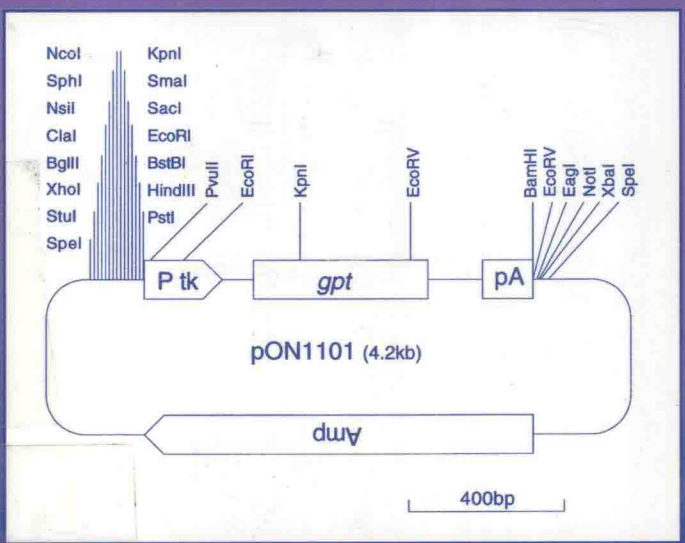
METHODS IN MOLECULAR MEDICINE™

Cytomegalovirus Protocols

Edited by
John Sinclair



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Preface

Human cytomegalovirus is an ubiquitous herpesvirus that rarely causes symptomatic disease in immunocompetent individuals. However, it is a major cause of disease in the immunocompromised, particularly those undergoing solid organ or bone marrow transplants, and infection *in utero* is a major cause of congenital malformation, often resulting in deafness and mental retardation. Clinically, then, cytomeglovirus is a major problem and an effective antiviral therapy is still in its infancy.

As well as being a major medical problem and an important target for antiviral research, cytomegalovirus also represents a viral system that is inherently scientifically interesting. As with all herpesviruses, cytomegalovirus persists in the host for life after primary infection. At least one site of carriage of the virus in the healthy seropositive individual is the peripheral blood monocyte, where the virus remains latent, but terminal differentiation of monocytes in vivo, to tissue macrophages, probably results in sporadic local reactivation in the healthy carrier. The mechanism by which latency and reactivation is controlled in these latently infected cells and just how the virus persists in the face of the host immune response are major areas of interest in cytomegalovirus research.

As with a number of other viruses, once productive infection occurs, cytomegalovirus "hijacks" many normal cellular control mechanisms in order to optimize the cell for viral DNA replication. The mechanisms by which HCMV modifies normal cellular gene expression are also of great current interest. It is clear that the virus has evolved many gene functions that target cellular transcription and DNA replication and these often involve viral proteins that act as transcriptional regulators of cellular genes, *per se*, but also act by interacting directly with cellular proteins by modifying their function.

The early chapters in *Cytomegalovirus Protocols* cover the culture and detection of cytomegalovirus using both immunological and biological techniques. The next four chapters describe techniques that analyze fundamental aspects of the infection cycle—binding and entry of the virus, transcriptional control of viral genes, and regulation of viral and cellular gene expression by direct DNA/protein or protein/protein interactions.

Cytomegalovirus infection is maintained in the infected host despite strong immune surveillance. Chapter 8 details the analysis of the T cell response to cytomegalovirus infection in the human host and the following chapter describes current methods in anticytomegalovirus research.

Much of our understanding of the molecular biology of cytomegalovirus has, to date, depended on the analysis of specific viral genes in isolation. The final chapter of the book describes techniques to generate cytomegalovirus recombinants. Such techniques will allow the generation of specific viral gene deletions and mutations, which are essential tools for investigating the structure and function of viral genes in the context of the whole viral genome.

The aim of the various chapters in *Cytomegalovirus Protocols* is to provide complete protocols in certain techniques that have already and will in the future help us further understand the biology and pathogenesis of this complex and intriguing human herpesvirus.

