Monoclonal Antibody CMV Conjugate – 5 ml (Catalogue Number B1029-81A)

Three affinity purified mononuclear antibodies detecting nuclear and cytoplasmic CMV antigen in infected cells have been directly conjugated to FITC. Conjugate is in phosphate buffered saline (0.15 M sodium chloride and 0.01 M sodium phosphate) with 0.75% protein stabilizer. Sodium azide and thimerosal are added at 0.1% as preservatives. Evan’s blue counterstain is included at a concentration of 0.002%. Store at 2-8°C in the dark.

Buffered Glycerol Mounting Medium – 5 ml. (Catalogue Number B1029-45B)

Buffered glycerol mounting medium is to be used in conjunction with coverslips to prepare antigen slides and patient samples for microscopic observation. Store at 2-30°C.

Phosphate Buffered Saline - 1 vial. (Catalogue Number B1029-45F)

Supplied powdered buffer, when reconstituted in 1 litre of distilled water, is a physiological saline solution buffered with 0.01 M sodium phosphate to a pH range of 7.0-7.8. Prior to reconstitution, store powder at 2-30°C. Phosphate buffered saline is stable for sixty days after reconstitution. Discard if solution becomes cloudy.

WARNINGS AND PRECAUTIONS

1. For in vitro diagnostic use only.

2. Any reagents containing sodium azide or thimerosal should be evaluated for proper disposal. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. If products containing sodium azide are discarded into a drain, flush with a large volume of water to prevent azide build-up. Check with regulatory agencies to determine at what concentration sodium azide may cause a product to be a hazardous waste. For disposal, thimerosal containing reagents should be handled as a hazardous waste. MSDS for sodium azide and thimerosal are available by phoning a Technical Service Representative in the U.S. at 1-800-329-3424, or outside the U.S. at +353-1-276 9800

3. Reagents are supplied at working strength. Dilution of reagents will decrease sensitivity.

4. Reagents should not be used beyond their expiration date.

5. Reagents should not be frozen prior to use.

6. Microbial contamination of reagents may cause a decrease in sensitivity.

7. All specimens should be considered potentially infectious and handled in a manner which prevents infection of laboratory personnel. Decontamination is most effectively accomplished with a 0.5% solution of sodium hypochlorite (1:10 dilution of household bleach).

8. Although control slides have been inactivated, they should be handled and disposed of as with other potentially infectious materials.

9. Never pipet reagents or clinical samples by mouth; use a pipetting aid.

10. Do not substitute reagents from other manufacturers.

11. Incubation at different temperatures and lengths of time may give false results.

12. Evan’s blue dye is a potential carcinogen. If skin contact occurs with the conjugate, flush with water immediately.

13. Once staining has been initiated, do not allow cells to dry.

14. WARNING TO RESIDENTS OF THE STATE OF CALIFORNIA: This product contains an ingredient listed under California Chapter 3, Safe Drinking Water and Toxic Enforcement Act of 1986, Section 12000, Chemicals Known to Cause Cancer or Reproductive Toxicity. The listed ingredient is thimerosal, known to the State of California to be a reproductive toxicant.

STABILITY AND STORAGE

Reagent components will retain their full potency until the expiration date shown on the label of each vial when stored at recommended temperatures. Slides stained with fluorescent conjugate will demonstrate quenching or fading if exposed to light. Slides should be protected as much as possible during the assay. The fluorescent antibody conjugate should not be left out in the light for prolonged periods.

SPECIMEN COLLECTION AND TRANSPORT

Collection of a proper specimen is an important factor in the successful laboratory diagnosis of CMV. While CMV can be isolated from a variety of body fluids, the following specimens are recommended. Potentially infectious agents should be transported according to Title 42 and 49 of the Code of Federal Regulations.

