Adeno-X™ Rapid Titer Kit User Manual

Cat. No. 631028
PT3651-1 (PR5X1070)
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I. Introduction & Protocol Overview

The **Adeno-X™ Rapid Titer Kit** provides a complete set of materials for the quantification of adenoviral stocks. Titration of Adenoviral stocks is important for maintaining consistency between experimental samples and achieving the correct level of transient expression. Also, when producing viral stocks it is important to know the titer of infectious particles for sucessful virus production. Results are obtained much more quickly with this kit (within ~48 hr) than with the endpoint dilution assay . The values obtained are comparable to endpoint dilution titration values, a method that normally takes up to 2 weeks to perform.

The Adeno-X Rapid Titer Kit takes advantage of production of viral hexon proteins for the quantification of viral stocks. Dilutions of the viral stock in question are used to infect HEK 293 cells. Just 48 hours later, these cells are fixed and stained with the antibody specific to the adenovirus hexon protein. Signal is detected after a secondary antibody conjugated with horseradish peroxidase (HRP) amplifies the signal of the antihexon antibody (Figure 1). Subsequent exposure to metal-enhanced DAB substrate turns only the infected cells dark brown (Figure 2). Then the titer of the stock in question can be determined by counting the number of brown cells in a given area. Each stained cell corresponds to a single infectious unit or event (ifu). This assay yields values that correlate well with endpoint and gene transducing unit assay measurements as well as with OD_{260} measurements of total viral particles (Bewig & Schmidt, 2000). For more information about different methods for adenoviral titration, refer to our Adeno-X Expression System User Manual (PT3414-1).

Applications

This kit has been developed for use with any first generation adenoviral system as long as the adenovirus is able to amplify in HEK 293 cells. It can be used to determine titers of the recombinant adenovirus created with our **Adeno-X™ Expression System** (Cat. No. 631513). With our Adeno-X Expression System, an early passage HEK 293 cell line is required to propagate and titrate recombinant adenoviruses derived from Adeno-X Viral DNA because the E1 elements have been eliminated (Graham *et al.*, 1977; Aiello *et al.*, 1979). HEK 293 cells stably express the Ad5 E1 genes that are essential for replication and transcription of Adeno-X Viral DNA. For more information on expression cassettes and the Adeno-X Viral genome, see our Adeno-X Expression System User Manual (PT3414-1).

