*.

## Quality Control

### Cell Culture

To ensure sensitivity to Chlamydiae, a *Chlamydia*-inoculated control monolayer should be included each time a new lot of cell culture is used. 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. 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.

### Reagents

A fresh set of Chlamydia Antigen Control Slides (1-positive and 1-negative slide) should be stained each time the staining procedure is performed to ensure proper test performance. The positive wells will show multiple infected cells with bright, apple-green fluorescent inclusions. Negative cells will fluoresce a dull red due to the included Evans Blue counter-stain. The negative well will contain only dull red fluorescing negative cells. Positive and negative controls must demonstrate appropriate fluorescence for specimen results to have validity. Antigen Control Slides may also aid in the interpretation of patient specimens.

## INTERPRETATION OF RESULTS

It is recommended that controls be examined first to ensure proper test performance before examination of the specimens. A positive reaction is one in which bright apple-green fluorescent inclusions are observed in the infected cells. Negative cells will fluoresce a dull red due to the Evans Blue counter-stain included in the Chlamydiae DFA Reagent. Technologists should not confuse cell clumps which may fluoresce due to entrapment of antibody with Chlamydiae-specific staining. Occasionally, dead, rounded cells due to specimen toxicity or improper cell storage may nonspecifically stain a dull olive green due to trapped antibody. Adequate washing between steps will help to eliminate this type of nonspecific staining.

The entire monolayer of cells must be examined for fluorescent inclusions within the cells. If no fluorescent inclusions are found, the results of testing of the specimen should be reported as, “No Chlamydiae isolated by cell culture.” If fluorescent inclusions within the cells are found, it should be reported as “Chlamydiae isolated by cell culture.”

## LIMITATIONS OF THE PROCEDURE

- Inappropriate specimen collection, storage, and transport may lead to false negative culture results.<sup>4</sup>
- Incubation times or temperatures other than those cited in the test instructions may give erroneous results.
- Isolation of Chlamydiae will vary greatly depending upon the specimen quality and subsequent handling. A negative result does not exclude the possibility of Chlamydiae 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.
- The monoclonal antibodies used in this kit are from hybridomas created using *Chlamydophila pneumoniae* infected cells as the immunogen. The specific antigen detected by the antibodies is the lipopolysaccharide molecule.
- The monoclonal antibodies used in this kit are not species-specific and therefore cannot be used to differentiate among the different species of Chlamydiae.
- A negative result in a cultured specimen does not rule out the presence of Chlamydiae infection.
- Performance of the kit can only be assured when components used in the assay are those supplied by Quidel.
- Prolonged storage of the Chlamydiae 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 bind the Fc portions of conjugated antibodies. Such binding can be distinguished from Chlamydial 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.

## EXPECTED VALUES

The clinical studies described in Section *Specific Performance Characteristics* were comprised of specimens collected and cultured for the presence of Chlamydiae. The specimen sources are described in Table 2 below.

**Table 2. Specimen Sources**

Site	Total	Unknown or Not Indicated	Genital	Penis	Vaginal	Cervical	Rectal	Perineum*	Eye	Urethral	Mouth**	Respiratory‡	Intra abdominal aspirate
1	396	9	15 5	5	35	177		2	5	6	1	1	
2	200			13	55	83	34		6	3	2	4	
3	278			13	146	32	5	2	64	7	2	6	1
4	120		12 0										

\*perineum: groin, pubic, perianal, pelvic, endocervix  
 \*\*mouth: mouth, throat  
 ‡respiratory: nasopharyngeal, nasal aspirate, tracheal aspirate, sputum

Both “fresh” (tested within 48 hours of receipt) or frozen (stored at –70°C or colder) specimens were used in the studies. The numbers of fresh and frozen specimens tested are summarized below (Table 3).

**Table 3. Numbers of Specimens per Study Site**

Sites	Fresh prospective	Frozen prospective	Archived (frozen)	Site Total
1	156	240	0	396
2	90	84	26	200
3	23	68	187	278
4	0	120	0	120

### SPECIFIC PERFORMANCE CHARACTERISTICS

This study included nine hundred and ninety-four (994) original specimens evaluated by D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit and three currently marketed Culture Confirmation Kits (“Comparison” tests) for the presence of Chlamydiae. These evaluations were conducted at three external laboratory sites and one in-house laboratory: (1) A reference laboratory in the southeastern United States; (2) A hospital laboratory in the mid-west United States; (3) A hospital laboratory in the southwestern United States; and (4) Quidel’s in-house virology laboratory. The patient demographics are listed below (Table 4).