John Sinclair

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Contents

1

Immunological Methods for the Detection of Human Cytomegalovirus

Andrew D. Yurochko and Eng-Shang Huang

1. Introduction

Human cytomegalovirus (HCMV), a double-stranded DNA virus in the herpesvirus family, is a ubiquitous virus that infects greater than 40–60% of the general population and up to 100% within some subpopulations and/or geographic areas *(1)*. HCMV has a complex pathobiology because infection of immunocompetent individuals is rarely associated with severe clinical symptoms and in most cases is simply asymptomatic, whereas HCMV infections can cause a wide range of severe diseases, including mononucleosis, mental retardation, deafness, chorioretinitis, and fatal diseases, such as interstitial pneumonia and disseminated virus infections in immunocompromised hosts *(1)*. As with other herpesviruses, HCMV is thought to establish latent or persistent infections. Reactivation of this infection is frequently encountered during pregnancy and in organ transplant and acquired immune deficiency syndrome (AIDS) patients *(1)*. In addition, HCMV has been implicated as a co-etiological agent in cervical cancer *(2)* and has been found associated with a wide range of other tumors *(1)*. More recently, HCMV has also been shown to be epidemiologically linked to restenosis *(3–5)* and atherosclerosis *(5,6)*. The severity of these HCMV-associated

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diseases warrants an accurate ability to detect and diagnose persons with HCMV, especially because of the clinical availability of the anti-HCMV agents, ganciclovir and foscarnet, which have been used successfully to treat patients with HCMV viremia. Early and accurate detection is needed especially in cases in which infected individuals are at risk of complications arising from HCMV infection; for example, in patients undergoing organ transplants or angioplasty, expectant mothers, or AIDS patients.

The detection or diagnosis of HCMV infection in the laboratory can be achieved by a number of different approaches, including both classical methods (i.e., virus isolation, some serologic tests, and the search for cytomegalic inclusions in histological sections) and more modern methods (i.e., Western blot analysis, enzyme linked immunosorbent assay [ELISA], nucleic acid hybridization, and the polymerase chain reaction [PCR]). The appropriate method to use depends on the type of technique used to collect the specimens and the type of answer that is desired. Because of limitations, we discuss only the modern immunological techniques for detecting HCMV in the laboratory setting. The focus of this discussion is the common immunological methods for the detection of HCMV, including ELISA, immunohistology (immunofluorescence and immunoperoxidase), and Western blot analysis as it pertains to the use of immunological reagents, antibodies. Since other methods exist *(7–10)*, we also included a brief synopsis of these additional methods and provided some references for the reader's convenience to allow for a more focused understanding of these additional techniques.

2. Materials

2.1. ELISA

1. As a source of HCMV antigen and control extracts, 2 × 850-cm^2 roller-bottle (Corning, Corning, NY) cultures of human fibroblasts are needed: (1) a roller bottle with 100% of the cells showing cytopathic effects (CPE), and (2) a roller bottle of uninfected cells as a control.
2. 0.15 *M* glycine buffer (pH 9.0), 100 mL is needed.
3. Microtiter plates (Nunc, Corning, or Falcon Elisa Plates [Fisher]).

4. 1–2 L of phosphate-buffered saline (PBS).
5. Blocking solution: 0.1 *M* Tris-HCl (Boehringer, Mannheim, Indianapolis, IN; pH 8.0), 2% protease free bovine serum albumin (BSA; Boehringer Mannheim) and 0.1% thimersol (Sigma, St. Louis, MO; ~50 mL is needed).
6. Human serum sample(s).
7. 100 mL of PBS containing 0.1% Triton X-100 (Sigma).
8. Alkaline phosphatase (AP)-conjugated or horseradish peroxidase (HRP)-conjugated anti-human IgG or IgM secondary antibody (i.e., Sigma, Santa Cruz, Santa Cruz, CA, or Vector Laboratories, Burlingame, CA). *See* **Note 1** for additional comments.
9. The colorimetric substrate (*see* **Note 2**).
   a. For AP-conjugated secondary antibodies, the substrate is *p*-nitrophenyl phosphate (NPP; Sigma) — a solution containing 3 m*M* NPP, 0.05 *M* Na_2CO_3 (Mallinckrodt, Paris, KY), and 0.05 m*M* $MgCl_2$ diluted in dH_2O is needed (10 mL is needed for each 96-well microtiter plate used — this solution should be stored at 4°C).
   b. For HRP-conjugated secondary antibodies, the substrate is 3,3',5,5'-tetramethylbenzidine (TMB) — two solutions should be made:

