AdEasy Viral Titer Kit

Instruction Manual

Catalog #972500

Revision D.0

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AdEasy Viral Titer Kit

MATERIALS PROVIDED

Materials Provided	Concentration	Quantity[a]
Mouse Anti-Hexon Antibody	1 µg/µl	60 µg
Secondary Goat Anti-Mouse Antibody	0.4 µg/µl	12 µg
DAB (Diaminobenzidine) Substrate	10×	2 ×1.5 ml
Peroxide Buffer	1×	30 ml

[a]Sufficient reagents are provided to assay 120 wells using 24-well plates.

STORAGE CONDITIONS

Mouse Anti-Hexon Antibody: –20°C
Secondary Goat Anti-Mouse Antibody: –20°C (Reagent contains glycerol.)
10× DAB Substrate: –20°C
The DAB substrate is packaged under nitrogen for long-term stability. For storage over 6 months, replace nitrogen by gently bubbling nitrogen into the DAB liquid.
Peroxide Buffer: +4°C

ADDITIONAL MATERIALS REQUIRED

Phosphate-buffered saline (PBS) (see *Preparation of Media and Reagents*)
Bovine serum albumin (BSA)
DMEM complete growth medium (see *Preparation of Media and Reagents*)
AD-293 cells (Catalog #240085) or HEK293 cells
12-well tissue-culture treated multiwell plates or collagen type I-coated multiwell plates
(Surface area = 2.0 cm^2/well) **OR**
24-well tissue-culture treated multiwell plates or collagen type I-coated multiwell plates
(Surface area = 3.8 cm^2/well)
Methanol

Revision D.0

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INTRODUCTION

The AdEasy Viral Titer Kit is a simple enzyme-linked immunoassay for the determination of adenoviral titers. The immunoassay detects an adenoviral capsid protein called hexon, which is required for adenoviral replication. Expression of the adenoviral hexon protein is dependent upon the E1 gene product. AD-293 and HEK293[1] cells are human embryonic kidney cells transformed by sheared adenovirus type 5 DNA. These cell lines produce the adenovirus E1 gene in *trans*, allowing the expression of hexon, and thus the production of infectious virus particles when cells are transfected with E1-deleted adenovirus vectors.

The AdEasy viral titer kit can determine the titer of your stocks in 24 to 48 hours, compared to two weeks for traditional endpoint dilution assays. Titer determination is achieved in three simple steps. First, AD-293 or HEK293 cells are fixed 24 to 48 hours post-adenoviral infection, and then expression of the hexon protein is detected with an anti-hexon antibody. Next, a horseradish peroxidase (HRP) conjugated secondary antibody is added to amplify the signal. The HRP enzyme catalyzes a colorimetric reaction with the third reagent, a metal-enhanced substrate diaminobenzidine (DAB), which produces a dark precipitate in the cells expressing the hexon protein. Since only infected cells express the hexon protein, these are the only cells in culture that contain the dark brown precipitate indicative of the HRP-catalyzed reaction. The dark-colored, infected cells can therefore be counted under a standard laboratory microscope and used to calculate viral titer.

ADENOVIRAL STOCK TITERING PROTOCOL

The adenoviral titering protocol provided is performed in a 24-well culture plate. If performing the titering using a 12-well culture plate, please double all of the values in the protocol.

The AD-293 cell line (Catalog #240085), included in the protocol below, is a derivative of the commonly used HEK293 cell line, with improved cell adherence and plaque formation properties.

Note *Regardless of the cell line used for titering experiments, it is important to minimize monolayer disruption during handling.*

Infecting the Cells

1. Plate AD-293 or HEK293 cells at a density of 2.2×10^5 cells per well in 0.5 ml complete DMEM growth medium (see *Preparation of Media and Reagents*) in a 24-well tissue culture plate. Ensure that cells are spread evenly in wells for accurate titer determination.

   Notes *Cells should ideally be passaged no more than 6 times before infection.*

   It is not necessary to wait until cells adhere to the plate.

2. Perform 10-fold serial dilutions of your viral stock in 1 ml complete DMEM over a dilution series of 10^{-2} to 10^{-6}.

3. Add 50 µl of each viral dilution drop wise to each of two corresponding tissue culture wells (prepared in step 1). Incubate the cells at 37°C in a 5% CO_2 humidified incubator for 24–48 hours.

