Takara Bio USA, Inc.

Adeno-X™ Mega Purification Kit User Manual

Cat. No. 631032
(082421)

Takara Bio USA, Inc.
2560 Orchard Parkway, San Jose, CA 95131, USA
U.S. Technical Support: technical_support@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

Table of Contents

Table of Figures

ATTENTION: The viral supernatants produced by transfecting Adeno-X 293 cells with recombinant Adeno-X Viral DNA could, depending on your DNA insert, contain potentially hazardous recombinant virus. Due caution must be exercised in the production and handling of recombinant adenovirus**. The user is strongly advised not to create adenoviruses capable of expressing known oncogenes.**

Appropriate NIH, regional, and institutional guidelines apply, as well as guidelines specific to other countries. NIH guidelines require that adenoviral production and transduction be performed in a Biosafety Level 2 facility. For more information, see appropriate HHS publications. Section IV in this User Manual contains a brief description of Biosafety Level 2 as well as other general information and precautions.

I. Introduction & Protocol Overview

The Adeno-X Mega Purification Kit is a complete chromatography-based system for purifying and concentrating recombinant adenovirus. It provides a superior alternative to cesium chloride (CsCl) density gradient centrifugation. Although centrifugation in CsCl is an extremely effective method for purifying adenovirus, it is also time-consuming, technically demanding, and toxic (Graham & Prevec, 1991). Furthermore, the procedure is restrictive in that it is not easily scaled up or down. The Adeno-X Mega Purification Kit, on the other hand, can bescaled up or down without difficulty. You simply wait until the cytopathic effect (CPE) is complete—when the viral titer is highest—harvest the cells and purify the virus. The Adeno-X Purification Kit is not only faster than CsCl methods, it is also easier, safer, and just as effective.

A chromatographic method

The Adeno-X Mega Purification Kit lets you purify adenovirus chromatographically, using an adsorbent membranethat selectively binds adenoviral particles based on their distinctive surface-associated properties. The membrane is housed in a small, single-use cartridge that fits securely on a disposable Luer-Lok syringe. For added convenience, the syringe-membrane ('purification') assembly comes completely pre-assembled and ready to use (Figure 1).

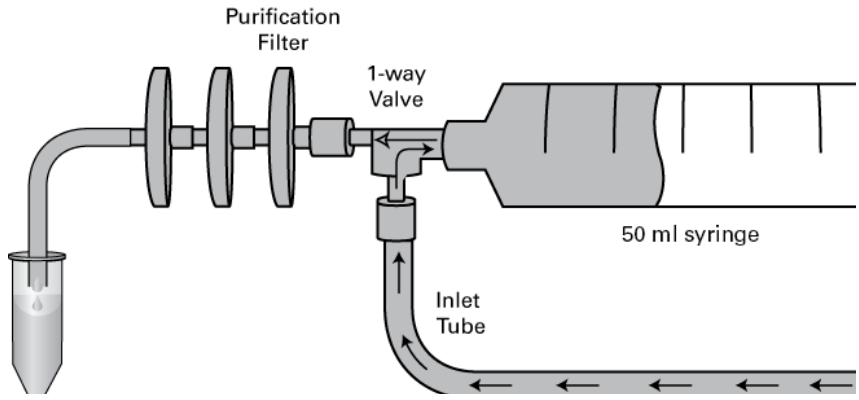

Figure 1. The Adeno-X Mega Purification Assembly. The apparatus comes pre-assembled.

The purification protocol

The procedure is simple. After the cytopathic effect is complete, the cells are harvested, lysed, and centrifuged. The resulting supernatant is then treated with Benzonase Nuclease to digest nucleic acids and cleared (clarified) through a bottle-top filter unit. This clarified, virus-containing lysate is then drawn into the purification assembly through a one-way valve. The assembly is placed into a syringe pump, and the lysate is pumped through the purification cartridge, where the adenoviral particles bind to the membrane. The bound particles are then eluted with a small volume of buffer. The entire protocol, from harvest to purification, takes just 1-1.5 hours to complete(Figure 2). Aliquots of the purified, high-titer adenoviral stock can be stored in Elution Buffer at –70°C. Please note: All of these steps must be conducted under sterile conditions in a Biosafety Level 2 certified hood.

The syringe pump

IMPORTANT: We strongly recommend the use of a continuous flow syringe pump (not a peristaltic pump) with the Adeno-X Mega Purification Kit (for example, the GenieTouch from Kent Scientific). The controlled flow rate and low back pressure produced by these pumps results in exceptional performance and highly

reproducible results. See the syringe pump's User Manual for installation and operating instructions.