I. Introduction & Protocol Overview *continued*

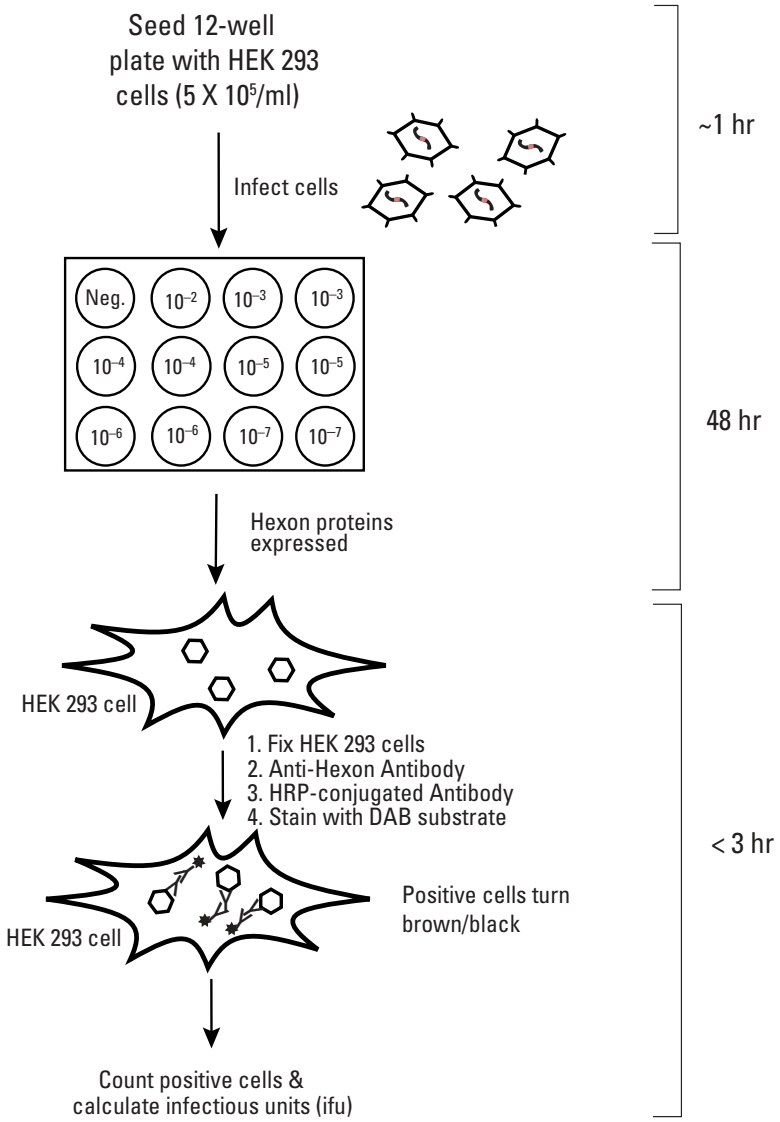

Figure 1. Adeno-X™ Rapid Titer Method. HEK 293 cells are infected with adenovirus at different dilutions. After the hexon proteins appear, the cells are fixed and treated with a hexon protein-specific antibody, HRP-conjugate antibody and developed with DAB Substrate. During development the positive cells turn brown so that they can be easily counted under a 20X objective. The ifu is calculated from the resulting average number of positive cells/unit dilution. Neg.= negative control

II. List of Components

- Store Rat Anti-Mouse Antibody (HRP conjugate) and Stable Peroxidase Buffer at 4°C. DO NOT FREEZE.
- Store Mouse Anti-Hexon Antibody and 10X DAB Substrate at –20°C.
- Store Adeno-X-LacZ Viral Stock at –70°C. Note: repeatedly freezing and thawing of the Adeno-X-LacZ Viral Stock may inactivate the virus and thus, could reduce the observed titer.

The following reagents are suitable for 60 titrations or 5 x 12-well plates.

Box 1:

- 30 µl Mouse Anti-Hexon Antibody
- 60 µl Rat Anti-Mouse Antibody (HRP conjugate)
- 30 ml 1X Stable Peroxidase Buffer

Box 2:

- 3.0 ml 10X DAB Substrate
- 100 µl Adeno-X-LacZ Viral Stock
  (For assaying purposes only. See Product Analysis Certificate for titer of the Viral Stock)

III. Additional Materials Required

The following materials are required but not supplied:

- **Phosphate buffered saline** (PBS; pH 7.5)
- **Phosphate buffered saline + 1% bovine serum albumin (PBS + 1% BSA)**

  Dissolve 5 g Bovine serum albumin (Sigma, Fraction V, Cat. No. A-3803) in 500 ml PBS (above). Store at 4°C.
- **12-well culture plates** (BD Falcon, Cat. No. 353043)
- **Laminar flow hood** (BL2)
- **Incubator** (humidified, 5% CO_2)
- **Microscope** (with a 20X objective)
- **Hemacytometer**
- **Cell culture medium** (e.g. DMEM + 10% fetal bovine serum + antibiotics)
- **Methanol**
- [Optional] **BD Biocoat Collagen Type I 12-well plates**
  (BD Biosciences - Discovery Labware Cat. Nos. 354500 & 356500)

  Note: Promotes stronger cell adhesion and helps prevent disruption of cell monolayer during rinses of the wells.
- [Optional] **Orbital Shaker**

[Optional] Required for X-gal Staining Procedure (Appendix C)

- **Fixative** (2% formaldehyde, 0.2% glutaraldehyde in PBS, 4°C)
- **X-gal** (40 mg/ml, dissolved in dimethyl formamide, stored at –20°C)
- **Potassium ferricyanide** (Sigma, Cat. No. P-3667)
- **Potassium ferrocyanide** (Sigma, Cat. No. P-3289)
- **Magnesium Chloride** (1 M solution)

IV. General Considerations

When gathering data for the Adeno-X Rapid Titer Kit, it is important that the counted fields be selected in an unbiased manner. Therefore, we recommend that you randomly select a minimum of three fields to count and that the counted fields contain 10–50 positive cells—assuming that the distribution of infected cells is random over the entire well. Fields with fewer positives (5–10 cells) can be counted; if you do so we suggest that you count more fields (6–10) to achieve the same degree of accuracy.

An Adeno-X-LacZ Viral Stock is included with the Adeno-X Rapid Titer Kit for use as a positive control. This Stock can also be used with the X-gal staining procedure provided in Appendix C.