**Table 4. Specimen Demographics**

Values are # pos/Total	Site 1		Site 2		Site 3		Site 4	
Age	F	M	F	M	F	M	F	M
Totals	362	32	170	30	215	63	98	22
<1m	0/1	0/3	0/2	2/4	9/15	14/19	0	0
1m to 2y	0	0	0/4	0/2	4/28	11/22	0	0
2y to12y	0/2	0	1/28	1/13	6/13	0	0	0
12y to18y	0/17	0	31/117	5/10	79/143	14/20	0/7	0
18y to 21y	6/56	1/4	4/19	1/1	4/13	0/2	9/30	3/7
>21y	9/283	3/25	0	0	0/3	0	8/61	4/15
Age not reported	1/3	0	0	0	0	0	0	0
Age/gender not reported	0/2		0		0		0	

### Performance Characteristics For Each Study Site

Percent Agreement between the D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit and Comparison test was calculated for results from Sites 1, 2, and 3 using prospectively collected specimens, and is reported below (Table 5).

**Table 5. Combined Percent Agreement Using Prospectively Collected Specimens**

		Comparison Test	
		+	–
D <sup>3</sup> DFA Chlamydiae Culture Confirmation Kit	+	<b>42</b>	4
	–	2	<b>613</b>

---

Positive Percent Agreement\* (PPA): 95.5%  
 95% CI\*\* - PPA: 84.5% to 99.4%  
 Negative Percent Agreement\*\*\*(NPA): 99.4%  
 95% CI – NPA: 98.3% to 99.8%

---

\*“Positive Percent Agreement”, or “PPA”, values were calculated according to {[Total Number of Positive Results in Agreement by both Subject and Predicate Tests) divided by [(Total Number of Positive Results in Agreement by both Subject and Predicate Tests) plus (Number of Results Positive by Predicate but Negative by Subject)]} multiplied by 100%.

\*\*“95% CI” refers to 95% Confidence Intervals, which were calculated according to Exact method (Clopper, C. and S. Pearson, Biometrika 26:404-413, 1934).

\*\*\*“Negative Percent Agreement”, or “NPA”, values were calculated according to {[Total Number of Negative Results in Agreement by both Subject and Predicate Tests) divided by [(Total Number of Negative Results in Agreement by both Subject and Predicate Tests) plus (Number of Results Negative by Predicate but Positive by Subject)]} multiplied by 100%.

### Study Site 1

A total of 396 specimens were tested for the presence of Chlamydiae (156 fresh and 240 frozen) using D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit and a comparison test. Briefly, 200 µL from the specimens were inoculated onto two 48/24-fill McCoy cluster plates. The inoculated cells were centrifuged at 700xg for 60 minutes, incubated at 35° to 37°C for either 48 or 72 hours, then stained in accordance with the respective product insert procedures. The results of this testing site are summarized below (Table 6).

**Table 6. Study Site 1 – D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit and Comparison Device Results in Multi-Well Plate Culture**

		Fresh Comparison Test		Frozen Comparison Test	
		+	–	+	–
D <sup>3</sup> DFA Chlamydiae Culture Confirmation Kit	+	0	0	18	3
	–	0	156	1	218

---

Fresh	Frozen
PPA: [>1 result required for calculation]	94.7%
95% CI – PPA: N/A	74.0% to 99.9%
NPA: 100%	98.6%
95% CI – NPA: 97.7% to 100%	96.1% to 99.7%

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### Study Site 2

A total of 200 specimens were tested for the presence of Chlamydiae using the same comparison test as Study Site 1 [90 fresh and 84 frozen (prospectively collected) and an additional 26 archival specimens.] Briefly, 200 µL from the specimens were inoculated into two McCoy shell vials. The inoculated cells were centrifuged at 700xg for 60 minutes, incubated at 35° to 37°C for either 48 or 72 hours, then stained in accordance with the respective product insert procedures. The results of this testing site are summarized below (Table 7).

**Table 7. Study Site 2 – D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit and Comparison Device Results in Shell-vial Culture**

D <sup>3</sup> DFA Chlamydiae Culture Confirmation Kit	Fresh Comparison Test		Frozen Comparison Test	
	+	-	+	-
	+	<b>11</b>	0	<b>8</b>
-	0	<b>79</b>	1	<b>74</b>

Fresh	Frozen
PPA: 100%	88.9%
95% CI – PPA: 71.5% to 100%	51.8% to 99.7%
NPA: 100%	98.7%
95% CI – NPA: 95.4% to 100%	92.8% to 100%

**Archival Specimens**

D <sup>3</sup> DFA Chlamydiae Culture Confirmation Kit	Comparison Test	
	+	-
+	<b>25</b>	0
-	0	<b>1</b>

