Product Manual

Rapid RCA Assay Kit

Catalog Number

VPK-111	30 assays
VPK-111-5	5 x 30 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Introduction

Recombinant adenoviruses have tremendous potential in both research and therapeutic applications. They provide numerous advantages when introducing genetic material into host cells. The permissive host cell range is very wide. Adenovirus has been used to infect many mammalian cell types (both replicative and non-replicative) for high expression of the recombinant protein. Recombinant adenoviruses are especially useful for gene transfer and protein expression in cell lines that have low transfection efficiency with liposome. After entering cells, the virus remains epichromosomal (i.e. does not integrate into the host chromosome so does not activate or inactivate host genes). Recently, recombinant adenoviruses have been used to deliver RNAi into cells.

HEK 293 cells or their variants are used as host cells for viral amplification. Recombinant adenoviruses can be grown at high titer, e.g. 10^{10} VP (viral particles)/mL, which can be concentrated up to 10^{13} VP/mL, and subsequently purified by Cell Biolabs ViraBind™ Adenoviral Purification Kit or traditional CsCl ultracentrifugation.

During adenovirus vector production, particles may be generated which are replication competent. The probability of producing replication competent adenovirus (RCA), although low, increases with each successive amplification. RCA is thought to be produced via homologous recombination. Traditionally, RCA is measured in permissive cells by a plaque-forming unit (PFU) assay that scores the number of viral plaques as a function of dilution. These methods are time-consuming (10-14 days), require a long infection period, and suffer from a high degree of inter-assay variability and are affected by virus-cell interactions. Cell Biolabs' Rapid RCA Assay Kit utilizes an antibody against adenovirus hexon proteins to visualize infected cells by immunocytochemistry staining, the kit antibody against hexon protein recognizes all serotypes of adenovirus. The hexon proteins are the largest and most abundant of the structural proteins in the adenovirus capsid, and they are distributed symmetrically to form capsid facets.

Cell Biolabs Rapid RCA Assay Kit provides a quick and complete system to measure the titer of replication-competent virus in your viral prep. In contrast to the 10-day infection of a classical plaque assay, the kit only requires 2-day infection. The kit provides sufficient reagents for up to 30 tests in 6-well culture plates.

Assay Principle

Seed A549 cells in a 6-well plate

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Prepare Adenovirus Dilutions and Infect A549 cells for 48 hours

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IHC Staining with Anti-Hexon Antibody

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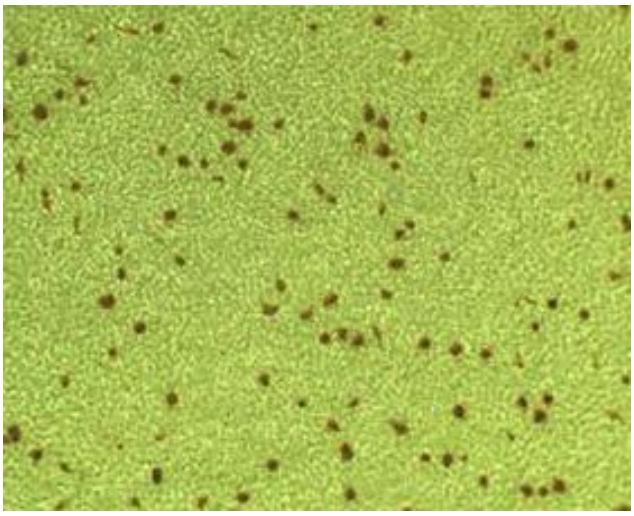
Count Positive Cells and Calculate Viral RCA



Related Products

1. AD-100: 293AD Cell Line
2. AD-200: ViraDuctin™ Adenovirus Transduction Reagent
3. VPK-106: QuickTiter™ Adenovirus Quantitation Kit
4. VPK-109: QuickTiter™ Adenovirus Titer Immunoassay Kit
5. VPK-110: QuickTiter™ Adenovirus Titer ELISA Kit
6. VPK-252: RAPAd® CMV Adenoviral Expression System
7. VPK-099: ViraBind™ Adenovirus Miniprep Kit
8. VPK-112: QuickTiter™ Lentivirus Quantitation Kit
9. VPK-120: QuickTiter™ Retrovirus Quantitation Kit

Kit Components

1. Anti-Hexon Antibody (1000X) (Part No. 10901): One 30 µL tube.
2. Secondary Antibody, HRP Conjugate (1000X) (Part No. 10902): One 50 µL tube.
3. DAB Substrate (25X) (Part No. 10903): One 1.5 mL tube.
4. Diluent (10X) (Part No. 10905): One 4.5 mL bottle.

Materials Not Supplied

1. Recombinant adenovirus of interest
2. A549 cells and cell culture growth medium
3. Methanol

4. 1% BSA/PBS
5. H_2O_2
6. Light Microscope
7. (optional) Wild type adenovirus

Storage

Store all 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 Reagents

The table below is suggested for tests in 6-well plate.

- 1X Anti-Hexon antibody solution: Prepare a 1X anti-hexon antibody solution by diluting the provided 1000X Anti-Hexon 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 dilute 10X diluent, use ddH_2O. Heavy metals in impure H_2O will cause DAB precipitation.