To greatly simplify large scale virus purification, we have developed a protocol that allows you to:

- Purify virus directly from your cell pellet, thus reducing the need to handle large volumes of medium.
- Use a syringe pump to obtain purified virus quickly and easily.

II. List of Components

Store Benzonase Nuclease at –20°C. Store all other components at room temperature. The Adeno-X MegaPurification Kit (Cat. No. 631032) contains sufficient reagents for 2 Mega purifications:

- 2 x 15 ml 1X Equilibration Buffer
- 50 ml 1X Dilution Buffer
- 60 ml 1X Wash Buffer
- 2 x 12 ml 1X Elution Buffer
- 4 Stericup Filter Units
- 2 x 2 Pre-Filter Discs
- 2 each Adeno-X Mega Purification Assembly
- 2 each 10 ml Syringe
- 40 µl Benzonase Nuclease (25 U/µl)

III. Additional Materials Required

The following materials are required but not supplied.

- Syringe pump

  IMPORTANT: We strongly recommend the use of a continuous flow syringe pump (not a peristaltic pump) with the Adeno-X Mega Purification Kit (for example, the GenieTouch from Kent Scientific). The controlled flow rate and low back pressure produced by these pumps results in exceptional performance and highly reproducible results. See the syringe pump's User Manual for installation and operating instructions.

- Tissue culture plates and flasks
  (e.g. 10 or 15 cm plates, T75 or T175 flasks, or roller bottles)
- Centrifuge
  (Swinging-bucket and fixed-angle rotors compatible with 15 ml, 50 ml, and if needed, 100 ml centrifugetubes)
- Sterile 50 ml centrifuge tubes
- **Optional:** 1X Formulation Buffer
  2.5% glycerol (w/v), 25 mM NaCl, and 20 mM Tris-HCl, pH 8.0 (GTS buffer; Hoganson, *et al*., 2002).
- **Optional:** For adenoviral stock titration, we recommend using Clontech's Adeno-X Rapid Titer Kit (632250),Adeno-X qPCR Titration Kit (Cat. No. 632252), or Adeno-X GoStix™ (Cat. No. 632270).
- Adeno-X 293 Cells (Cat. No. 632271)
- **Optional:** If you plan to perform buffer exchange from elution buffer into formulation buffer, you may use PD10 Desalting Columns (GE Healthcare), Millipore Centriprep YM-50 Centrifugal Filter Unit (50,000 NMWL) or dialysis using Slide-A-Lyzer Cassette MW-10K MWCO (Pierce) according to the manufacturer'sinstructions.

IV. Safety Guidelines for Working with Adenoviruses

The protocols in this User Manual require the production, handling, and storage of infectious adenovirus. It is imperative to fully understand the potential hazards of, and necessary precautions for, the laboratory use of adenoviruses.

The National Institute of Health and Center for Disease Control have designated adenoviruses as Level 2 biological agents. This distinction requires the maintenance of a Biosafety Level 2 facility for work involving thisvirus and others like it. The viruses packaged by transfecting Adeno-X 293 cells with the adenoviral-based vectors described here are capable of infecting human cells. These viral supernatants could, depending on your gene insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human genetherapy trials, attesting to their potential ability to express genes *in vivo*.

For these reasons, due caution must be exercised in the production and handling of any recombinant adenovirus.

The user is strongly advised not to create adenoviruses capable of expressing known oncogenes.

For more information on Biosafety Level 2, see the following reference:

> *Biosafety in Microbiological and Biomedical Laboratories*, (BMBL), 5th Edition (December 2009) U.S. Department of Health and Human Services, CDC, NIH.
>
> Available at http://www.cdc.gov/biosafety/publications/bmbl5/index.htm

Biosafety Level 2:

The following information is a brief description of Biosafety Level 2. It is neither detailed nor complete. Details of the practices, safety equipment, and facilities required for Biosafety Level 2 are available in the above publication. If possible, observe and learn the practices described below from someone who has experience working with adenoviruses.

- **Practices:**
  - perform work in a limited access area
  - post biohazard warning signs
  - avoid generating aerosols
  - decontaminate potentially infectious wastes before disposal
  - take precautions with sharps (e.g., syringes, blades)
- **Safety equipment:**
  - biological safety cabinet, preferably Class II

  (i.e., a laminar flow hood with microfilter [HEPA filter] that prevents release of aerosols)
  - protective laboratory coats, face protection, double gloves
- **Facilities:**
  - autoclave for decontamination of wastes
  - unrecirculated exhaust air
  - chemical disinfectants available for spills.

