

## ELISA on Virus-Infected Cells

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**[Abstract]** The gammaherpesvirus murid herpesvirus 4 (MuHV-4) enters cells by endocytosis from the cell surface and fusion of the viral envelope with the membrane of late endosomes. The viral envelope glycoproteins undergo antigenic changes both upon virion endocytosis and upon fusion of the viral envelope with the endosomal membrane. These changes in virion antigenicity during virus entry were first described by immunofluorescence of infected cells. Although immunofluorescence provides valuable information on the subcellular distribution of the viral glycoproteins, the quantification of immunofluorescence signals in a large number of cells is not only dependent on relatively expensive microscopy equipment, but is also relatively time-consuming. In order to quantify the antigenicity of MuHV-4 virions entering NMuMG epithelial cells in a reliable, as well as time- and cost-effective way, we have developed an ELISA with infected cells as the solid phase. In this assay, cells are grown on 96-well tissue culture plates, exposed to virions at 4 °C, followed by incubation at 37 °C allowing virion endocytosis. Cells are fixed either directly after virion binding at 4 °C or after incubation at 37 °C. After subsequent permeabilization, the cells are incubated with monoclonal antibodies specific for the viral envelope glycoproteins, followed by detection with an alkaline phosphatase-coupled secondary antibody. Upon incubation of cells with *p*-nitrophenyl phosphate substrate, the absorbance is measured on a conventional ELISA microplate reader. The different ways of data interpretation are discussed.

### **Materials and Reagents**

1. NMuMG cells (ATCC, catalog number: CRL-1636)
2. BAC-derived MuHV-4 strain 68 (Adler *et al.*, 2000)
3. Glutamine (PAA Laboratories GmbH, catalog number: E15-883)
4. Fetal bovine serum (FBS) (Amimed, catalog number: 2-01F10-I)
5. 100 U/ml penicillin and 100 µg/ml streptomycin (PAA Laboratories GmbH, catalog number: P11-010)
6. Trypsin-EDTA (PAA Laboratories GmbH, catalog number: L11-003)
7. Trypan blue solution (Sigma-Aldrich, catalog number: T8154)
8. Paraformaldehyde (Sigma-Aldrich, catalog number: P6148)

9. Glycine (Sigma-Aldrich, catalog number: 50046)
10. Triton-X100 (Sigma-Aldrich, catalog number: X100)
11. Tween 20 (Sigma-Aldrich, catalog number: P1379)
12. Bovine serum albumin (BSA) (Fraction V) (Amresco, catalog number: 0332)
13. MuHV-4 envelope glycoprotein-specific monoclonal antibodies (hybridoma supernatants) (Glauser *et al.*, 2012b; Glauser *et al.*, 2013)
14. Alkaline phosphatase-conjugated goat anti-mouse IgG ( $\gamma$  chain specific) polyclonal antibody (SouthernBiotech, catalog number: 1030-04)
15. *p*-nitrophenyl phosphate tablets (Sigma-Aldrich, catalog number: N2770)
16. Complete medium (see Recipes)
17. 10x PBS (pH 7.4) (see Recipes)
18. 1x PBS (pH 7.4) (see Recipes)
19. Trypsin-EDTA (see Recipes)
20. PBS (pH 7.4)
  - a. PBS containing 4% formaldehyde (see Recipes)
  - b. PBS containing 0.1 M glycine (see Recipes)
  - c. PBS containing 0.1% Triton-X100 (see Recipes)
  - d. PBS containing 0.1% Tween 20 and 2% BSA (see Recipes)
  - e. PBS containing 0.1% Tween 20 (see Recipes)
21. ELISA substrate (*p*-nitrophenyl phosphate) (see Recipes)

## **Equipment**

1. 150 cm<sup>2</sup> tissue culture flasks (TPP Techno Plastic Products, catalog number: 90150)
2. 96-Well tissue culture plates (F-base) (TPP Techno Plastic Products, catalog number: 92096)
3. Standard tissue culture equipment
4. Cooled tabletop microfuge
5. Fume hood
6. 37 °C, 5% CO<sub>2</sub> incubator
7. 4 °C cold room
8. Sunrise microplate reader (Tecan Trading AG)
9. Neubauer improved hemocytometer
10. 10, 20, 200, and 1,000  $\mu$ l volume pipettes and tips
11. 200  $\mu$ l volume 12-channel pipette
12. 5 ml Bijoux tubes (Sigma-Aldrich, catalog number: Z645346)
13. Disposable sterile buffer reservoirs for multichannel pipettes