   Note *Time of infection used for titering experiments should correspond to infection time used in subsequent experiments.*

   Each dilution of virus should be assayed in duplicate to ensure accuracy.

   Include wells with no virus as negative controls.

Fixing and Staining the Cells

1. Following the 24–48 hour infection, remove the medium from the cells by aspiration, without disrupting the cells. Incubate the plate in a hood for 5–10 minutes to dry.

2. Fix the cells by adding 0.5 ml of ice-cold 100% methanol to each well. Incubate at –20°C for 10 minutes. If desired, plates can be kept at –20°C overnight.

   Note *Be sure to add the methanol to the side of the well and not directly onto the cells to avoid disrupting the cell monolayer.*

3. Aspirate the methanol. Carefully wash cells twice with 0.5 ml 1× PBS containing 1% BSA (see *Preparation of Media and Reagents*).

4. Dilute the mouse anti-hexon antibody 1:500 in 1× PBS containing 1% BSA. Aspirate the final wash from the wells. Add 0.25 ml of the diluted antibody to each well and incubate for 1 hour at 37°C.

5. Aspirate the mouse anti-hexon antibody. Carefully wash the cells twice with 0.5 ml PBS containing 1% BSA.

6. Dilute the HRP-conjugated goat anti-mouse antibody 1:1000 in 1× PBS containing 1% BSA. Aspirate the final wash from the cells, and then add 0.25 ml of the diluted secondary antibody to each well. Incubate for 1 hour at 37°C.

7. During this incubation, prepare a 1× working solution from the 10× DAB substrate solution provided. The assay requires 0.25 ml/well. Remove the 10× DAB substrate from storage at –20°C and add one part 10× DAB substrate to nine parts peroxide buffer to make the 1× working solution. Mix well. Immediately return the 10× DAB to –20°C.

   Notes *Do not bring the 10× DAB substrate to room temperature. Keep the 10× substrate solution capped when possible.*

   The 1× solution is stable over a few hours. To achieve the best results, store the 1× solution at 4°C when not in use, but bring to room temperature before adding to cells.

8. Aspirate the goat anti-mouse secondary antibody. Wash the cells twice gently with 0.5 ml 1× PBS containing 1% BSA. Aspirate the wash buffer completely.

9. Add 0.25 ml room temperature DAB substrate 1× working solution to each well and incubate at room temperature until the desired staining color develops.

   Note *Positive cells should appear dark brown to black. Typical substrate development takes about 5–15 minutes. The color can be intensified by incubating at 4°C overnight.*

10. Aspirate DAB substrate and add 0.25 ml 1× PBS to each well.

Quantitating the Viral Stock

1. Choose the dilutions with about 10% positive cells (dark brown to black) to count. Randomly choose 10 fields to count in each chosen well using a 20× microscope objective. Compute the average number of positive cells per field.

   Note *Fields containing between 10 to 50 positive cells give more accurate titers.*

2. Calculate the infectious units (IFU) per ml for each well using the following formula:

$$\frac{\text{Average number of positive cells/field} \times \text{fields/well}}{\text{Volume of diluted virus used in each well (ml)} \times \text{dilution factor}}$$

Example #1: 24-well tissue-culture plate

If the average number of positive cells per field = 20 at 10^{-4} dilution,

IFU/ml = (20 cells/field) × (314 fields/well) / (0.05 ml) × (10^{-4})

= 1.26×10^9 IFU/ml

Example #2: 12-well tissue-culture plate

If the average number of positive cells per field = 20 at 10^{-4} dilution,

IFU/ml = (20 cells/field) × (597 fields/well) / (0.1 ml) × (10^{-4})

= 1.19×10^9 IFU/ml

Determination of the Fields/Well Value

The number of total fields when using a 20× objective is derived as follows:

Radius of a standard 20× objective = 0.45 mm

The area per field = 0.045 cm × 0.045 cm × 3.1415 = 6.36×10^{-3} cm^2.

Since the area of the well of a 24-well plate is 2.0 cm^2, the total number of the fields per well = 2 cm^2 / 6.36×10^{-3} cm^2 = 314 fields / well. The area of the well of a 12-well plate is 3.8 cm^2, so the total number of fields per well is 597.

