User Manual

Adeno-X™ Rapid Titer Kit User Manual

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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 expression. Also, when producing viral stocks, it is important to know the titer of infectious particles for successful virus production. Results are obtained much more quickly with this kit (within ~48 hr) than with standard assays. The values obtained are comparable to values obtained with other infectious assay methods that normally take up to one week to perform.

The Adeno-X Rapid Titer Kit takes advantage of the production of viral hexon protein in infected cells 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 for 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 (ifu). This assay yields values that correlate well with plaque assay 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 ourAdeno-X Expression System User Manuals, which are available at **www.clontech.com/mamuals**:

- Adeno-X Expression System 1 User Manual (PT3414-1)
- Adeno-X Adenoviral System 3 User Manual (PT5177-1)).

Applications

This kit has been developed for use with any adenoviral system, provided that the hexon protein is being expressed. The kit can be used to determine titers of the recombinant adenovirus created with our **Adeno-X Expression System 1** (Cat. No. 631513) or one of our **Adeno-X Adenoviral Systems 3** (Cat. Nos. 632264, 632265, 632266, 632267, 632268, 632269, and 631180). Adeno-X Expression Vectors carry a deletion in the E1-region of the adenovirus genome. A cell line complementing missing E1 elements is required for amplification and titration of the E1-deleted adenoviruses. One of the most commonly used E1-region complementing cell lines is human embryonic kidney 293 (HEK 293) cell line, which carries integrated in its genome constitutively expressed human adenovirus type 5 E1 sequences (Graham *et al.*, 1977; Aiello *et al.*, 1979). For more information on expression cassettes and the Adeno-X Viral genome, see our Adeno-X Expression System1 and System 3 User Manuals, which are available at **www.clontech.com/mamuals**.

We recommend the use of our Adeno-X 293 Cell Line (Cat. No. 632271), but this kit can be used with any cell line that complements the E1 elements missing from our Adeno-X Expression Vectors (e.g., HEK 293 cells).