V. Adenovirus Purification Protocol

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. Test the Titer of the Adenoviral Stock

1. Determine the optimal amount of viral stock needed to infect your cells. To do this, test the viral titer in a small-scale cytopathic effect assay, using culture conditions that closely approximate those that will be used for the actual adenoviral prep.
   a. Seed low passage Adeno-X 293 cells (Cat. No. 632271) on a 12-well tissue culture dish at a density of $1x10^5$ cells/cm^2, using 0.28 ml/cm^2 medium. At 1 ml/well, this is equivalent to ~$3.57x10^5$ cells/well.
   b. Place the cells at 37°C and 5% CO_2 while you dilute the adenovirus.
   c. Infect the cells with a range of adenoviral concentrations. If you know the titer of your stock, aim for a multiplicity of infection (M.O.I.) of 1–2. If you don't know the titer, infect the cells with a range of dilutions, starting with 1 ml of stock and serially diluting 3X from there.

      NOTE: We recommend using our Adeno-X Rapid Titer Kit (Cat. No. 632250) to quickly and easily titrate your viral stock.
   d. Incubate the cells at 37°C and 5% CO_2 until the cytopathic effect is complete; this should take approximately 3-5 days.

      NOTES:
      - **Adeno-X GoStix** (Cat. No. 632270) can be used to monitor the culture prior to cytopathic effect (CPE) formation.
      - The cytopathic effect (CPE) refers to the morphological changes that the cells undergo after infection. Infected cells typically remain intact but round up and may detach from the dish individually or in “grape-like” clusters that float in the medium. Optimally, the purification assay should be performed when 50% of the cells are detached, and the remainder are attached but rounded.
2. Use the results from the test titer to determine the optimal amount of virus to use in the actual purification prep. The full-scale prep requires seeding up to twenty-five 150 mm tissue culture plates with $1.46x10^7$ cells/plate. Therefore, if 0.1 ml of adenoviral stock optimally infected the $3.57x10^5$ cells used to seed the test titer, you would need 4 ml of virus for each 150 mm dish inthe actual prep.

B. Amplify Adenovirus in Adeno-X 293 Cells

1. Detach low passage Adeno-X 293 cells with trypsin, wash, and count.
2. In a sterile flask, dilute the cells to a final density of $1.83x10^6$ cells/ml in a total of 200 ml.
3. Add virus to the cells and mix.

   NOTE: Perform the test titer in Part A to determine the optimal amount of virus to add in this step.
4. Aliquot 33 ml of fresh medium onto each of twenty-five 150 mm cell culture dishes.
5. Aliquot 8 ml of virus-infected cells (from step 3) onto each of the twenty-five 150 mm cellculture dishes; make sure the cells are evenly distributed.

   NOTE: This is equivalent to $1.46x10^7$ cells/plate.
6. Incubate the cells at 37°C and 5% CO_2 until the CPE is 80–100% complete; this should takeapproximately 3-5 days.

NOTE: Optimally, the purification assay should be performed when 50% of the cells are detached, and the remainder are attached but rounded.

