Product Manual

Influenza A Immunoplaque Assay Kit

Catalog Numbers

VPK-5189 60 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

The influenza virus is an enveloped virus that can be divided into three classes: A, B, and C, largely based upon conserved antigenic differences in the internal nucleoprotein (NP). Only Influenza A and B are clinically relevant for humans.

Plaque assays in cell culture monolayers represent the most common method for quantification of infectious viruses and antiviral substances. In these assays, each infectious virus particle multiplies under conditions that result in a localized area of infected cells known as a plaque. The plaques are revealed as areas of dead or destroyed cells detected by general cellular stains.

Cell Biolabs' Influenza A Immunoplaque Assay Kit provides an accurate system to functionally titer virus infectivity. In contrast to the classical plaque assay, a specific anti-Influenza A Nucleoprotein antibody is used in immunostaining to reveal the plaques as areas of infected cells. The kit provides sufficient reagents for up to 60 titrations in a 12-well plate.

Assay Principle

Day 1: Seed MDCK cells in a 12-well or 6-well plate

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Day 2: Infect MDCK cells with Influenza Virus Serial Dilutions and Add Overlay Media

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Day 5: Stain with Anti-Influenza Virus Nucleoprotein Antibody

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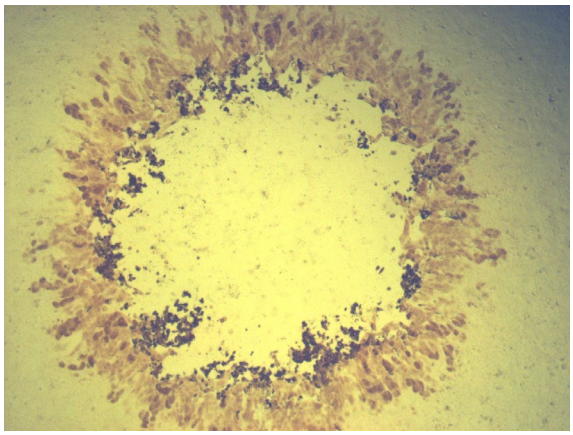
Count Positive Plaques and Calculate Viral Titer



Related Products

1. VPK-5170: RSV Fusion Protein ELISA Kit
2. VPK-5171: RSV Nucleoprotein ELISA Kit
3. VPK-5174: Influenza A Nucleoprotein ELISA Kit
4. VPK-5175: Influenza B Nucleoprotein ELISA Kit
5. VPK-5190: Influenza B Immunoplaque Assay Kit

Kit Components (shipped at room temperature)

1. 5X DMEM Medium (Part No.51901B): One 30 mL sterile bottle containing 5X DMEM, sodium bicarbonate, non-essential amino acids and 5 mg/mL BSA.
2. CytoSelect™ Agar Powder (Part No. 113001): One 1.2 g bottle.
3. TPCK-Trypsin (100X) (Part No. 51902C): One 1 mL sterile tube containing 100 µg/mL TPCK-Trypsin, 1 mg/mL BSA.
4. Anti-Influenza A Nucleoprotein Antibody (1000X) (Part No. 51891C): One 30 µL tube.
5. Secondary Antibody, HRP Conjugate (1000X) (Part No. 10902): One 50 µL tube.
6. DAB Substrate (25X) (Part No. 10903): One 1.5 mL tube.
7. Diluent (10X) (Part No. 10905): One 4.5 mL bottle.

Materials Not Supplied

1. Influenza Virus A samples
2. MDCK cells, cell culture growth medium and sterile DPBS
3. Sterile cell culture grade water
4. Microwave or Heating Block
5. Water bath
6. 10% Neutral Buffered Formalin
7. 0.1% Triton X-100 in PBS
8. 1% BSA/PBS
9. H_2O_2
10. Light Microscope

Storage

Upon receipt, store the TPCK-Trypsin at -20ºC. Store all other kit components at 4ºC.

Safety Considerations

Remember that you will be working with samples containing infectious virus. Follow the recommended NIH guidelines for all materials containing BSL-2 organisms.

Preparation of Overlay Media

Note: The table below is suggested for tests in a 12-well plate. Use twice the volume of each reagent if using a 6-well plate.

1. Preparation of 1% Agar Solution: Place 1.0 g of Agar Powder in a sterile bottle, add 100 mL of sterile cell culture grade water. Autoclave or microwave until agar is completely dissolved. Aliquot and store the 1% Agar Solution at 4ºC.

2. Melt 1% Agar Solution in a 56ºC water bath or microwave, and then cool to 37ºC in a water bath.
3. Warm the 5X DMEM medium to 37ºC in a water bath. Allow at least 30 minutes for the temperature to equilibrate.
4. According to Table 1 (below), prepare the desired volume of Overlay Media:
    a. In a sterile tube, add the appropriate volume of 5X DMEM Medium.
    b. Next, add the corresponding volume of warm Sterile H_2O and TPCK-Trypsin (100X). Mix well.
    c. Finally, add the corresponding volume of 1% Agar Solution. Mix well.