I. Introduction & Protocol Overview, continued

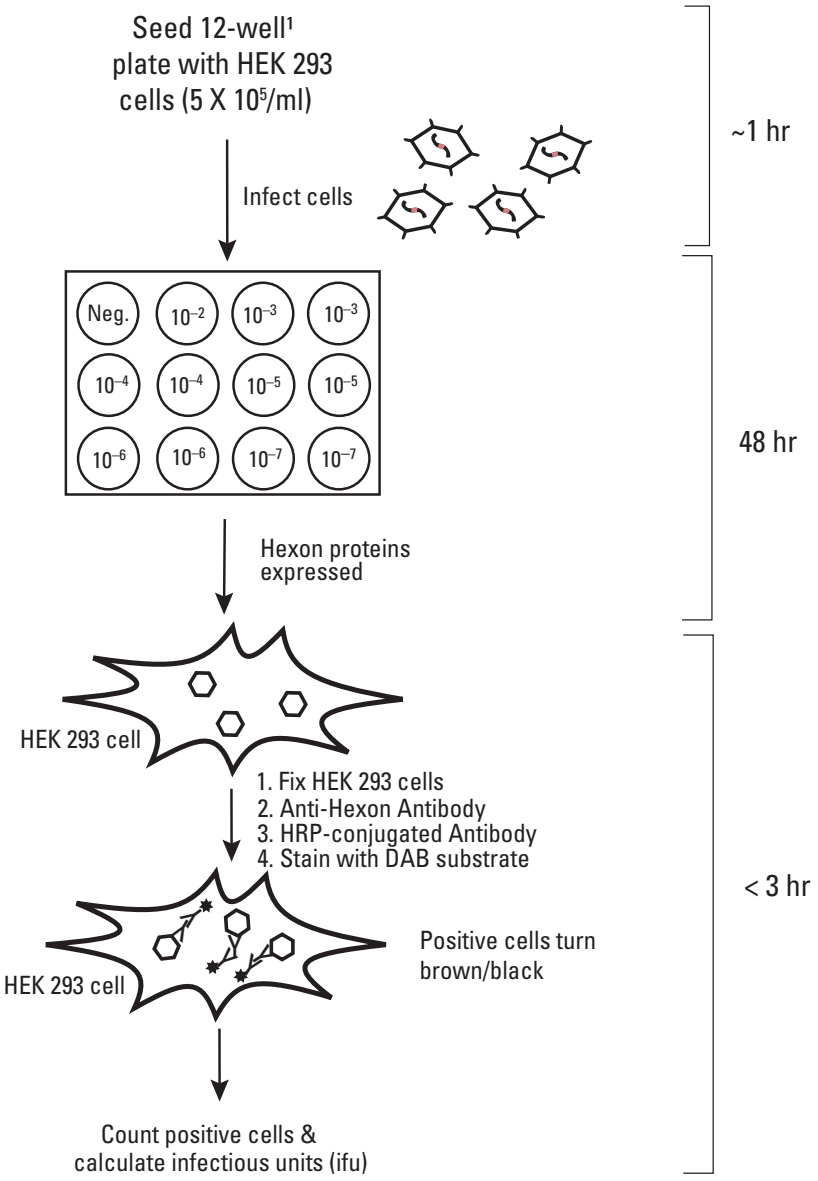

Figure 1. Adeno-X™ Rapid Titer Method. HEK 293 cells are infected with serial dilutions of adenovirus. After the hexon proteins appear, the cells are fixed and treated with a hexon protein-specific antibody, HRP-conjugate antibody and developed with DAB Substrate. During development the positive cells 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.

[1] The procedure to follow when using a 24-well plate is similar except for quantities; please see Section V: Adeno-X™ Rapid Titer Procedure.

I. Introduction & Protocol Overview, continued

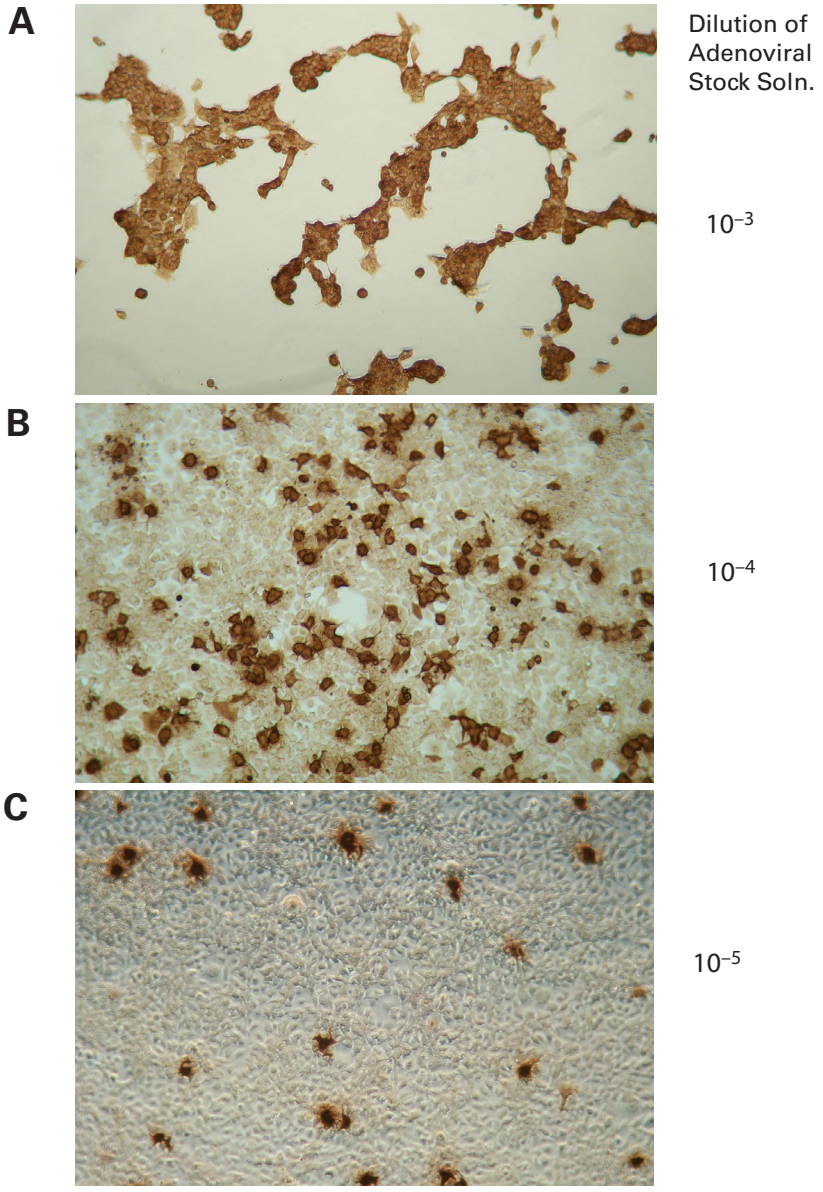

Figure 2. Fields of positive cells. Different dilutions of adenovirus were used to infect HEK 293 cells and developed by the Adeno-X Rapid Titer method. A cytopathic effect is evident in **Panel A** (10^{-3}). In this example, the 10^{-5} dilution **(Panel C)** would provide the field of cells most ideal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

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.