14. Polystyrene boxes

**Software**

1. Microsoft Excel 2010

**Procedure**

**Part I. Considerations for the experimental design**

1. Cells are fixed at 3 different stages in the viral entry process: (i) after virion binding at 4 °C, (ii) after cell entry at 37 °C for 1 h, and (iii) after cell entry at 37 °C for 2 h. For each of these conditions, a separate tissue culture plate is used.
2. At least 6 wells should be infected for each experimental condition (virus, treatment, antibody etc.).
3. On each plate, at least 6 wells should be left uninfected in order to determine the background signal for each virus-specific monoclonal antibody.
4. If the assay is used to study the entry process of viruses pretreated with neutralizing antibodies, it might be necessary to use IgG subclass-specific secondary antibodies in order to avoid binding of the secondary antibody to the neutralizing antibody (Glauser *et al.*, 2012a).

**Part II. Experimental procedure**

**Day 1**

A. Passage and seed cells

1. Grow NMuMG cells in a 150 cm<sup>2</sup> tissue culture flask until they form a 90-100% confluent monolayer. One 150 cm<sup>2</sup> tissue culture flask yields approximately 2-3 x 10<sup>7</sup> cells, the experiment described below requires 6 x 10<sup>6</sup> cells. Wash cells 1x with 10 ml sterile PBS, then overlay with 5 ml Trypsin-EDTA and incubate for 10 min at 37 °C. Detach the cells by hitting the flask, then add 15 ml complete medium. Re-suspend cells by pipetting up and down, centrifuge 3 min at 350 x g at 4 °C and discard the supernatant. Re-suspend cells in 10 ml fresh complete medium, mix a small aliquot 1:1 with trypan blue and count with hemocytometer.
2. Seed cells into 96-well tissue culture plates (20,000 cells in 100 µl complete medium/well) and incubate overnight at 37 °C.

**Day 2**

B. Binding of virus to cells (work in 4 °C cold room)

1. Dilute virus (MOI of 5 PFU or 15 eGFP units/cell) in complete medium (83  $\mu$ l/well) in 5 ml Bijoux tubes.
2. Pre-cool the 96-well tissue culture plates containing the cells and the tubes containing the diluted viruses for 1 h on ice at 4 °C.
3. Gently tap off the cell medium and overlay cells with 83  $\mu$ l/well diluted viruses or complete medium (uninfected controls), incubate for 2 h on ice at 4 °C.
4. Gently wash the cells 3x with ice-cold PBS (approximately 300  $\mu$ l/well).

C. Fixation of cells after cell binding at 4 °C

1. Take one plate and overlay the cells with ice-cold PBS containing 4% formaldehyde (100  $\mu$ l/well), incubate for 1 h at room temperature (RT).
2. In order to stop the fixation, gently tap off the fixative and incubate the cells with PBS containing 0.1 M glycine (100  $\mu$ l/well) for 15 min at RT.
3. Wash the cells 3x with PBS (approximately 300  $\mu$ l/well).
4. Permeabilize the cells by incubation with PBS containing 0.1% Triton-X100 (100  $\mu$ l/well) for 30 min at RT.
5. Block the cells by overnight incubation with PBS containing 0.1% Tween 20 and 2% BSA (100  $\mu$ l/well) at 4 °C.

D. Fixation of cells after cell entry at 37 °C

1. Take the remaining two plates and overlay the cells with ice-cold complete medium (83  $\mu$ l/well), then incubate one plate for 1 h and the other for 2 h at 37 °C.
2. Wash the cells 1x with ice-cold PBS (approximately 300  $\mu$ l/well), then fix and treat as described in steps C1-5.

### Day 3

E. Detection of virions by ELISA

*Remark: For the primary antibodies use a concentration which gives a strong signal in immunofluorescence (IF). In our experience, hybridoma supernatants diluted 1:2 give good signals in IF and ELISA.*

1. Dilute primary antibodies (hybridoma supernatants) in PBS containing 0.1% Tween 20 and 2% BSA, add to cells (50  $\mu$ l/well) and incubate 3 h at RT.
2. Wash cells 3x with PBS containing 0.1% Tween 20 (approximately 300  $\mu$ l/well).
3. Centrifuge secondary antibody (alkaline phosphatase conjugate) for 10 min at 16,000  $\times$  g in a cooled tabletop microfuge (4 °C) to remove any potential antibody aggregates.
4. Dilute secondary antibody 1:1,000 in PBS containing 0.1% Tween 20 and 2% BSA, add to cells (100  $\mu$ l/well) and incubate 3 h at RT.
5. Wash cells 6x with PBS containing 0.1% Tween 20 (approximately 300  $\mu$ l/well).