Urine

Collect clean voided urine in a sterile container. For viral isolation, transfer 1 to 2 ml of the specimen to viral transport medium. It is not necessary to concentrate specimens prior to adding to viral transport medium, and to do this may increase toxicity to cell culture.2 Collection of multiple urine specimens has been reported to increase the isolation rate of CMV since viral shedding by the patient may be intermittent.1,3,7 For direct examination, the sample is processed directly.

Throat swabs or washings

For very young patients, a throat culture collected with a sterile cotton or Dacron™ swab is acceptable. The swab should be premoistened with sterile physiological saline, then used to rub the throat and posterior pharynx. The swab is then placed into viral transport medium, the shaft broken off, and the cap tightly secured. For older children and adults, a throat wash obtained by gargling with 10-20 ml sterile PBS or physiological saline generally allows the collection of more cells than swab cultures.2,7-9 One to two ml of the washing is then added to viral transport medium.
Bronchoalveolar lavage
Cells obtained from flexible fiberoptic bronchoscopy and subsequent lavage with sterile PBS or physiological saline should be added to viral transport medium. 22,23

Biopsy tissue
Place tissue in sterile container and send to the laboratory for specimen processing.

Buffo coat cells from heparinized peripheral blood
Collect peripheral blood into a heparin tube and send to the lab for immediate processing. EDTA is an optional anticoagulant that is reported for use in the successful isolation of CMV. 8

SPECIMEN STORAGE
Successful viral isolation depends on rapid specimen processing. Specimens should be held at room temperature or refrigerated for 30 minutes after collection to allow antigens and anticoagulants to act upon contaminants. If specimens cannot be processed immediately after collection, they should be maintained at 2-8ºC for up to 48 hours prior to freezing. Freezing CMV specimens prior to initial isolation attempts is not recommended, but if necessary, they may be frozen at -70ºC. Direct specimen examination slides should be held at 2-8ºC in an airtight container until staining.

PROCEDURE
Materials supplied
● 10 prepared antigen control slides containing one positive control well with CMV-infected Human Newborn Foreskin culture cells and one negative control well with uninfected Human Newborn Foreskin culture cells.
● 5ml anti-CMV FITC-labelled mouse monoclonal antibody conjugate with Evan’s blue counterstain.
● 5ml buffered glycerol mounting medium.
● 1 vial phosphate buffered saline for reconstitution to one litre.

Materials required but not supplied
Cell culture for cytomegalovirus isolation: Human newborn foreskin (HNF) or MRC-5 human lung fibroblast cell cultures are recommended for isolation. 23 A suitable combination would be 16 x 125 mm glass culture tubes and glass dram vials with 12 mm coverslips.

Viral transport medium which is noninhibitory to CMV or fibroblast culture cells: Catalogue Number B1029-35C and B1029-35D, B1029-58C and B1029-56D, or B1029-90C and B1029-95D, or a suitable equivalent such as Hank’s balanced salt solution plus antibiotics and a protein stabilizer, may be utilized.

Sterile Pasteur pipettes.
Sterile graduated pipettes: 10 ml, 5 ml, 1 ml.
Acetone, reagent grade. Note: Acetone fixative contaminated with water or saline may cause a hazy appearance on the substrate in fluorescence assays. Anhyd glass slides. 5

Specimen preparation
If the transport tube contains a swab, it should be handled with sterile forceps. Rotate the swab in the transport medium, then press the swab against the inside of the tube to allow excess fluid to drain back into the transport medium. Discard the swab in 0.5% sodium hypochlorite solution. If specimens are to be used for both direct detection and culture isolation/confirmation, half of the cells should be removed by centrifugation at 300 to 500g and used for the direct examination. Supernatant and remaining cells from the specimen should be used for processing specimens for cell culture isolation.