The following reagents are suitable for 60 titrations with 5 x 12-well plates, or for 120 titrations with 5 x 24-well plates.

- 60 µl Rat Anti-Mouse Antibody (HRP conjugate)
- 30 ml 1X Stable Peroxidase Buffer
- 30 µl Mouse Anti-Hexon Antibody
- 3.0 ml 10X DAB Substrate

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% CO2)
- 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
- We recommend the use of our Adeno-X 293 Cell Line (Cat. No. 632271), but this kit can be used with any cell line that complements the E1 elements missing from our Adeno-X Expression Vectors (e.g., HEK 293 cells).

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.

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. Two important factors in making the dilutions and infecting the cells are to be consistent in the amount of viral dilution added to the well (0.1 ml in our procedure) and to be sure to use a new pipet tip for each dilution.

V. Adeno-X Rapid Titer Procedure

Protocol 30 min hands-on; 48 hr overall

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.
Each dilution of virus should be assayed in duplicate to ensure accuracy.

A. Protocol: Infect Cells

	12 well	24 well
1. Seed 1 ml of healthy, log-phase 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).	5 x 10^5 cells	2.5 x 10^5 cells

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). Use a fresh pipet tip for each step in the dilutions.

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

3. Add 100 µl of viral dilution dropwise to each well.	100 µl	50 µl

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. Do not overdry.

Note: While it is also possible to use the 96-well format, this is not recommended because the geometry of the well together with infection kinetics and staining procedures can combine to cause issues with the consistency of the assay.

V. Adeno-X™ Rapid Titer Procedure, continued

Protocol 30 min hands-on; 3 hr overall

B. Protocol: Fix Cells and Add Antibodies

	12 well	24 well
1. Fix cells by VERY GENTLY adding 1 ml ice-cold 100% methanol to each well. **Note:** Add methanol very gently. Do this by adding the methanol to the wall of the well. Do not dislodge the cell monolayer. The monolayer can be easily dislodged until cells are fixed. **Using a Pipetman and not a 5 ml pipet can help**. This is particularly important for the 24-well plate.	1 ml	0.5 ml
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.	1 ml	0.5 ml
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).	0.5 ml	0.25 ml
6. Aspirate Mouse Anti-Hexon Antibody. Then gently rinse wells three times with 1 ml PBS + 1% BSA.	1.0 ml	0.5 ml
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).	0.5 ml	0.25 ml
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 1X DAB working solution to come to room temperature. **Note**: Do not allow 10X DAB Substrate to come to room temperature.	500 µl	250 µl
10. Aspirate Rat Anti-Mouse Antibody (HRP conjugate) dilution. Gently rinse each well three times with 1 ml PBS + 1% BSA.	1 ml	0.5 ml

V. Adeno-X™ Rapid Titer Procedure, continued

Protocol 30 min hands-on; 30 min overall

C. Protocol: Develop Color and Quantitate

	12 well	24 well
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.	500 µl	250 µl
2. Aspirate DAB and add 1 ml PBS to each well.	1 ml	0.5 ml

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. An ideal field should contain 5 to 50 positive (black/brown) cells. Adjust your objective as needed. See Figure 2.

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

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

Protocol

D. Protocol: Example Calculation:

- Mean positive cells/ field = 10 at 10^{-5} dilution
- Fields/well (20X objective) = 594 fields
- Amount dilution added = 0.1 ml

  Therefore,
- ifu/ml = (10 cells/field) x (594 fields/well) / (0.1 ml) x (10^{-5}/ml)
  = 5.94 X 10^{9} ifu/ml

Note: This example calculation is for a 12-well plate. See Table I below.

Table I. Derivation of Area Counted in Fields/Well

Objective Lenses	Eyepiece Lenses (10X)			Fields/Well		
	Total Magnification	Field Diameter	Field Area (mm^2)	12-Well Plate area = 3.8 cm^2	24-Well Plate area=2.0 cm^2	96-Well Plate area=0.32 cm^2
4X	40X	5 mm	19.6	19	10	1.6
5X	50X	4 mm	12.5	30	16	2.6
10X	100X	1.8 mm	2.54	150	79	12.6
20X	200X	0.9 mm	0.64	594	313	50

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.

  Note: The HRP enzyme is sensitive to freeze-thaw cycles.

- Did not infect for a full 48 hr before fixing cells. As a result hexon expression did not reach detection threshold.

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 Required 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. USA* **84**(1):156–160.

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).

Using filter tips, make serial dilutions of the adenoviral stock in question in diluent (PBS or sterile medium). For example:

Note: Change filter tips at each serial dilution

- 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 x 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 x 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 x 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 x 10^{-5} ml,

  Add 100 µl to the 1 ml of cells.