PPA: 100%
95% CI – PPA: 86.3% to 100%
NPA: 100%
95% CI – NPA: [>1 result required for calculation]

**Study Site 3**

A total of 278 specimens were tested for the presence of Chlamydiae using the same comparison test as Study Site 1 [23 fresh and 68 frozen (prospectively collected) and an additional 187 archival specimens]. Briefly, 200 µL from the specimens were inoculated into two McCoy shell vials. The inoculated cells were centrifuged at 700xg for 60 minutes, incubated at 35° to 37°C for either 48 or 72 hours, then stained in accordance with the respective product insert procedures. The results of this testing site are summarized below (Table 8).

**Table 8. Study Site 3 – D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit and Comparison Device Results in Shell-vial Culture**

	Fresh Comparison Test		Frozen Comparison Test			
	+	-	+	-		
D <sup>3</sup> DFA Chlamydiae Culture Confirmation Kit	+	<b>0</b>	0	+	<b>5</b>	0
	-	0	<b>23</b>	-	0	<b>63</b>

Fresh	Frozen
PPA: [>1 result required for calculation]	100%
95% CI – PPA: N/A	47.8% to 100%
NPA: 100%	100%
95% CI – NPA: 85.2% to 100%	94.3% to 100%

**Archival Specimens**

	Comparison Test	
	+	-
D <sup>3</sup> DFA Chlamydiae	134	0
Culture Confirmation Kit	0	53

---

PPA: 100%  
 95% CI – PPA: 97.3% to 100%  
 NPA: 100%  
 95% CI – NPA: 93.3% to 100%

---

**Study Site 4a**

A total of 120 frozen specimens were tested for the presence of Chlamydiae using a second comparison test. Briefly, 200 µL from the specimens were inoculated onto two 48/24-fill McCoy multi-well plates. The inoculated cells were centrifuged at 700xg for 60 minutes, incubated at 35° to 37°C for either 48 or 72 hours, then stained in accordance with the respective product insert procedures. The results of this testing site are summarized below (Table 9).

**Table 9. Study Site 4a – D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit and Comparison Device Results in Multi-well Plate Culture**

	Frozen Comparison Test	
	+	-
D <sup>3</sup> DFA Chlamydiae	24	0
Culture Confirmation Kit	0	96

---

PPA: 100%  
 95% CI – PPA: 85.8% to 100%  
 NPA: 100%  
 95% CI – NPA: 96.2% to 100%

---

**Study Site 4b**

The same 120 frozen specimens from above were tested for the presence of Chlamydiae using a third comparison test. Briefly, 200 µL from the specimens were inoculated onto two 48/24-fill McCoy multi-well plates. The inoculated cells were centrifuged at 700xg for 60-minutes, incubated at 35° to 37°C for either 48 or 72 hours, then stained in accordance with the respective product insert procedures. The results of this testing site are summarized below (Table 10).

**Table 10. Study Site 4b – D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit and Comparison Device Results in Multi-well Plate Culture**

		Frozen Comparison Test	
		+	-
D <sup>3</sup> DFA Chlamydiae Culture Confirmation Kit	+	<b>23</b>	1
	-	0	<b>96</b>

---

PPA: 100%

95% CI – PPA: 85.2% to 100%

NPA: 99%

95% CI – NPA: 94.4% to 100%

---

### Specificity Testing

The D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit was characterized for its ability to specifically detect *Chlamydiae* species. The FITC-conjugated MABs used in the kit exhibited characteristic staining patterns on *Chlamydiae* infected cells, with fluorescent inclusions in infected cells. The conjugated MABs reacted with *Chlamydomphila pneumoniae*, *Chlamydomphila psittaci*, as well as 15 serovars of *Chlamydia trachomatis*.

The D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit was tested for cross-reactivity against a wide variety of cells and microorganisms. No cross-reactivity was observed for 20 host culture cell types or for 57 virus strains (cultured and processed for staining). Twenty-five (25) bacterial cultures, as well as one protozoan and one yeast specimen, were stained and examined for cross-reactivity. **The protein-A produced by *Staphylococcus aureus* bound the F<sub>c</sub> portion of the MAB's and appeared as small points of fluorescence (see *Limitations of Procedure*) while all other bacterial cultures were negative.**

To test this product against Chlamydiae species, cell cultures (McCoy, BGMK, or HEp-2) were inoculated with approximately 1.5 x 10<sup>6</sup> infective units of *Chlamydia trachomatis*, *Chlamydomphila psittaci*, or *Chlamydomphila pneumoniae*, and incubated for 2 days to yield a 3+ to 4+ infection. Cultures were processed and stained with the Chlamydiae DFA Reagent or specially prepared DFA reagents containing only one of the Chlamydia MABs.