1. Direct specimen examination

a. Urine sediments - Centrifuge specimen for 5 minutes at greater than 1000g to pellet cells. Discard the supernatant, resuspend the cells in 5 ml of sterile PBS, and centrifuge at 300 to 500g. Remove and discard all but 0.1 ml PBS. Using a Pasteur pipette, resuspend and spot the cells on acetone-cleaned glass slides. Allow slides to air dry and fix for 5 minutes in prechilled acetone (2-8ºC).

b. Throat swabs/washings – Centrifuge specimen at 300 to 500g to pellet cells. Discard the supernatant and resuspend the cells in 5 ml of sterile PBS. Centrifuge the specimen again to pellet cells and discard all but 0.1 ml PBS. Using a Pasteur pipette, resuspend and spot the cells on acetone-cleaned glass slides. Allow slides to air dry and fix for 10 minutes in prechilled acetone (2-8ºC).

c. Bronchoalveolar lavage - Centrifuge specimen at 300 to 500g to pellet cells. 22,23 Discard the supernatant and resuspend the cells in 5 ml of sterile PBS. Centrifuge the specimen again to pellet cells and discard all but 0.1 ml PBS. Using a Pasteur pipette, resuspend and spot the cells on acetone-cleaned glass slides. Allow slides to air dry and fix for 10 minutes in prechilled acetone (2-8ºC).

d. Biopsy tissue - Frozen sections are best for most tissues. Impression smears are acceptable, but do not give as many cells for examination. Touch preparations, prepared by blotting fresh tissue against a slide and then pressing firmly with a sliding motion, are also acceptable for direct examination. It is recommended that tissues not be fixed with formalin since the antigen will be denatured. 22 Allow slides to air dry and fix for 10 minutes in prechilled acetone (2-8ºC).

e. Buffo coat cells from heparinized peripheral blood - Add 6% (w/w) Dextran in normal saline to a syringe containing 2 ml of heparinized blood at a ratio of 1 ml Dextran to 2 ml blood. Place syringe in an inverted position at 37ºC for 30 to 60 minutes. The upper leukocyte fraction may be expelled through a needle and the cells washed twice with 5 ml volumes of sterile PBS and low speed centrifugation (300g). 22 Resuspend in viral transport medium. Alternatively, separation of mononuclear and polymorphonuclear leukocytes using a Ficoll-Hypaque gradient has been shown to give increased isolation of the virus over conventional methods.23 Heparinized blood diluted with an equal volume of 0.9% saline is layered onto the gradient in a ratio of 3 ml of specimen to 1 ml of gradient. Specimens are then centrifuged at room temperature at 400g for 30 minutes. The mononuclear fraction is found above the gradient. The mononuclear and polymorphonuclear leukocytes which sediment below the gradient are then mixed with 6% Dextran in normal saline in a ratio of 2 ml of specimen to 1 ml of Dextran and drawn into a syringe. The solution is allowed to settle at room temperature for 60 minutes with the syringe in an inverted position. The polymorphonuclear supematant which separates from sedimented erythrocytes is removed via a needle. Both the mononuclear and polymorphonuclear fractions are washed with 5 ml volumes of sterile PBS. Centrifuge the specimen again to pellet cells and discard all but 0.1 ml PBS. Using a Pasteur pipette, resuspend and spot cells on an acetone-cleaned glass slide. If the cell density is too high, dilute with an appropriate volume of PBS. Allow slides to air dry and fix for 10 minutes in prechilled acetone (2-8ºC).

Note: Use of a cytocentrifuge in the preparation of direct examination slides may aid in interpretation of the specimen and help to avoid nonspecific trapping of reagents associated with

INCIDENT LIGHT SOURCE

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Figure 1A. Filter combinations for FITC fluorescence assay observation with a transmitted light source.

Figure 1B. Filter combinations for FITC fluorescence assay observation with an incident light source.

Incubator, 35-37ºC (CO2 or non CO2).
Sterile phosphate buffered saline (0.15 M sodium chloride and 0.01 M sodium phosphate, pH 7.0-7.6) for specimen collection and processing.
Copelin Jar
Vortex
Centrifuge
clumping of cells. Cell count is typically adjusted to 2 to 4 x 10^6 cells/ml and slides centrifuged at 500g on poly-L-lysine treated glass slides.

