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# A novel high-throughput neutralization assay for supporting clinical evaluations of human cytomegalovirus vaccines

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ABSTRACT

Neutralizing antibodies are considered an important component of protective immunity against congenital infection of human cytomegalovirus (HCMV), a frequently cited cause of birth defects. An effective HCMV vaccine is desired to induce potent neutralizing antibodies in seronegative females, so that the viral transmission to fetus would be blocked if the women contracted HCMV infections during their pregnancies. We describe a novel microneutralization assay to measure antiviral activities against HCMV in serum samples. The assay is based on detection of a dominant HCMV antigen expressed in cells, using near infrared dye-labeled immune reagents. Since the detection is independent of viral cytopathic effects, this assay format has the appeal of a short turn-around time and a read-out that is not subject to operator experience and judgment, making it a promising platform to support large scale clinical studies. In a serological survey of a cohort of 200 healthy females, we showed that the neutralizing titers measured in this assay are highly comparable to those from a neutralization assay based on an enzyme-linked immunostaining method. Lastly, to demonstrate the utility of this assay to support HCMV vaccine study, we presented the results of neutralizing titers from a rhesus macaque vaccination study.



## 1. Introduction

Human cytomegalovirus (HCMV) is the leading cause of *in utero* viral infections [1]. The congenital HCMV infection may lead to severe neurological and developmental defects of fetuses, especially those born to the mothers who contracted primary HCMV infections during their pregnancies. Ten to twenty percent of the infected children may suffer permanent disabilities later in life, including sensorineural hearing loss or mental retardation [2,3]. Because of the high impact on the affected families, as well as the children, and the high economic cost associated with the medical care of children suffering from congenital HCMV infections, the Institute of Medicine has assigned the highest priority for developing a prophylactic vaccine for an indication against congenital HCMV infection [4].

There are several lines of evidence indicating that humoral immunity is important for prevention against congenital HCMV infection. HCMV seropositive mothers can provide protection for their fetuses against congenital HCMV infection, likely due to the combined effect of humoral and cellular immunity [5,6]. A non-randomized clinical study showed that the adoptive transfer of purified HCMV immune IgG from seropositive donors was effective in prevention against congenital HCMV infection in pregnant women diagnosed with primary HCMV infections [7]. A double-blind, randomized and placebo-controlled Phase II trial recently showed that an experimental vaccine of HCMV glycoprotein B (gB), designed to elicit neutralizing Abs against HCMV, was able to prevent HCMV infection in a phase II trial of seronegative women with about 50% efficacy [8]. These studies highlight the importance of humoral immunity for an effective HCMV vaccine, and the necessity of serological assays that can assess the functional component in immune sera, i.e., neutralizing activity, in HCMV vaccine development.

During natural infection, HCMV can infect multiple cell types including endothelial cells, epithelial cells, and leukocytes [9]. Recent findings have suggested that HCMV tropisms for endothelial and epithelial cells are mediated by a pentameric glycoprotein H (gH) complex [10,11], so-called because of its composition of five viral proteins (gH, gL, pUL128, pUL130 and pUL131). Laboratory HCMV AD169 and Towne strains lack this pentameric gH complex due to various mutations accumulated in UL128, UL130 or UL131 open reading frames [11]. For this reason, in order to characterize the immune responses to HCMV through natural infection or by vaccination, an assay must be able to quantitatively measure

*Abbreviations:* IRD, near-infrared dye; HCMV, human cytomegalovirus; IE, immediate early; NT50, serum dilution titers to neutralize 50% input virions; IMS, immunostaining; GMT, geometric mean titers; CPE, cytopathic effect.

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the potential of serum samples to block the entry to epithelial or endothelial cells using a virus restored of the pentameric gH complex.

In order to conduct reliable immune monitoring for large-scale clinical trials of candidate vaccines, an assay must be sensitive and robust, wherein all sources of potential variability are minimized; fairly rapid and amenable to high throughput automation, and able to measure the immune responses which are relevant to the biological mechanism of the intervention. In the case of HCMV neutralization, there are several types of assays which have been previously described. The traditional plaque reduction assay measures the ability of serum in dilutions to block viral induced cytopathic effects (CPE) on host cells. Although this assay directly assesses the antiviral activity of the testing serum samples, it is technically challenging to perform, and requires a long incubation time (14–21 days) for complete CPE [12]. Also, HCMV-induced plaques are typically small and irregular in shape, and the counting of the plaques must be aided with magnification and requires experience and judgment from assay operators. A modified microplaque assay in 96-well plate format reduces the assay turn-around time, but it involves plaque counting under a microscope [13]. These limitations and inconsistent assay read-outs, subjective in nature, render the plaque-based assay impractical to support large scale clinical studies. Another type of HCMV neutralization assay involves the immunostaining of HCMV proteins such as immediate early proteins (IE) expressed in the nuclei of infected cells in 96-well format [14,15]. The IE expression can be detected as early as 8 h post infection [9]. The infected cells are visualized by immunostaining (IMS) with either enzyme-based color development or fluorescent dye labeled reagents [14,16], and enumerated under a microscope. An alternative to this approach is to use a surrogate virus recombinantly constructed to express enhanced green fluorescent protein (EGFP). The neutralizing activities of the tested serum samples can be determined through the reduction of EGFP intensity in viral infected cultures [17]. These approaches shorten the assay time and ultimately improved the assay throughput.