In addition, the degree of error introduced in each serial dilution may affect the result. Therefore, in order to maximize the accuracy, measure samples in duplicate. One important factor in making the dilutions and infecting the cells is to be consistent in the amount of viral dilution added to the well (0.1 ml in our procedure).

V. Adeno-X™ Rapid Titer Procedure

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Each dilution of virus should be assayed in duplicate to ensure accuracy. If desired, you may also assay the provided Adeno-X-LacZ Viral Stock of known titer as a positive control.

A. Infect Cells

1. Seed 1 ml of healthy HEK 293 cells (5 x 10^5 cells/ml) in each well of a 12-well plate. Use standard growth medium (e.g., DMEM + 10% FBS + antibiotics).

   Note: Cells will not completely adhere to the plate during infection.

2. Using PBS or medium as diluent, prepare 10-fold serial dilutions of your viral sample from 10^{-2} to 10^{-6} ml. For example, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} (See Figure 1; see Appendix A for suggestions on how to dilute adenoviral stock solutions).

   Note: To improve accuracy, you may need to adjust dilutions to 5x 10^{-3}, 5x 10^{-4}, etc., depending on the expected viral titer (see Appendix A).

3. Add 100 µl of viral dilution dropwise to each well.

   Note: Perform duplicate infections to ensure accurate assay results.

4. Incubate cells at 37°C in 5% CO_2 for 48 hr.
5. Aspirate medium. Allow cells to dry in hood for 5 min.

B. Fix Cells and Add Antibodies

1. Fix cells by **very gently** adding 1 ml ice-cold 100% methanol to each well.

   Note: Add methanol gently. Do not dislodge cell monolayer. The monolayer can easily dislodge until cells are fixed.

2. Incubate the plate at –20°C for 10 min.
3. Aspirate methanol. Gently rinse the wells three times with 1 ml PBS + 1% BSA.
4. Dilute Mouse Anti-Hexon Antibody 1:1,000 in PBS + 1% BSA.
5. Aspirate final rinse from the wells. Then add 0.5 ml of Anti-Hexon Antibody dilution to each well. Incubate 1 hr at 37°C on an orbital shaker (orbital shaker optional).
6. Aspirate Mouse Anti-Hexon Antibody. Then gently rinse wells three times with 1 ml PBS + 1% BSA.
7. Dilute Rat Anti-Mouse Antibody (HRP conjugate) 1:500 in PBS + 1% BSA.
8. Aspirate final rinse from the wells. Then add 0.5 ml Rat Anti-Mouse Antibody (HRP conjugate) dilution to each well. Incubate 1 hr at 37°C on an orbital shaker (orbital shaker optional).

V. Adeno-X™ Rapid Titer Procedure *continued*

9. Prior to removing the Rat Anti-Mouse Antibody (HRP conjugate), prepare DAB working solution by diluting 10X DAB Substrate 1:10 with 1X Stable Peroxidase Buffer (you will need 500 µl DAB working solution per assay well). Allow the DAB working solution to come to room temperature.

   Note: Do not allow 10X DAB Substrate to come to room temperature.

10. Aspirate Rat Anti-Mouse Antibody (HRP conjugate) dilution. Gently rinse each well three times with 1 ml PBS + 1% BSA.

C. Develop Color and Quantitate

1. After removing the final PBS + 1% BSA rinse, add 500 µl DAB working solution to each well. Incubate at room temperature for 10 min.
2. Aspirate DAB and add 1 ml PBS to each well.
3. Count a minimum of three fields of brown/black positive cells using a microscope with a 20X objective, and calculate the mean number of positive cells in each well.

   Note: Count dilutions with 10% or fewer positive cells. Ideal field should contain 5 to 50 positive (black/brown) cells. See Figure 2.