2. Direct specimen examination
   a. Urine sediment – Centrifuge specimen for 5 minutes at greater than 1000g to pellet cells. Suspend the cells in 5ml of sterile PBS and then centrifuge at 300 to 500g. Remove and discard all but 0.1ml PBS. Using a Pasteur pipette, spot cells on acetone cleaned glass slides. Allow slides to air dry and then fix for 10 minutes in prechilled acetone (2-8°C).
   b. Throat Swab – Centrifuge specimen at 300 to 500g to pellet cells. Resuspend the cells in 1ml of fresh PBS. Centrifuge the specimen again to pellet cells and discard all but 0.1ml PBS. Using a Pasteur pipette, spot cells on acetone cleaned glass slides. Allow slides to air dry and then fix for 10 minutes in prechilled acetone (2-8°C).
   c. Bronchoalveolar lavage – Centrifuge specimen at 300 to 500g to pellet cells.10,11,12 Using a Pasteur pipette, spot cells on acetone-cleaned glass slides. Allow slides to air dry and then fix for 10 minutes in prechilled acetone (2-8°C).
   d. Biopsy tissue – Frozen sections are best for most tissues. Impression smears are acceptable but do not give as many cells for examination. Tissue preparations, prepared by blotting fresh tissue against a slide and then pressing firmly with a sliding motion, are also acceptable for direct examination. It is recommended that tissues are not fixed with formalin since the antigen will be denatured.32 Allow slides to air dry and then fix for 10 minutes in prechilled acetone (2-8°C).
   e. Buffy coats – Refer to the preceding specimen processing section for viral isolation using buffy coats. At the last step, the cells may be washed with either 5ml volumes of tissue culture refeeding medium or sterile PBS. Then, using a Pasteur pipette, spot cells on an acetone-cleaned glass slide. Allow slides to air dry and then fix for 10 minutes in prechilled acetone (2-8°C).

Note: Use of a cytocentrifuge in the preparation of direct examination slides may aid in interpretation of the specimen and help to avoid nonspecific trapping of reagents associated with clumping of cells. Cell count is typically adjusted to 2 to 4 x 10^6 cells/ml and slides centrifuged at 500g on poly-L-lysine treated glass slides.

Tissue culture isolation and confirmation Specimen inoculation
1. If the specimen contains a swab, agitate the swab to release all cellular material and discard the swab in 0.5% sodium hypochlorite solution.
2. Disrupt host cellular material by vortexing the transport medium with sterile glass beads or by sonication at 10 k/sec for 30 seconds to enhance the release of infectious virus into the inoculum.
3. Clarify the specimen by centrifugation at 1500 to 2000g for 10 minutes.
4. Human Newborn Foreskin (HNF) or MRC-5 human lung cells are among the most sensitive culture cells available for CMV isolation.27,33 Prior to inoculation, cells should be examined microscopically to insure cell quality. Using a sterile pipette, remove medium from tubes or vials and inoculate with 0.2 ml to 0.5 ml of supernatant. It is recommended that specimens be inoculated in duplicate for maximum sensitivity.
5. Incubate for one hour at 35-37°C. Alternatively, it has been reported that a 60 minute centrifugation of vials at 700g enhances isolation.12,32-34
6. Remove inoculum, rinse with 1-1.2 ml of sterile PBS, and add 2 ml of fresh, prewarmed (35-37°C) tissue culture refeeding medium to tubes and 1 ml to drum vials. If toxicity is observed with urine or buffy coat cultures, cells may be refed the day after inoculation.
7. To insure viral sensitivity, a CMV inoculated control tube should be included each time a new tissue culture lot number is used. Also, an un inoculated tube from each lot should be kept and refed weekly to observe for normal growth and to be utilized as a negative control when observing for cytopathic effect (CPE). A negative control further allows the user to observe any adverse storage conditions.
8. Incubate all specimens and controls at 35-37°C in a stationary position with media bathing the cells. Cell culture tubes may be rotated on a roller drum at a rate of one revolution per minute to enhance virus isolation.
9. Monitor cell cultures daily for observation of CPE. Developing CPE can be detected from several weeks post-inoculation. Typical effects are rounding and syncytium formation. For early screenings of specimens (40 to 96 hours), cultures may be stained prior to development of typical CPE.12,13,14,25