Here, we report an alternative HCMV microneutralization assay based on staining viral IE proteins with near-infrared fluorescent dye (IRD)-labeled reagents. Our assay format uses a laboratory strain AD169 with its epithelial tropism restored and human epithelial cell line, ARPE-19, which could serve as a surrogate substrate for primary epithelial cells. The method of detection has the ability to quantify the antigen content through fluorescence detection. It has been applied to measuring intracellular kinase activities in high throughput drug-screening [18]. We systematically optimized this assay for measuring HCMV neutralizing activity in human sera. Furthermore, we demonstrated the comparability of this assay to the commonly used enzymatic IMS staining assay. A significant advantage of this assay format is its suitability for high throughput automation. Firstly, the format can be easily adapted to 384-well plates and liquid handling systems. Secondly, the incubation for staining is optimized for operations at ambient temperature. Thirdly, because of the favorable physical features of IRD, there is little concern for fluorescence quenching even under visible light, making this assay more flexible when adapted in high-throughput liquid handling platforms. Feasibility of this assay to support clinical studies is demonstrated by a serological survey and a rhesus monkey vaccination study.

## 2. Materials and methods

### 2.1. Cells, media and virus

ARPE-19 and MRC-5 cells were obtained from the American Tissue Culture Collection (ATCC) and maintained in DMEM:F12 medium and DMEM medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS) from HyClone (Logan, UT). Laboratory strain AD169 was obtained from ATCC, and was propagated in MRC-5 cells according to a standard protocol [12]. The procedure for generating a revertant virus with its epithelial tropism restored was modified from a method described previously [19]. Briefly, ARPE-19 cells in a 75 $cm^2$ flask were infected with AD169 virus at moi of 0.1. The cells were passed every 3–4 days and were closely monitored for CPE. After 4 passages, CPE became prominent, and the viruses were isolated from supernatant. The isolates were shown to have similar viral growth kinetics in both MRC-5 and ARPE-19 cells, and the virus was shown to contain pUL130 using a monoclonal Ab specific to the protein and it has repaired the frame-shift mutation in UL131 region reported to affect viral epithelial tropism of AD169 (data not shown) [11]. The revertant virus was cultured in ARPE-19 cells as described [11], and purified from the viral cultures by two rounds of ultracentrifugation though 20% Sorbitol cushion.

### 2.2. Serum samples

Aliquots of serum samples from 200 healthy female subjects (18–32 years old) were obtained from local blood banks with donor consent. Serum aliquots were treated at 56 °C for 30 min to inactivate complement. Serum samples were tested for HCMV reactivity using a commercial CMV IgG Enzyme Immunoassay Kit (BioCheck, Inc., Foster City, CA), and grouped into a seropositive cohort ($n = 136$) and seronegative cohort ($n = 64$) based on the cut-off value recommended by manufacturer. The sensitivity and specificity of the kit are reported to be 95% and 96%, respectively.

### 2.3. Reagents

Monoclonal antibody to HCMV immediate early (IE) antigen was purified from a mouse hybridoma (clone L-14) [20]. Biotinylated horse anti-mouse IgG (H + L) and Vectastain ABC Kit were purchased from Vector Laboratories (Burlingame, CA). IRDye® 800CW Streptavidin, Sapphire700 and Odyssey Blocking Buffer were obtained from Li-Cor Biosciences (Lincoln, NE). DRAQ5 was obtained from Biostatus Limited (Shepshed, Leicestershine, UK). Sapphire 700 and DRAQ5 were used to stain host cellular proteins and DNAs, respectively.

### 2.4. Immunostaining (IMS) neutralization assay

The IMS CMV neutralization assay was developed based on a previously published procedure [14]. Briefly, one day prior to assay, ARPE-19 and MRC-5 cells were seeded in 50 μl of DMEM:F12 or DMEM supplemented with 10% FBS in the flat bottom 96-well plates. On assay day, serum samples were serially diluted in the cell culture media 2-fold for ten to twelve dilution points starting 1:20. AD169 revertant virus stock was thawed in a 37 °C water bath and diluted in complete cell culture media. A 50 μl serum sample in dilutions was then mixed with an equal volume of viral dilution containing approximately 800 pfu, determined by IMS method. The mixture was incubated at room temperature for 1 h with gentle rocking. Afterwards, the mixture was added at 50 μl per well onto ARPE-19 or MRC-5 cell cultures. The first well in each row serves as a positive control (containing virus without serum, designated 100%) and the last well in each row serves as a negative control (containing medium only without virus, designated 0%). Plates were incubated at 37 °C with 5% $CO_2$ overnight (~20 h). On day two, supernatants from cell culture plates were gently aspirated, and the cells were fixed with methanol for 30 min. Plates were blocked with 100 μl per well of 3% milk in PBS/0.05% Tween 20 for 1 h, and incubated with 50 μl per well of mAb L-14 at 10 μg/ml for 2 h, followed by