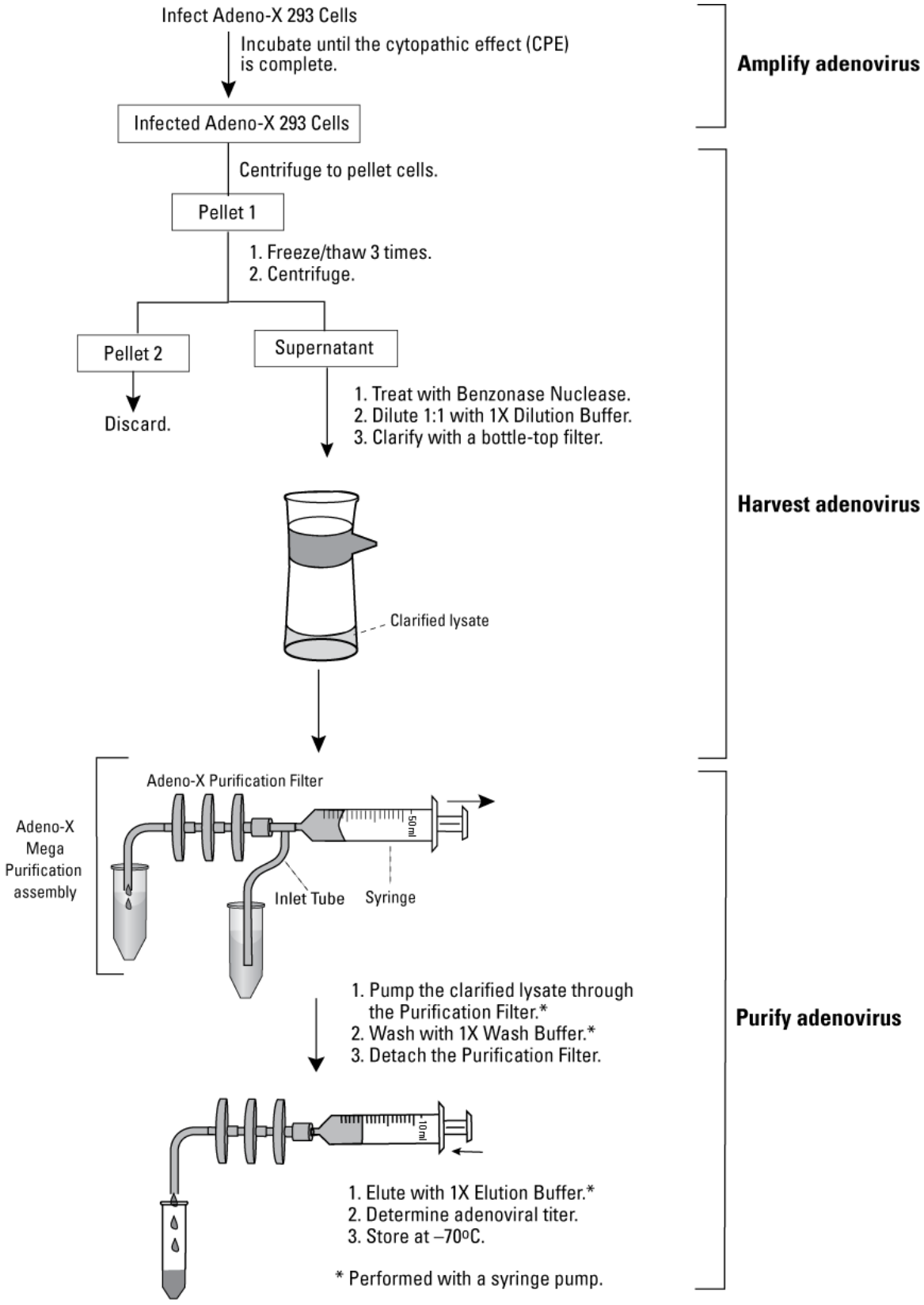

Figure 2. Overview of the Adeno-X Mega Purification Protocol.

C. Harvest Adenovirus

(See Figure 2 for a procedural diagram)

1. Pellet the cells by centrifugation in a Beckman GH3.8 (or equivalent) swinging-bucket rotor at ~1,500 rpm (500 x g) for 10 min.
2. Discard the supernatant.
3. Resuspend the pellet in 25 ml of fresh, serum-free medium.

   NOTE: Do not use PBS, as it inhibits some endonucleases. Endonuclease is used below to remove contaminating cellular nucleic acids from the filter-clarified cell lysate.
4. Lyse the cells with three consecutive freeze-thaw cycles; briefly freeze the cells in a dry ice/ethanol bath, then thaw them in a 37°C water bath.
5. After thawing for the final time, centrifuge the lysate in a Beckman GH3.8 (or equivalent)swinging-bucket rotor at 3,000 rpm (1,500 x g) for 10 min to pellet the debris.
6. Collect and save the supernatant in a sterile centrifuge tube. Discard the pellet.
7. Add 20 µl Benzonase Nuclease and incubate for 20 min at 37°C.

   NOTE: Nuclease treatment decreases the viscosity of the solution so that it can be drawn more easily through the Adeno-X Purification Assembly.
8. Measure the volume of the lysate with a pipet, then add an equal volume of 1X Dilution Buffer(approximately 25 ml).

   NOTE: The lysate should turn a light shade of purple.
9. Clarify the lysate by filtering it through a Stericup Filter Unit (provided).

   NOTE: Before filtering the lysate, place a Pre-filter Disc in the top portion of the filter unit.
10. After filtering the lysate, wash the Stericup Filter Unit by placing 5–10 ml of Equilibration Buffer in the upper portion unit, and filter. Combine the wash with the clarified lysate from step 9.

D. Set up the Syringe Pump

IMPORTANT: We strongly recommend the use of a continuous flow syringe pump (**not** a peristaltic pump) with the Adeno-X Mega Purification Kit (for example, the GenieTouch from Kent Scientific). Thecontrolled flow rate and low back pressure produced by these pumps results in exceptional performance and highly reproducible results. See the syringe pump's User Manual for installation and operating instructions.