Fixation of cell culture vials
1. When CPE is observed, cells should be fixed and stained to confirm microscopic detection. The following is a description of fixation and staining of a drum vial containing a coverslip, which allows for the easy fixation and removal of an intact cell culture monolayer. If necessary, cells may be rinsed with sterile PBS and scraped from a tube for spotting on an acetone-cleaned glass slide. Slides are allowed to air dry and then fixed for 10 minutes in prechilled acetone (2-8°C). Cell cultures prepared by scraping are more difficult to interpret.
2. Remove culture medium from the cell culture container using a sterile pipette and save in a sterile tube until staining has been completed. If the culture is destroyed during fixation or staining, the reserved culture medium can be used to subculture the specimen. As a secondary alternative, the original frozen specimen may be thawed in an attempt to reisolate the virus.
3. Rinse 2-3 times with 1 ml volumes of PBS. Discard rinses into a 0.5% sodium hypochlorite solution.
4. As soon as the final rinse has been removed, add 1 ml of prechilled acetone (2-8°C).
5. Remove and add 1 ml of fresh, prechilled acetone (2-8°C).
6. Fix at 2-8°C for 10 minutes, remove acetone and allow vial to air dry.
7. Rinse 2-3 times with 1 ml volumes of PBS. Vials may be stored in PBS at 2-8°C if not stained immediately.

Staining of cell culture vials
1. A control slide from the kit should be removed and stained each time the test is performed to insure reagent activity. The procedure for staining control slides is described in the “staining of direct specimen examination and antigen control slides” section below.
2. Remove the last rinse of PBS and add enough anti-CMV FITC-labelled mouse monoclonal antibody conjugate to cover the cell monolayer (2-3 drops). Replace vial cap to prevent drying during incubation.
3. Incubate at 35-37°C for 30 minutes.
4. Rinse briefly with 1 ml aliquots of PBS to remove nonreactive material. Remove 0.5 ml of the final rinse leaving a small amount of PBS to cover the cell monolayer.
5. Using a bent-tip needle attached to a 1 ml tuberculin syringe, pull the coverslip up on one side and rest the coverslip against the side of the vial. Use fine-tipped forceps to remove the coverslip from the vial.
6. Wipe dry the side of the coverslip which does not contain cells to remove excess reagent.
7. Using buffered glycero! mount medium, mount the coverslip cell side down on a glass microscope slide. When mounting, avoid trapping air bubbles which may cause hazy fluorescence.

Staining of direct specimen examination and antigen control slides
1. Add enough anti-CMV FITC-labelled mouse monoclonal antibody conjugate to cover the slide wells.
2. Incubate slides in a humid chamber for 30 minutes at 35-37°C.
3. Rinse slides briefly with phosphate buffered saline, avoiding forceful streams pointed directly at the wells.
4. Add a drop of mounting medium to the centre of each well and mount with a coverslip. Avoid trapping air bubbles, which may cause hazy fluorescence.

Observation
Observe coverslips and slides with a fluorescence microscope at 100-400X magnification. Slides may be stored in the dark at 2-8°C for 24 hours without significant fading, but immediate observation of slides after staining is recommended. For longer storage, slides may be held in the freezer at -20°C or colder. Slides which have been held under refrigerated or frozen temperatures must be brought to room temperature before reading to allow condensed moisture to evaporate from the slide surface, or reading will be obscured.