6. Dilute *p*-nitrophenyl phosphate in H<sub>2</sub>O according to manufacturer's instructions, add to cells (100  $\mu$ l/well) and incubate overnight at RT and protected from light.
7. Measure absorbance at 405 nm on an ELISA microplate reader. If the microplate reader can measure at a reference wavelength, use 650 nm.

### Part III. Data interpretation

1. The absorbance values should not be considered an absolute measure of virion antigenicity, instead they should only be used to compare the antigenicity of virions between the different stages of virus entry, *i.e.*, virus binding at 4 °C and virus entry at 37 °C for 1 or 2 h.
2. The absorbance signal which is measured on uninfected cells can be considered the background signal of the respective monoclonal antibody and can be subtracted from the absorbance values measured on infected cells (for an example, see Figure 1A-B).
3. If the aim of the experiment is to compare the virion antigenicity of a single virus at different entry stages, the absolute absorbance values can be shown (Glauser *et al.*, 2012b).
4. If the aim of the experiment is to compare the entry kinetics of different virus mutants (Glauser *et al.*, 2013), the absorbance values for each virus mutant should be normalized to the values measured after virion binding at 4 °C (for an example, see Figure 1C).
5. If the aim of the experiment is to compare the effect of different neutralizing antibodies on virus entry (Glauser *et al.*, 2012a), the absorbance values for each virus-antibody combination should be normalized to the values measured after virion binding at 4 °C, because virus neutralization can either increase or decrease cell binding.

**A Raw data**

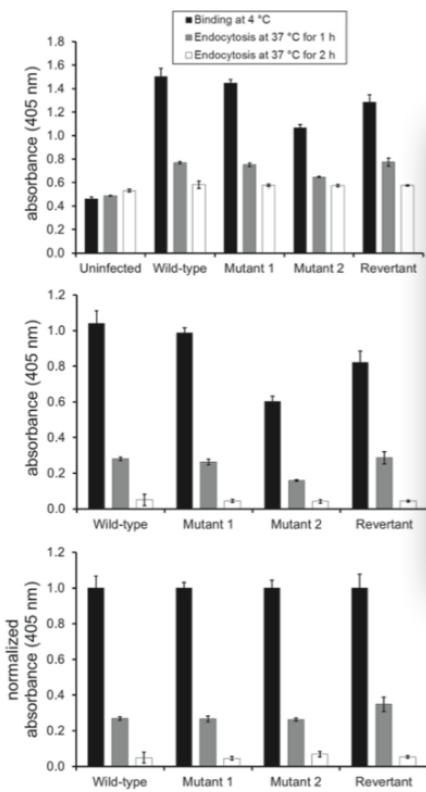
| Virus      | Binding at 4 °C | Endocytosis at 37 °C for 1 h | Endocytosis at 37 °C for 2 h |
|------------|-----------------|------------------------------|------------------------------|
| Uninfected | 0.46            | 0.49                         | 0.53                         |
| Wild-type  | 1.50            | 0.77                         | 0.58                         |
| Mutant 1   | 1.45            | 0.75                         | 0.58                         |
| Mutant 2   | 1.07            | 0.65                         | 0.57                         |
| Revertant  | 1.28            | 0.77                         | 0.57                         |

**B Background subtracted**

| Virus     | Binding at 4 °C | Endocytosis at 37 °C for 1 h | Endocytosis at 37 °C for 2 h |
|-----------|-----------------|------------------------------|------------------------------|
| Wild-type | 1.04            | 0.28                         | 0.05                         |
| Mutant 1  | 0.99            | 0.26                         | 0.04                         |
| Mutant 2  | 0.60            | 0.16                         | 0.04                         |
| Revertant | 0.82            | 0.29                         | 0.04                         |

**C Background subtracted & normalized**

| Virus     | Binding at 4 °C | Endocytosis at 37 °C for 1 h | Endocytosis at 37 °C for 2 h |
|-----------|-----------------|------------------------------|------------------------------|
| Wild-type | 1.00            | 0.27                         | 0.05                         |
| Mutant 1  | 1.00            | 0.27                         | 0.05                         |
| Mutant 2  | 1.00            | 0.26                         | 0.07                         |
| Revertant | 1.00            | 0.35                         | 0.05                         |