Reagents	6 tests	12 tests	24 tests
1000X Anti-hexon Antibody	6 µL	12 µL	24 µL
1000X Secondary Antibody	6 µL	12 µL	24 µL
25X DAB	240 µL	480 µL	960 µL
Final Volume (Each Reagent)	**6 mL**	**12 mL**	**24 mL**

Table 1. Preparation of Antibody and DAB solutions for use in a 6-well Plate.

Preparation of Adenoviral Samples

Immediately before infection, dilute your adenovirus sample to 1 x 10^9 VPs/mL in culture medium. Prepare 2 mL for each viral sample. For example, if you have a viral prep of 1 X 10^{11} VPs/mL, add 20 µL into 2 mL of culture medium.

Note: A wild type adenovirus, such as wild type Ad5, should be used as an assay positive control. Prepare serial dilutions of the wild type adenovirus in culture medium.

Assay Protocol

Note: The instructions below are suggested for assays performed in 6-well plate.

I. Virus Infection

1. Harvest fresh, healthy A549 cells and resuspend cells in culture medium at 2.5 x 10^5 cells/mL. Seed 2 mL in each well of a 6-well plate and incubate at 37ºC, 5% CO_2 for 1 hr.
2. Dropwise add 0.5 mL of adenoviral sample (1 x 10^9 VPs/mL) to each well of the 6-well plate. To ensure accuracy, perform each sample in duplicate.

   Note: a positive control, such as wild type Ad5, should be tested simultaneously.
3. Incubate infected cells at 37ºC, 5% CO_2 for 48 hrs.

II. Immunostaining

1. Slowly remove medium from the wells by tilting the plate and aspirating from the edge, then fix infected A549 cells by gently adding 2 ml of cold methanol to each well of the 6-well plate. Incubate 20 minutes at -20ºC.
2. Gently wash the fixed cells three times with 1X PBS, five minutes each wash.
3. Block for 1 hr with 1% BSA in PBS at room temperature on an orbital shaker.
4. Add 1 mL of diluted 1X Anti-Hexon antibody solution to each well and incubate for 1 hr at room temperature on an orbital shaker.
5. Gently wash the fixed cells three times with 1X PBS, five minutes each wash.
6. Add 1 mL of diluted 1X Secondary antibody solution (HRP-conjugated) to each well and incubate for 1 hr at room temperature on an orbital shaker.
7. Gently wash the fixed cells five times with 1X PBS, five minutes each wash.
8. Add 1 mL of freshly diluted 1X DAB working solution to each well and incubate for 15-30 minutes at room temperature on an orbital shaker.
9. Aspirate DAB, wash once with 1X PBS and add 2 mL of 1X PBS to each well.
10. Count positive stained cells (brown) for at least ten separate fields per well using a light microscope and 10X objective.
11. Calculate the average number of positive cells per field and RCA.

Example of Results

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

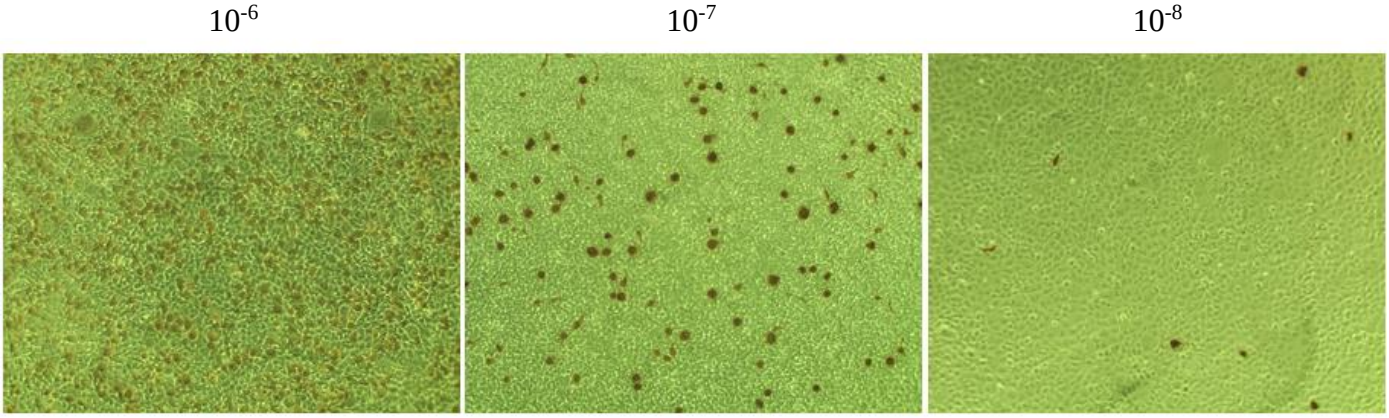


Figure 1: Rapid RCA Assay. Serial 10-fold dilutions of wild type Ad5 (2.0×10^{12} VPs/mL) were used to infect A549 cells in 6-well plate for 48 hrs. Anti-Hexon immunostaining was performed as described in the Assay Protocol.