5X DMEM Medium (mL)	Warm Sterile H_2O (mL)	100X TPCK-Trypsin (mL)	1% Agar Solution (mL)	Total Volume of Overlay Media (mL)	**# of Tests in 12-well Plate (1.5 mL/test)**
1.2	1.74	0.06	3.0	6.0	**4**
2.4	3.48	0.12	6.0	12.0	**8**
3.6	5.16	0.24	9.0	18.0	**12**

Table 1. Preparation of Overlay Media

5. After mixing, maintain the Overlay Media at 37ºC to avoid gelation.

Preparation of Immunostaining Reagents

- 1X Anti-Influenza A Nucleoprotein antibody solution: Prepare a 1X Anti-Influenza A Nucleoprotein antibody solution by diluting the provided 1000X Anti-Influenza A Nucleoprotein antibody stock 1:1000 in 1% BSA/PBS. Store the diluted solution on ice.
- 1X Secondary antibody solution: Prepare a 1X Secondary antibody solution by diluting the provided 1000X stock 1:1000 in 1% BSA/PBS. Store the diluted solution on ice.
- 1X DAB working solution: Prior to use, FRESHLY prepare a 1X DAB working solution. First dilute the provided 10X Diluent to 1X with ddH_2O, and add H_2O_2 to a final concentration of 0.01%. Then dilute the 25X DAB stock to 1X with 1X Diluent/ H_2O_2 and use the 1X DAB working solution immediately.

  Note: When diluting the 10X Diluent, use ddH_2O. Heavy metals in impure H_2O will cause DAB precipitation.

Assay Protocol

Note: The instructions below are suggested for assays performed in a 12-well plate. Use twice the volume of cells and reagents for assays performed in a 6-well plate.

I. Virus Infection

1. Harvest MDCK cells and resuspend cells in culture medium at 2.5 x 10^5 cells/mL. Seed 2 mL in each well of a 12-well plate and incubate at 37ºC, 5% CO_2 overnight or until 90-100% confluent.
2. Prepare a 5- or 10-fold serial dilution of your viral sample in culture medium. Rinse MDCK cells once with DPBS, and add 200 µL of diluted viral sample to each well of the 12-well assay plate. Each sample should be performed in duplicate, and a negative control should be performed simultaneously.
3. Incubate infected cells at 37ºC, 5% CO_2 for 45-60 minutes. Gently rock the plate every 10 minutes to ensure even coverage and prevent the cell monolayer from drying.
4. Aspirate the viral inocula and wash once with DPBS.
5. Gently add 1.5 mL of warmed Overlay Media, and allow the plate to sit at room temperature for 20 minutes until the Overlay Media solidifies.
6. Incubate the infected cells at 37ºC, 5% CO_2 for 3 days.

II. Immunostaining

1. Add 1.5 mL of 10% Neutral Buffered Formalin directly on top of the Overlay Media to fix the MDCK cells and inactivate the virus. Incubate 1 hr at room temperature on an orbital shaker.
2. Aspirate the Formalin fixation solution and remove the semisolid Overlay Media with slowly running water. Gently wash the cells two times with 1X PBS.
3. Permeabilize the MDCK cells by adding 0.5 mL of 0.1% Triton X-100 in PBS for 5 min. Gently wash cells two times with 1X PBS.
4. Block for 1 hr with 1.0 mL of 1% BSA in PBS at room temperature on an orbital shaker.
5. Add 0.5 mL of diluted 1X anti-Influenza A NP antibody solution to each well and incubate for 1 hr at room temperature on an orbital shaker.
6. Gently wash the cells three times for 5 minutes each with 1X PBS.
7. Add 0.5 mL of diluted 1X Secondary antibody solution (HRP-conjugated) to each well and incubate for 1 hr at room temperature on an orbital shaker.
8. Gently wash the cells three times for 5 minutes each with 1X PBS.
9. Add 0.5 mL of freshly diluted 1X DAB working solution to each well and incubate for 10-20 minutes at room temperature on an orbital shaker.

   Note: Influenza virus-infected cells should show brown staining within 5 minutes. During incubation, excess DAB starts to form light precipitates in solution; this will not affect the staining results.
10. Aspirate the DAB solution, wash twice with 1X PBS, and add 1 mL of 1X PBS to each well.
11. Count positive stained plaques (brown).

Calculation of Influenza Virus Titer (pfu/mL)

Count the plaques in each well. Disregard wells with fewer than 5 or greater than 100 plaques. The negative control should have a uniform monolayer and can be used as a reference control.

Tests in a 12-well plate:

$$\text{Viral Titer (pfu/mL)} = \frac{\text{(average positive plaques per well) x (dilution factor)}}{\text{(0.2 mL)}}$$

Tests in a 6-well plate:

$$\text{Viral Titer (pfu/mL)} = \frac{\text{(average positive plaques per well) x (dilution factor)}}{\text{(0.4 mL)}}$$

Example of Results

The following figures demonstrate typical titration results. One should use the data below for reference only. This data should not be used to interpret actual results.