**Figure 1. Wild-type, mutant 1, mutant 2, and mutant 1 revertant MuHV-4 (MOI 15 eGFP units/cell) were bound to NMuMG cells for 2 h at 4 °C.** After 3 washes with ice-cold PBS to remove unbound virions, the cells were either fixed directly or after further incubations at 37 °C to allow virion endocytosis. The cells were then incubated with mAb BN-1A7 recognizing the pre-fusion conformation of MuHV-4 glycoprotein B and bound antibody detected with an alkaline phosphatase-conjugated secondary antibody and incubation with *p*-nitrophenyl phosphate substrate. The bars show mean values from 6 wells  $\pm$  standard error of the mean. (A) Mean absorbance readings at 405 nm (raw data). (B) The signal measured on uninfected cells was taken as background and subtracted from the signals measured on the infected cells. (C) After subtraction of the background signal, all values were normalized to the values measured after virus binding at 4 °C. This allows better comparison of the entry kinetics different virus mutants.

## Recipes

1. Complete medium
 

|  |        |
|--|--------|
| DMEM, 4.5 g/L glucose, with stable glutamine | 500 ml |
| Heat-inactivated fetal bovine serum          | 50 ml  |
| 100x Penicillin-Streptomycin                 | 5.5 ml |
| Stored at 4 °C                               |        |

## 2. 10x PBS (pH 7.4)

Deionized H<sub>2</sub>O 800 ml

NaCl 80 g

K<sub>2</sub>Cl 2 gNa<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O 26.6 gKH<sub>2</sub>PO<sub>4</sub> 2.4 g

Stir with magnetic stirrer until completely dissolved

Adjust to pH 7.4 with HCl

Adjust volume to 1 liter with H<sub>2</sub>O

Sterilize by autoclaving

Stored at RT

## 3. 1x PBS (pH 7.4)

Deionized H<sub>2</sub>O 900 ml

10x PBS (pH 7.4) 100 ml

Sterilize by autoclaving

Stored at RT

## 4. Trypsin-EDTA

Sterile PBS (pH 7.4) 90 ml

10x Trypsin-EDTA 10 ml

Stored at 4 °C

## 5. PBS containing 4% formaldehyde

PBS (pH 7.4) 100 ml

Paraformaldehyde 4 g

Boil PBS in microwave oven

*Caution: Do not close the lid of the bottle.*

Let PBS cool down for approximately 5 min, then add paraformaldehyde to warm PBS.

*Caution: Work in fume hood and wear gloves and eye protection.*

Stir with magnetic stirrer until completely dissolved

Stored at -20 °C

## 6. PBS containing 0.1 M glycine

PBS (pH 7.4) 100 ml

Glycine 0.75 g

Mix until completely dissolved

Sterilize by autoclaving

Stored at RT

## 7. PBS containing 0.1% Triton-X100

PBS (pH 7.4) 100 ml

Triton-X100 0.1 ml

Mix until completely dissolved

Stored at RT

8. PBS containing 0.1% Tween 20 and 2% BSA

|              |        |
|--------------|--------|
| PBS (pH 7.4) | 100 ml |
| Tween 20     | 0.1 ml |
| BSA          | 2 g    |

Mix until completely dissolved

Sterilize by filtration (0.2  $\mu$ m filter)

Stored at 4 °C

9. PBS containing 0.1% Tween 20

|              |          |
|--------------|----------|
| PBS (pH 7.4) | 1,000 ml |
| Tween 20     | 1 ml     |

Mix until completely dissolved

Stored at RT

10. ELISA substrate (*p*-nitrophenyl phosphate)

|                            |       |
|----------------------------|-------|
| Deionized H <sub>2</sub> O | 20 ml |
| Buffer tablet              | 1 pc. |
| Substrate tablet           | 1 pc. |

Mix until completely dissolved

Prepare immediately before use

### Acknowledgments

This protocol is adapted from Glauser *et al.* (2012a); Glauser *et al.* (2012b); and Glauser *et al.* (2013).

### References

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4. Glauser, D. L., Milho, R., Frederico, B., May, J. S., Kratz, A.-S., Gillet, L. and Stevenson, P. G. (2013). [Glycoprotein B cleavage is important for murid herpesvirus 4 to infect myeloid cells.](#) *J Virol* 87(19): 10828-10842.