4. Calculate infectious units (ifu)/ml for each well as follows:

$$\frac{\text{(infected cells/field) x (fields/well)}}{\text{volume virus (ml) x (dilution factor)}}$$

D. Example Calculation:

- Mean positive cells/ field = 10 at 10^{-5} dilution
- Fields/well (20X objective) = **573 fields** (See Appendix B for derivation)
- Amount dilution added = 0.1 ml

  Therefore,

- ifu/ml = (10 cells/field) x (573 fields/well) / (0.1 ml) x (10^{-5}/ml)

  = 5.73 X 10^{9} ifu/ml

V. Adeno-X™ Rapid Titer Procedure *continued*

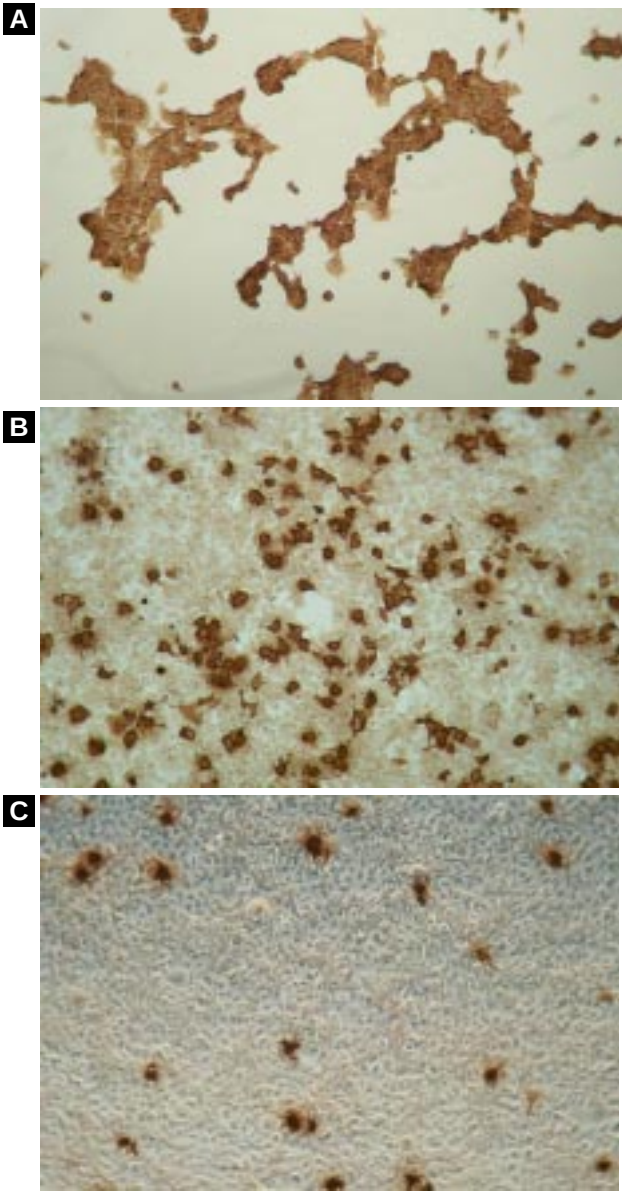

Figure 2. Fields of positive cells. Different dilutions of adenovirus were used to infect HEK 293 cells and developed by the Adeno-X Rapid Titer method. A cytopathic effect is evident in Panel A (10^{-3}). In this example, the 10^{-4} and 10^{-5} dilutions (Panels B & C) would provide the fields of cells most ideal for counting. These photos were taken using a 5X objective.

VI. Troubleshooting Guide

A. No positive cells

After completing the protocol, no brown or black cells can be observed in any wells at any dilution.

- Anti-Hexon or Rat Anti-Mouse Antibody (HRP conjugate) was inadvertently omitted.
- Rat Anti-Mouse Antibody (HRP conjugate) was inadvertently frozen.

  The HRP enzyme is sensitive to freeze-thaw cycles.
- Rat Anti-Mouse Antibody (HRP conjugate) expired.

  Check storage and shelf life of Rat Anti-Mouse Antibody (HRP conjugate).
- Did not infect for a full 48 hr before fixing cells. As a result hexon expression did not reach detection threshold.
- Check your technique using the Adeno-X-LacZ Viral Stock and the X-gal staining procedure provided in Appendix C.

B. All cells are positive (Brown/Black)

- Inadequate rinsing steps
- Incorrect or insufficient dilution of adenovirus stock
- Incorrect dilution of Rat Anti-Mouse Antibody (HRP conjugate), or incorrect preparation of DAB working solution
- Did not use PBS + 1% BSA for washing steps

C. Cell monolayer is disrupted or comes off during fixing step

- Use collagen-coated plates for growing cells (See Additional Materials Section).
- Add methanol **very** gently to the well
- Rinses not gentle enough

VII. References

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Bewig, B. & Schmidt, W. E. (2000) Accelerated Titering of Adenoviruses. *BioTechniques* **28**:871–873.