RESULTS
Quality control of reagents
A control slide from the kit should be stained each time the test is performed to insure reagent activity. The positive well of the control slide will show multiple areas of CMV-infected human diploid fibroblast culture cells. Cytoplasmic and nuclear inclusion staining will be evident. Negative well staining will show an absence of fluorescence. Negative cells will stain dull red due to the Evan’s blue counterstain included in the conjugate. All clinical results are invalid if controls fail to stain properly.

Interpretation of cell culture isolates
Nuclear and cytoplasmic staining in CMV-infected cells fluoresces a bright apple-green colour. Stained cells may appear multinucleated or as single cells in early stages. Normal uninfected cells will not fluoresce and should be reported as “No virus isolated on specimen submitted. However, this does not rule out the possibility of a CMV infection.” Trapping of conjugate by rounded cells will not fluoresce and should be reported as “No virus isolated on specimen submitted. However, this does not rule out the possibility of a CMV infection.” Trapping of conjugate by rounded cells will not fluoresce and should be reported as “No virus isolated on specimen submitted. However, this does not rule out the possibility of a CMV infection.”

Interpretation of direct examination slides
Specimens exhibiting specific apple-green nuclear and/or cytoplasmic staining are considered positive for CMV. Specimens without any specific fluorescent staining and an insufficient number of epithelial cells or white blood cells (buffy coat preparations) per field of view at 400X magnification should be reported as an inadequate specimen for testing. Adequate specimens without specific staining are reported as “No virus detected. However, this does not rule out the possibility of a CMV infection.” Trapping of conjugate by mucus, specimen debris or lymphocytic cells should be considered nonspecific. Negative cells will appear dull red due to the Evan’s blue counterstain included in the conjugate. A lab technologist with prior fluorescence experience should interpret the results.

Reasons for nonspecific immunofluorescence in patient specimens
1. Hazy glow throughout with a yellow-green colour. Staining is not identified with specific morphology. Possible sources include immersion oil, dirty lens, or unclean slide. Use glycerol in place of immersion oil being used, clean lens, re-rinse both sides of slide, and replace with a new coverslip.

Page 3 of 5 - EN
2. Dull yellow-green fluorescence associated with grouping of cells. Source is cell piling. Avoid observation of this area of the slide since specimen is too thick and conjugate has become trapped.

3. Apple-green fluorescence only at the very edge of specimen. Staining may appear specific but is due to drying of conjugate during staining. When staining, the volume of reagents used should be increased and humid chamber checked to assure that proper humidity is maintained during staining.

LIMITATIONS OF THE PROCEDURE

1. CMV isolation is greatly dependent upon specimen quality and subsequent handling.

2. Performance of this kit can only be assured when components used in the assay were supplied by Triotech Biotech.

3. Negative specimens should be reported as “No virus isolated on specimen submitted. This does not rule out the possibility of a CMV infection.”

EXPECTED VALUES

During clinical evaluation of the Bartels® CMV Fluorescent Antibody Test, an eastern regional women's hospital reported an isolation rate of 9.1% (24/263), while confirmation of cell cultures showed cytopathic effect in a children's hospital in the same region demonstrated a detection rate of 12% (33/87). Further testing in an immunocompromised population in the northwestern section of the United States yielded a detection rate of 38% (33/87). Most studies have historically shown an incidence of 0.5 to 2.5% of the newborn population infected with CMV. 11,23,31,32

SPECIFIC PERFORMANCE CHARACTERISTICS

Cross reaction study

Viral and bacterial detection studies performed with the Bartels® CMV Fluorescent Antibody Test yielded the following results when prepared antigen slides were stained.

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<th>Agent Tested</th>
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Clinical accuracy for culture isolation confirmation

Study #1
Testing of urine, buffy coat, respiratory, and biopsy specimens in an eastern regional women's hospital, yielded

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