      Solution A (urea hydrogen peroxide; Sigma): 0.054 g urea hydrogen peroxide, diluted in 100 mL of 0.1 *M* sodium citrate (adjusted to pH 5.0 with H_3PO_4; Fisher)

      Solution B (the colorimetric substrate): 30 mg of TMB, dissolved in 1 mL of DMSO and then diluted to a final concentration of 0.3 mg/mL in a 100-mL solution containing 10% glycerol (10 mL), 30% methanol (30 mL), and dH_2O (59 mL). This solution should be stored in the dark.
10. 0.5 *M* Ethylenedinitrilotetraacetic acid (EDTA).
11. ELISA plate reader.
12. These reagents are generally very stabile and can be stored for months.

2.2. Immunofluorescence and Immunoperoxidase Assays

2.2.1. IFA

1. A freshly prepared paraformaldehyde solution containing 3% paraformaldehyde (3 g), 2% sucrose (2 g) in 100 mL of PBS. The paraformaldehyde solution must be neutralized with 2 N NaOH to a

pH 7.0 and heated to 60°C to allow it to go into solution. Care should be exerci Èd when heating this product because formaldehyde is a carcinogen (this step should be performed in a chemical hood).
2. Wash buffer (PBS).
3. 10% normal goat serum (Accurate Chemical and Scientific Corporation, Westbury, NY; bovine serum albumin [BSA] or the serum from the species of animal in which the primary or secondary antibody was prepared) diluted in PBS.
4. Permeabilization buffer (0.5% Triton X-100 and 300 m*M* sucrose diluted in PBS). Make up 100 mL of this solution and store at 4°C.
5. An appropriate primary antibody targeting the HCMV gene product of interest; some are available commercially (e.g., Vancouver Biotech, British Columbia, Canada; Rumbaugh-Goodwin Institute for Cancer Research, Plantation, FL; Dupont, Boston, MA), but most have been developed in the laboratories of individual researchers (*see* **Note 1**).
6. The appropriate fluorescently-conjugated (usually fluorescein isothiocyanate [FITC]) secondary antibody (e.g., Sigma, Vector Laboratories, etc; *see* **Notes 3** and **4**).
7. 50% glycerol (Fisher) in PBS.
8. A fluorescent microscope (*see* **Note 4**).

2.2.2. IPA

1. PBS.
2. A primary antibody targeting the protein of interest (*see* **Note 1**).
3. The appropriate HRP-labeled secondary antibody (*see* **Note 3**).
4. 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB)—a solution of 0.05% DAB (5 mg DAB in 10 mL of PBS) should be prepared fresh (*see* **Note 5**).
5. 30% hydrogen peroxide (Fisher, Pittsburgh, PA).
6. 0.05 *M* EDTA in PBS.
7. A microscope with the required magnification.

2.3. Western Blot Analysis

1. Harvested infected cell lysates in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer *(7)*.
2. Gel electrophoresis and transfer equipment.

3. Nitrocellulose (Immobilon-P; Millipore, Bedford, MA).
4. Ponceau S (0.5 g of Ponceau S is first dissolved in 1 mL of acetic acid and then diluted in 100 mL of dH_2O).
5. Blocking solution (5% skim milk (Carnation nonfat dry milk) and 0.1% Tween-20 diluted in PBS), around 100 mL of blocking solution is needed for each mini-gel performed.
6. Primary anti-HCMV antibodies. There are many commercially available antibodies (Vancouver Biotech., Rumbaugh-Goodwin Institute for Cancer Research, Dupont), as well as a large number of antibodies that have been described in the literature (*see* **Note 1**).
7. 0.5–1 L of washing solution (PBS containing 0.1% Tween-20).
8. HRP-conjugated secondary antibody.
9. The enhanced chemiluminescence kit (ECL) (Amersham Life Sciences or equivalent system) or a colorimetric substrate such as TMB or DAB (*see* **Notes 2** and **5**).

3. Methods

3.1. Detection of Infectious Virus — Virus Isolation

During active infection, HCMV can be found in most tissues and organs, as well as in most bodily fluids (urine, blood, semen, milk, stool, saliva, cervical discharges). However, during in vitro cell culture, HCMV usually replicates only in human fibroblasts (e.g., HEL, WI–38, MRC-5, from American Type Culture Collection). Thus, any attempt to isolate infectious virus from a patient and subsequent culture in vitro, must be performed in the various available human fibroblast lines. Briefly, this simple yet descriptive technique involves the separation or isolation of infectious virus from the various patient samples, infection of human fibroblast cells by these viral isolates, and then the examination 3–4 d postinfection for the typical HCMV induced CPE in these cells (*see* **refs. *8–10*** for more comprehensive details about this technique).