1. Attach the syringe to the pump, with the barrel of the syringe in the Syringe Block and the plunger connected to the Pusher Block (Figure 3).
2. Program the Syringe Size (i.e. the inside diameter of the syringe; ID) into the pump (the ID of the10 ml syringe is ~15 mm; that of the 50 ml syringe is ~27 mm).
3. Program the Flow Rate to be 3 ml/min.
4. Press the Run/Stop key to start the pump.

Figure 3. Placement of the Adeno-X Purification Assembly on an infusion pump. A pump from Kent Scientific isshown, but any infusion syringe pump will work.

E. Purify Adenovirus

1. Equilibrate the Purification Assembly with 10 ml 1X Equilibration Buffer. To do this, place the inlet tube into the buffer and draw the buffer into the syringe by pulling back on the plunger. Disconnect the syringe from the Assembly and force the air out of the syringe by pressing on theplunger. Reconnect the syringe and place the entire Assembly into the pump (see Figure 2). Pump the solution through the Purification Assembly at a rate of 3 ml/min, until ~0.5–1 ml of solution remains in the syringe.

   NOTE: Do not allow residual air to be forced through the Purification Assembly.

2. Remove the Purification Assembly from the pump. Place the inlet tube into the clarified lysate(from Section V.C.10) and draw the lysate into the syringe.
3. Reconnect the Purification Assembly to the pump. Position a sterile 50 ml conical tube to

collectthe flow-through. Pump the lysate through the Purification Assembly at a rate of 3 ml/min, until ~0.5–1 ml of lysate remains in the syringe. Store the flow-through at –20°C.

4. Remove the Purification Assembly from the pump. Place the inlet tube into a sterile tubecontaining 30 ml 1X Wash Buffer and draw the buffer into the syringe.
5. Reconnect the Purification Assembly to the pump. Position a sterile 50 ml conical tube to collect thewash flow-through. Pump the Wash Buffer through the Purification Assembly at a rate of 3 ml/min, until ~0.5–1 ml of Buffer remains in the syringe. Store the wash flow-through at –20°C.
6. Remove the Purification Assembly from the pump. Separate the Purification Filters ('MembraneStack') from the 1-Way Valve (see Figure 2) and set aside.
7. Elute the adenovirus:
    a. Program the pump for a 10 ml syringe as described in Section V.D.2.
    b. Attach the Membrane Stack to a 10 ml syringe containing 10 ml 1X Elution Buffer.Connect this new assembly to the pump and position a sterile 15 ml conical tube tocollect the eluate.
    c. Pump the Elution Buffer through the assembly at a rate of 3 ml/min, until 3 ml has eluted.Stop the pump and allow the assembly to sit for 5 min.
    d. Restart the pump and collect the remaining 7 ml of eluate. Stop the pump and fill the syringe with air. Reconnect the syringe to the pump; use the air in the syringe to remove any remaining liquid from the Membrane Stack. You should have approximately 10 ml ofeluate in the tube.
8. Determine the adenoviral titer.

NOTE: We recommend using the Adeno-X Rapid Titer Kit (Cat. No. 632250) or the Adeno-X qPCR Titration Kit (Cat. No. 632252).

9. The adenovirus can be used immediately, or aliquoted and stored at –80°C.

NOTE: For improved long-term stability and proper tonicity for *in vivo* applications, we recommend a buffer exchange of the eluted adenovirus into 1X Formulation Buffer.

- **1X Formulation Buffer:** 2.5% glycerol (w/v), 25 mM NaCl, 20 mM Tris-HCl, pH 8.0 (GTS buffer; Hoganson, et al., 2002)

VI. References

1. Graham, F. L. & Prevec, L. (1991) Manipulation of adenovirus vectors. *In Methods in Molecular Biology, Vol. 7:Gene Transfer and Expression Protocols*. Ed. Murray, E. J. (Human Press Inc., Clifton, NJ), pp. 109–128.

2. Hoganson, D. K., Ma, J. C., Asato, L., Ong, M., Printz, M. A., Huyghe, B. G., Sosnowski, B. A. & D'Andrea, M. J. (2002) Development of a stable adenoviral vector formulation. *Bioprocessing J.* **1**(1):43–48.

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: 800.662.2566 (toll-free)	tel: 800.662.2566 (toll-free)
fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
web: http://www.takarabio.com/service	web: http://www.takarabio.com/support
e-mail: ordersUS@takarabio.com	e-mail: technical_support@takarabio.com

Notice to Purchaser

Our products are to be used for **Research Use Only**. They may not be used for any other purpose, including, but not limited to, use in humans, therapeutic or diagnostic use, or commercial use of any kind. Our products may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without our prior written approval.

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product's web page at takarabio.com. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements

This document has been reviewed and approved by the Quality Department.