Calculation of Replication Competent Adenovirus

1. Randomly count at least 10 fields. Calculate the average number of positive cells per field.
2. Determine the number of fields per well.

For most microscopes, a standard 10X objective lens with 10X eyepiece lens has a field diameter (D) of 1.8 mm, then:

Area per field = $3.14 \times (D/2)^2 = 3.14 \times 0.9^2 = 2.54\ mm^2$

For a standard 6-well plate, the surface area is 9.5 cm^2/well, therefore:

Fields/each well of 6-well plate = $9.5\ cm^2/2.54\ mm^2 = 9.5\ cm^2/2.54 \times 10^{-2}\ cm^2$ = **374**

Note: If you are not sure about the field diameter of the 10X objective lens you are using or you are using objective lenses other than 10X, the field diameter can be determined by aligning with the grids of the hemocytometer (Figure 3), or referring to Table 2.

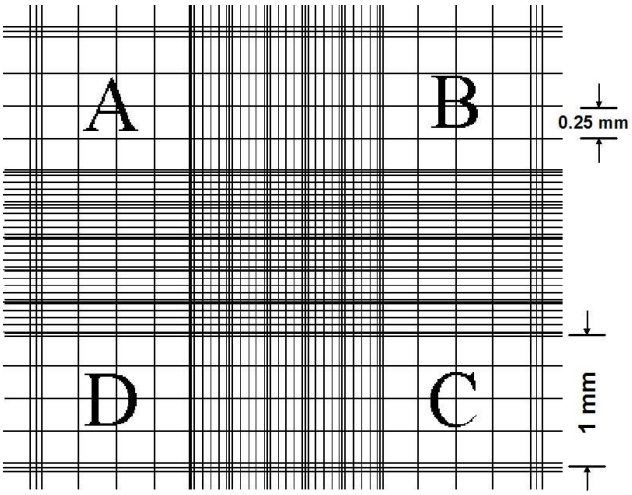


Figure 3. Hemocytometer Grid Dimensions.

Objective Lens	10X Eyepiece Lens		Fields/Well (6-well Plate)
	Total Magnification	Field Diameter	
4X	40X	5 mm	48
10X	100X	1.8 mm	374
20X	200X	0.9 mm	1494

Table 2. Field Sizes of Objective Lenses.

3. Calculate RCA (Replication-competent Adenovirus/VP)

For wild type Ad5:

$$\text{RCA} = \frac{\text{(average positive cells/field) x (374 fields/well) x (dilution factor)}}{\text{Total VPs in 0.5 mL viral sample}}$$

For recombinant adenovirus:

$$\text{RCA} = \frac{\text{(average positive cells/field) x (374 fields/well)}}{\text{Total VPs in 0.5 mL viral sample}}$$

Calculation Examples:

Sample #1: wild type Ad5

A series of 10-fold dilutions of the wild type Ad5 (2.0×10^{12} VP/mL) were made and RCAs were determined in a 6-well plate as described in assay instruction. At $1/10^7$ dilution, ten fields were counted and the average positive cells/field is 40 under a standard 10X objective, therefore:

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$$RCA = \frac{\text{(average positive cells/field) x (374 fields/well) x (dilution factor)}}{\text{Total VPs in 0.5 mL viral sample}}$$

$$RCA = \frac{\text{(40/field) x (374 fields/well) x } (10^7)}{1.0 \times 10^{12} \text{ VP}} = 1.5 \times 10^{11} \text{ RCA in } 10^{12} \text{ VP or 15 RCA in 100 VP}$$

Sample #2: Recombinant Ad-β gal

Ad-β gal viral stock (1.2×10^{12} VPs/mL) was diluted to 1×10^9 VPs/mL and its RCA was determined in a 6-well plate as described in assay instruction. Ten fields were counted and no positive stained cells are visible under a standard 10X objective, therefore:

$$RCA = \frac{\text{(average positive cells/field) x (374 fields/well)}}{\text{Total VPs in 0.5 mL viral sample}}$$

$$RCA = \frac{\text{(0/field) x (374 fields/well)}}{0.5 \times 10^9 \text{ VPs}} = 0 \text{ RCA in } 0.5 \times 10^9 \text{ VPs or } <1 \text{ RCA in } 0.5 \times 10^9 \text{ VPs}$$

Note: If there is no positive stained cell in your viral sample, it indicates that the titer of RCA is <1 RCA in 0.5×10^9 VPs in your viral prep. For details on adenovirus RCA safety, please follow NIH guidelines.

References

1. Bewig, B., and W. E. Schmidt (2000) Accelerated titering of adenoviruses. *BioTechniques* 28:870-873.

Recent Product Citations

1. Chappell, C.L. et al. (2016). Adenovirus 36 antibody detection: improving the standard serum neutralization assay. *J. Vir. Methods* **239**:69-74.
2. Dubuisson, O. et al. (2015). Accurate identification of neutralizing antibodies to adenovirus Ad36, a putative contributor of obesity in humans. *J Diabetes Complications.* **29**: 83-87.

Warranty

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