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

Depending on the particular bacteria, the number of CFU tested ranged from 6.4x10<sup>4</sup> to 2.9x10<sup>7</sup>. Depending on the virus, 150 to 2100 TCID<sub>50</sub> viruses were inoculated into shell vial culture and incubated for 24 to 48 hours, to yield a 1+ to 3+ infection, processed and stained with the 1.5X DFA according to the procedure detailed in the product insert. Additionally, for some viruses and other microorganisms, commercial slides containing the particular agent were used to test 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.

Organisms and cell lines which were tested against the Chlamydiae DFA Reagent are listed below (Table 11).

**Table 11. Organisms Tested for Reactivity with D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit**

<b>MICROORGANISMS (BACTERIA, YEAST, PROTOZOA)</b>			
<b>Result (Reactive = +; Negative = -)</b>			
<b>Chlamydiae</b>	<b>Serovar</b>	<b>Inoculum (Infective units per culture)</b>	<b>Result (Reactive = +) (Negative = -)</b>
Chlamydia trachomatis	A	1.5 x 10 <sup>6</sup>	+
	B	1.5 x 10 <sup>6</sup>	+
	C	1.5 x 10 <sup>6</sup>	+
	D	1.5 x 10 <sup>6</sup>	+
	E	1.5 x 10 <sup>6</sup>	+
	F	1.5 x 10 <sup>6</sup>	+
	G	1.5 x 10 <sup>6</sup>	+
	H	1.5 x 10 <sup>6</sup>	+
	I	1.5 x 10 <sup>6</sup>	+
	J	1.5 x 10 <sup>6</sup>	+
	K	1.5 x 10 <sup>6</sup>	+
	L1	1.5 x 10 <sup>6</sup>	+
	L2	1.5 x 10 <sup>6</sup>	+
	L3	1.5 x 10 <sup>6</sup>	+
Ba	1.5 x 10 <sup>6</sup>	+	
<b>Chlamydiae (cont.)</b>		<b>Inoculum</b>	<b>Result</b>
Chlamydophila pneumoniae		1.5 x 10 <sup>6</sup>	+
Chlamydophila psittaci		1.5 x 10 <sup>6</sup>	+
<b>Other Bacteria</b>		<b>CFU Tested</b>	<b>Result</b>
Acholeplasma laidlawi		~1.0 x 10 <sup>7</sup>	-
Acinetobacter calcoaceticus		9.7 x 10 <sup>5</sup>	-
Bordetella bronchiseptica		1.8 x 10 <sup>5</sup>	-
Bordetella pertussis		4.7 x 10 <sup>6</sup>	-
Corynebacterium diphtheriae		2.5 x 10 <sup>6</sup>	-
Escherichia coli		2.6 x 10 <sup>5</sup>	-
Gardnerella vaginalis		5.0 x 10 <sup>5</sup>	-
Haemophilis influenzae type A		9.3 x 10 <sup>5</sup>	-
Klebsiella pneumoniae		6.4 x 10 <sup>6</sup>	-
Legionella pneumophila		6.5 x 10 <sup>4</sup>	-
Moraxella cartarrhalis		6.4 x 10 <sup>4</sup>	-
Mycoplasma hominis		~1.0 x 10 <sup>4</sup>	-
Mycoplasma orale		~1.0 x 10 <sup>4</sup>	-
Mycoplasma pneumoniae		~1.0 x 10 <sup>4</sup>	-
Mycoplasma salivarium		~1.0 x 10 <sup>7</sup>	-
Neisseria gonorrhoeae		1.3 x 10 <sup>6</sup>	-
Proteus mirabilis		2.1 x 10 <sup>6</sup>	-
Pseudomonas aeruginosa		1.0 x 10 <sup>3</sup>	-
Salmonella enteriditis		2.5 x 10 <sup>6</sup>	-
Salmonella typhimurium		1.8 x 10 <sup>6</sup>	-
Staphylococcus aureus		1.0 x 10 <sup>7</sup>	+
Streptococcus agalactiae		9.6 x 10 <sup>6</sup>	-