Graham, F. L., Smiley, J., Russel, W. C. & Nairn, R. (1977) Characterization of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**:59–72.

Price, J., Turner, D., Cepko C. (1987) Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proc. Natl. Acad. Sci. U S A* **84**(1):156–160.

VIII. Related Products

For the latest and most complete listing of all Clontech products, please visit **www.clontech.com**

Products	Cat. No.
• Adeno-X™ Expression System	631513
• Adeno-X™ Tet-Off Expression System	631022
• Adeno-X™ Tet-On Expression System	631050
• Adeno-X™ Viral DNA (PI-SceI/I-CeuI digested)	631026
• Adeno-X™ Accessory Kit	631027
• Adeno-X™ PCR Primer Set 2	631030

Appendix A: Diluting Adenoviral Stock Solutions

There are many ways to make dilutions of adenoviral stocks. One important factor in making the dilutions and infecting the cells is to be consistent in the amount of volume added to the well (0.1 ml in our procedure).

Make serial dilutions of the adenoviral stock in question in diluent (PBS or sterile medium). For example:

10 µl Adenoviral Stock diluted in 990 µl diluent = 10^{-2} ml,
Add 100 µl to the 1 ml of cells.

100 µl of 10^{-2} dilution in to 900 µl of diluent = 10^{-3} ml,
Add 100 µl to the 1 ml of cells.

100 µl of 10^{-3} dilution in to 900 µl of diluent = 10^{-4} ml,
Add 100 µl to the 1 ml of cells.

100 µl of 10^{-4} dilution in to 900 µl of diluent = 10^{-5} ml,
Add 100 µl to the 1 ml of cells.

100 µl of 10^{-5} dilution in to 900 µl of diluent = 10^{-6} ml,
Add 100 µl to the 1 ml of cells.

If you need to adjust the dilutions to 1/2 log increments, it can be done as follows,

500 µl of 10^{-1} dilution in to 500 µl of diluent = 5×10^{-2} ml,
Add 100 µl to the 1 ml of cells.

500 µl of 10^{-2} dilution in to 500 µl of diluent = 5×10^{-3} ml,
Add 100 µl to the 1 ml of cells.

500 µl of 10^{-3} dilution in to 500 µl of diluent = 5×10^{-4} ml,
Add 100 µl to the 1 ml of cells.

500 µl of 10^{-4} dilution in to 500 µl of diluent = 5×10^{-5} ml,
Add 100 µl to the 1 ml of cells.

Appendix B: Derivation of Area counted in Fields/Well

Radius of a standard 20X objective = 0.5 mm

Since area = πr^2

Then area of cells counted per field = 0.7853 mm^2

= 7.853 x 10^{-3} cm^2

- Area of a well (for an average BD Falcon 12-well plate) = 4.5 cm^2

  Therefore, fields/well = 4.5 cm^2 / 7.853 x 10^{-3} cm^2

  fields/well = **573 fields**

Appendix C: X-gal Staining Procedure

This procedure, which is based on a method published by Price *et al.*, (1987), can be used to determine the titer of Adeno-X-LacZ Viral Stocks. Note, however, that the titer determined by this method may differ by as much as threefold from the titers that are determined using the Adeno-X Rapid Titer method. The HEK 293 cells would first need to be seeded in the plates, and then infected with Adeno-X-LacZ Viral Stock as in the Adeno-X Rapid Titer Procedure.

A. Prepare Staining Solution

10	ml	PBS (see Additional Materials Required)
250	µl	X-gal (40 mg/ml)
33	mg	Potassium ferricyanide
42.5	mg	Potassium ferrocyanide
20	µl	Magnesium Chloride (1 M)

B. Stain Cells

1. Rinse cells twice with 1 ml PBS. Aspirate PBS from the well.
2. Fix cells by incubating in 1 ml Fixative for 5 min at 4°C.
3. Remove Fixative. Repeat Step 1.
4. Add 0.5 ml Staining Solution to the well. Incubate 14–18 hr at 37°C.

   Note: Sometimes the positive cells turn blue within 1 hr after adding the Staining Solution.
5. Count the blue-stained positive cells.
6. Calculate infectious units (ifu)/ml for each well as follows:

$$\frac{\text{(blue infected cells/field) x (fields/well)}}{\text{volume virus (ml) x (dilution factor)}}$$