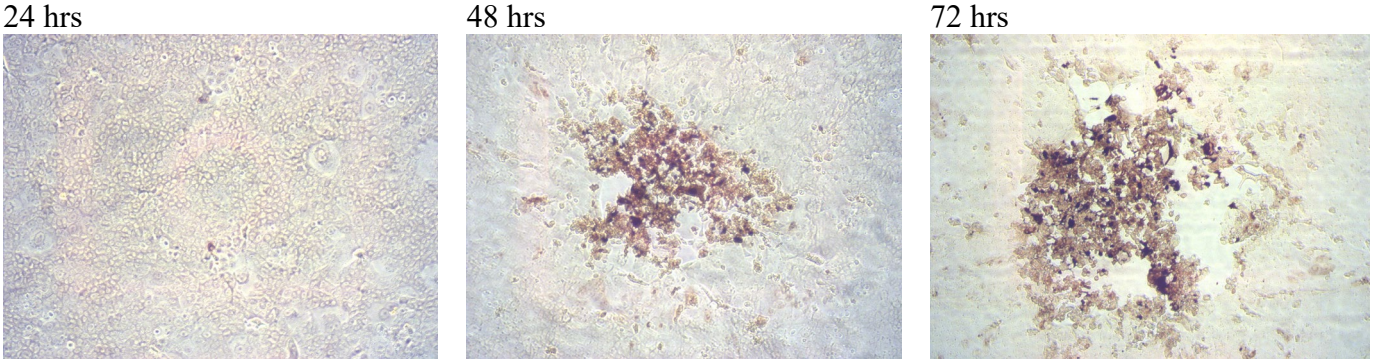


Figure 1: Plaque Formation in Influenza A Virus-Infected MDCK Cells. Influenza A virus was used to infect MDCK cells for 24 to 72 hrs. Post-infection 24 hrs, 48 hrs and 72 hrs, anti-Influenza A nucleoprotein immunostaining was performed as described in the Assay Protocol.

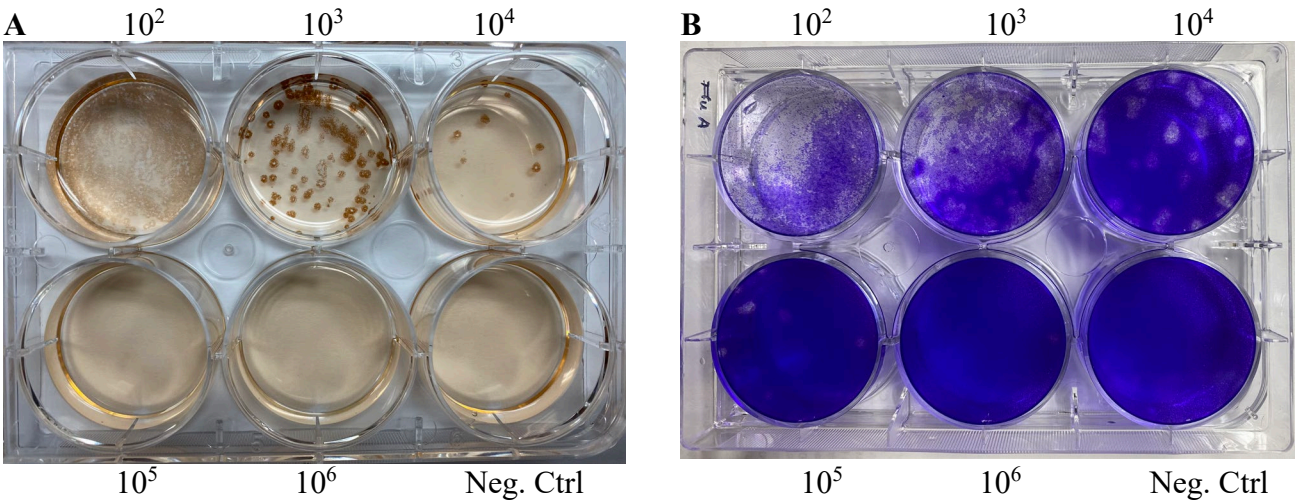


Figure 2: Immunoplaque Assay Titration of Influenza A Viruses in MDCK Cells.
Different dilutions of Influenza A Virus Culture Fluid were used to infect MDCK cells for 72 hrs. Anti-Influenza A nucleoprotein immunostaining (A) and Crystal Violet staining (B) were performed.

References

1. Herz C, Stavnezer E, Krug R, Gurney T. (1981) Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. *Cell* **26**, 391–400.
2. Jackson DA, Caton AJ, McCready SJ, Cook PR. (1982) Influenza virus RNA is synthesized at fixed sites in the nucleus. *Nature* **29**6, 366–368.
3. Baer, A. & Kehn-Hall, K. (2014) Viral concentration determination through plaque assays: using traditional and novel overlay systems. *J. Vis. Exp.* **93**, 52065.

Warranty

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