<b>MICROORGANISMS (BACTERIA, YEAST, PROTOZOA)</b>			
<b>Result (Reactive = +; Negative = -)</b>			
Streptococcus pneumoniae	8.0 x 10 <sup>5</sup>	-	
Streptococcus pyogenes	2.9 x 10 <sup>7</sup>	-	
Ureaplasma urealyticum	~1.0 x 10 <sup>4</sup>	-	
<b>Yeast</b>	<b>CFU Tested</b>	<b>Result</b>	
Candida glabrata	8.7 x 10 <sup>6</sup>	-	
<b>Protozoan</b>	<b>CFU Tested</b>	<b>Result</b>	
Trichomonas vaginalis	Commercially available control slide*	-	
*Test material is from commercially available prepared slides. Each positive well contains 10 to 50% reactive cells.			
<b>Virus Strains</b>	<b>Inoculum (TCID<sub>50</sub>)</b>	<b>Result</b>	
Adenovirus	Type 1	725	-
	Type 3	725	-
	Type 6	725	-
	Type 7	725	-
	Type 8	725	-
	Type 10	725	-
	Type 13	725	-
	Type 14	725	-
Type 18	725	-	
<b>Virus Strains (cont.)</b>	<b>Inoculum (TCID<sub>50</sub>)</b>	<b>Result</b>	
Adenovirus	Type 31	725	-
	Type 40	725	-
	Type 41	725	-
Influenza A	Aichi	2.1 x 10 <sup>3</sup>	-
	Malaya	2.1 x 10 <sup>3</sup>	-
	Hong Kong	2.1 x 10 <sup>3</sup>	-
	Denver	2.1 x 10 <sup>3</sup>	-
	Port Chalmers	2.1 x 10 <sup>3</sup>	-
	PR	2.1 x 10 <sup>3</sup>	-
Influenza B	Victoria	2.1 x 10 <sup>3</sup>	-
	Hong Kong	2.1 x 10 <sup>3</sup>	-
	Maryland	2.1 x 10 <sup>3</sup>	-
	Mass	2.1 x 10 <sup>3</sup>	-
	Taiwan	2.1 x 10 <sup>3</sup>	-
	GL	2.1 x 10 <sup>3</sup>	-
RSV	Russia	2.1 x 10 <sup>3</sup>	-
	Long	2.1 x 10 <sup>3</sup>	-
	Wash	2.1 x 10 <sup>3</sup>	-
Parainfluenza 1	9320	2.1 x 10 <sup>3</sup>	-
	C-35	Commercially available control slide*	-
	Greer	Commercially available control slide*	-
Parainfluenza 2	C 243	Commercially available control slide*	-

<b>MICROORGANISMS (BACTERIA, YEAST, PROTOZOA)</b>			
<b>Result (Reactive = +; Negative = -)</b>			
HSV-1	1F	150	-
	MacIntyre	150	-
HSV-2	MS	150	-
	Strain G	150	-
CMV	Towne	700	-
	AD169	700	-
	Davis	700	-
VZV	Ellen	500	-
Echovirus	4, 6, 9, 11, 30, 34	Commercially available control slide*	-
Coxsackievirus	B1, B2, B3, B4, B5, B6	Commercially available control slide*	-
Mumps		Commercially available control slide*	-
Measles (Rubeola)		Commercially available control slide*	-
Poliovirus	Types 1, 2, 3	Commercially available control slide*	-
Epstein-Barr		Commercially available control slide*	-
*Test material is from commercially available prepared slides. Each positive well contains 10 to 50% reactive cells.			
<b>HOST CELL CULTURE TYPES (shell vial monolayers were stained)</b>			
<b>Cell Type</b>	<b>Result</b>	<b>Cell Type</b>	<b>Result</b>
A549	-	NCI-H292	-
BGMK	-	pCMK	-
HEp-2	-	pRhMK	-
HFF (Hs27)	-	RD	-
LLC-MK2	-	RhMK II	-
McCoy	-	RK (passage 1)	-
MDCK	-	R-Mix	-
MRC-5	-	Vero	-
MRHF	-	Vero 76	-
Mv1Lu	-	WI-38	-

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REF

01-040000 – D<sup>3</sup> DFA Chlamydiae Culture Confirmation Kit

IVD



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PI2220002EN00 (07/19)

## GLOSSARY

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**REF**

Catalogue number



CE mark of conformity

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**EC REP**

Authorized Representative  
in the European Community

**LOT**

Batch code

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Use by



Manufacturer

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Temperature limitation



Intended use

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**R<sub>x</sub> ONLY**

Prescription use only



Consult e-labeling  
instructions for use

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Do not reuse

**IVD**

For *In Vitro* diagnostic use

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20 to 30

Contains sufficient for 20 to 30 determinations

**CONT NaN<sub>3</sub>**

Contents/Contains

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**NaN<sub>3</sub> 4%**

Contents/Contains

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