3.2. Serologic and Immunologic Tests for CMV Infections

The diagnosis of HCMV infection can also be rapid and accurately achieved by examining either the patients serum for the pres-

ence of specific antibodies or clinical specimens for the presence of viral antigens. The various tests that can be used to accomplish this task include the complement fixation test, the viral neutralization test, and the ELISA. These techniques have been commonly used for years to measure serum anti-HCMV titers. In addition, several additional procedures can be performed to detect the presence of HCMV and/or specific HCMV antigens in various cell types infectable by HCMV. These procedures include: the IFA, the IPA, and Western blot analysis. This chapter discusses only the more modern immunological tests, the ELISA, IFA and IPA, and Western blot analysis in detail.

3.2.1. Complement Fixation Test

The complement fixation test uses hemolysin-sensitized sheep red blood cells (RBCs) to measure the amount of complement bound by specific antigen–antibody complexes in a defined assay condition ***(8–11)***. The theory behind this technique is simple: if a specific HCMV antigen–antibody reaction occurs, the complement in the serum will bind to the complex and thus not lyse the sensitized RBCs; if no antigen–antibody complex is formed, lysis of the sensitized RBCs will occur. This method was used to measure antibody titers using defined HCMV antigen, or to detect the existence of HCMV antigen using anti-HCMV antibodies, but is now generally outdated because of the use of the newer and more sensitive techniques (ELISAs and Western blot analyses).

3.2.2. ELISA

ELISAs are a very sensitive, straightforward, easy-to-perform assay for detecting either specific antibodies or antigens in human serum. Another advantage of this technique is that no radioactive materials are used and most of the reagents are stable at 4°C for months. This assay is a very valuable tool for the semiquantitative detection of HCMV-specific IgG and IgM titers in the serum of infected patients and for the detection of HCMV antigens in the serum or other body fluids. This assay has been used for the detection of specific antibodies against HCMV in nearly all types of

patients (e.g., AIDS patients, organ transplant patients, expectant mothers, neonates). The theory behind this technique involves the unique and specific association of antigens and antibodies. In the procedure described below, which is an indirect ELISA, microtiter plates are first coated with HCMV antigens and then incubated with the patient's serum. Other procedures are described in detail elsewhere *(7)*. Patients who are seropositive will possess anti-HCMV specific antibodies that will bind to the HCMV antigens coating the wells. Next, the plates are incubated with an enzymatically labeled secondary antibody that is targeted against human immunoglobulins. Finally, a colorimetric substrate is added that changes color as it is hydrolyzed by the enzyme conjugated to the secondary antibody, thus allowing for the quantitation of the levels of anti-HCMV antibodies in the serum. For measuring HCMV-specific IgG or IgM titers in serum samples, the procedure is as follows (*see* **Note 6** for potential problems):

1. The HCMV-specific antigens that are used for coating the wells are harvested from the infected fibroblasts (100% of the cells should have developed CPE) and the control extract is harvested from the uninfected control cultures (*see* **Note 7**). First the roller-bottle cultures are gently washed (2 times) with PBS, once all the PBS has been removed, 10 mL of a 0.15 *M* glycine buffer is added, the cells are carefully removed with a cell scraper and placed in a 15-mL tube. Purified overexpressed HCMV-specific antigens such as the immediate-early gene products, viral membrane glycoproteins, or other viral products that elicit an immune response can also be used (if these products are used, skip to **step 3**).
2. The cells are lysed by sonication and centrifuged at 12,000*g* for 10 min to remove the insoluble cellular debris. The supernatant is the source of the soluble HCMV antigens. These soluble antigens can be stored at –70°C for years.
3. The infected cell extracts, control extracts, and/or purified proteins are then diluted in PBS to a final concentration of approx 100 µg/mL and then added to the microtiter plates at 100 µL per well and incubated overnight at 4°C with constant agitation.
4. The plates are washed three times in PBS after the overnight absorption (*see* **Note 8**).