Notes *We have provided the radius of a standard 20× objective. If the radius of the 20× objective of your particular microscope is different, adjust the fields/well value used in the IFU/ml formula accordingly.*

The surface area of each well of a 24-well plate can vary among plate manufacturers. Determine the surface area per well for your particular plate and adjust the fields/well value accordingly.

TROUBLESHOOTING

Observation	Suggestion
No brown or black cells at any dilution	Confirm anti-hexon antibody and goat anti-mouse antibody (HRP-conjugate) were added and diluted accurately.
	Use low-passage HEK293 or AD-293 cells. The detection is very sensitive to the freshness of the cell line.
All cells are brown/ black	Rinse cells gently but thoroughly.
	Confirm that adenoviral stock was diluted sufficiently.
	Verify that 1× DAB working solution was prepared correctly, and that 10× DAB stock is stored at -20°C.
	Perform first wash step with PBS + 1% BSA.
	Ensure that the goat anti-mouse antibody (HRP conjugate) was diluted sufficiently.
HEK293/ AD-293 cells detach during fixing or washing step	Carry plates carefully to minimize the disturbance to the monolayer.
	When exchanging solutions, gently pipette down the side of the well and not directly onto the cells to prevent disruption of the cell monolayer.
	Pipette methanol down the side of the well and not directly onto the cells.
	Insufficient dilution of the viral stock may cause cells to detach from the plate. Try using collagen type I-coated 12- or 24-well plates for growing cells, which promote cell adhesion.

Preparation of Media and Reagents

PBS	PBS + 1% BSA
137 mM NaCl 2.6 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4 Adjust the pH to 7.4 with HCl	Dissolve 1 g bovine serum albumin (BSA) in 100 ml PBS Store at 4°C
Complete DMEM Growth Medium DMEM (containing 4.5 g/L glucose, 110 mg/L sodium pyruvate, and 2 mM L-glutamine) supplemented with 10% (v/v) heat-inactivated fetal bovine serum	

References

1. Graham, F. L., Smiley, J., Russell, W. C. and Nairn, R. (1977) J Gen Virol 36(1):59-74.

MSDS Information

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

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QUICK-REFERENCE PROTOCOL

Infecting the Cells

- Inoculate 24-well tissue culture plates with 2.2×10^5 cells/well in 0.5 ml DMEM.
- Perform serial dilutions of viral stock in 1 ml DMEM from 10^{-2} to 10^{-6}.
- Add 50 μl of each viral dilution to the appropriate cell culture wells (in duplicate).
- Incubate cells at 37°C in a 5% CO_2 humidified incubator for 24–48 hours.

Fixing and Staining the Cells

- Remove the culture medium from the cells by aspiration.
- Incubate the plate in a hood for 5–10 minutes to dry.
- Add 0.5 ml ice-cold methanol to side of each well.
- Incubate at –20°C for 10 minutes.
- Aspirate methanol. Wash cells ***gently*** with 0.5 ml 1× PBS + 1% BSA twice.
- Dilute mouse anti-hexon primary antibody 1:500 in 1× PBS + 1% BSA.
- Add 0.25 ml diluted primary antibody to each well. Incubate 1 hour at 37°C.
- Aspirate the mouse anti-hexon antibody and gently wash the cells twice with 0.5 ml 1× PBS + 1% BSA.
- Dilute the goat anti-mouse secondary antibody (HRP conjugate) 1:1000 in 1× PBS + 1% BSA.
- Add 0.25 ml diluted secondary antibody to each well. Incubate 1 hour at 37°C.
- Prepare 1× DAB substrate by adding 1 part 10× DAB to 9 parts peroxide buffer. Mix well.
- Aspirate the secondary antibody. Wash cells twice ***gently*** with 0.5 ml 1× PBS+ 1% BSA. Aspirate completely.
- Add 0.25 ml room temperature 1× DAB substrate. Incubate at room temperature 5–15 minutes.
- Aspirate DAB substrate and add 0.25 ml 1× PBS to each well.

Determining Viral Titer

- Identify wells containing ~10% stained cells.
- Count the stained cells in 10 fields in each of the chosen wells, and then determine the average number of stained cells/field.
- Calculate the viral titer using the following formula:

$$\frac{\text{Average number of positive cells/field} \times \text{fields/well}}{\text{Volume of diluted virus used per well (ml)} \times \text{dilution factor}}$$