Takara Bio USA, Inc.

Lenti-X™ p24 Rapid Titer Kit User Manual

Cat. No. 632200
(111616)

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I. Introduction

Principle of the Lenti-X p24 Rapid Titer Assay

The Lenti-X p24 Rapid Titer Kit allows you to quickly determine the titer of any HIV-1-based lentiviral supernatant using standard ELISA methods. The wells of the included microtiter plate (12 x 8-well strips) are coated with an anti-HIV-1 p24 capture antibody, which quantitatively binds the HIV-1 p24 in your test samples (p24 is an abundant HIV-1 virus core/capsid protein [Figure 1]). Specifically-bound p24 is detected in a typical "sandwich" ELISA format using a biotinylated anti-p24 secondary antibody, a streptavidin-HRP conjugate, and a color producing substrate (Figure 2). Color intensity is measured spectrophotometrically to indicate the level of p24 in the samples, which can then be precisely quantified against a p24 standard curve. p24 values can then be correlated to virus titer of packaging cell supernatants.

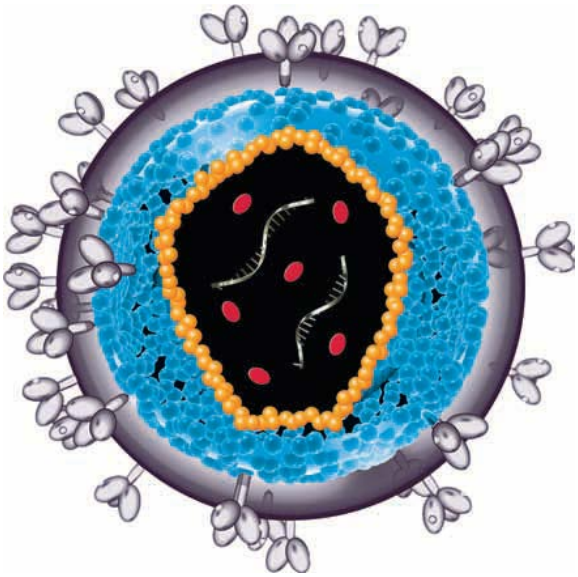

Figure 1. Lentiviral p24 is a virus core/capsid protein. The HIV-1 gag gene encodes p24, which is the major lentiviral capsid protein. The lysis of viral particles is necessary to generate soluble p24 which can then be measured by the Lenti-X p24 Rapid Titer Kit.

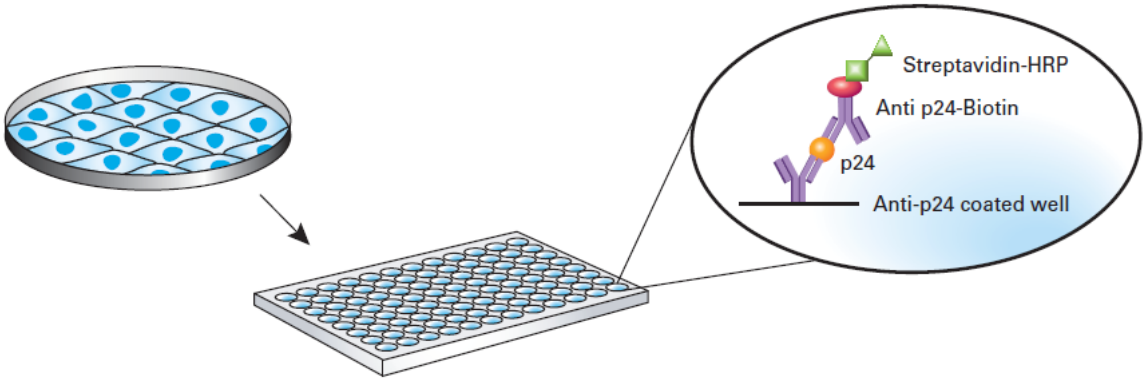

Figure 2. The Lenti-X p24 Rapid Titer Kit. Lentiviral (HIV-1) p24 core protein in packaging cell supernatants is bound to wells of a microtiter plate coated with HIV-1 p24 capture antibody. The presence of bound p24 in the wells is detected using a biotinylated secondary anti-p24 antibody, a streptavidin-horseradish peroxidase conjugate, and a color-producing substrate. Quantitation is performed by comparing test samples to a p24 standard curve.

II. List of Components

A. Lenti-X p24 Rapid Titer Kit (Cat. No. 632200)

- 1 each Anti-p24 Coated Plate (96 wells)
- 100 µl p24 Control (10 ng/ml)
- 6 ml Lysis Buffer
- 12 ml Anti-p24 (Biotin Conjugate)
- 12 ml Streptavidin HRP
- 120 ml Wash Buffer (20X)
- 12 ml TMB Substrate
- 12 ml Stop Solution

Other

- Lenti-X p24 Rapid Titer Kit User Manual (PT5002-1)
- Lenti-X p24 Rapid Titer Kit Protocol-at-a-Glance (PT5002-2)

III. Storage and Stability

- **All reagents should be stored at 2–8°C.** Do not use reagents beyond the expiration date on the label.
- **Microtiter Strips.** Once opened, microtiter strips may be stored at 2–8°C until the expiration date on the label. Strips must be stored under desiccated conditions. Return unused strips to their original foil pouch along with the sachet of desiccant, and securely reseal the pouch by folding over the open end and securing it with adhesive tape.
- **Wash Buffer.** The working concentration (1X) of wash buffer should not be stored for longer than 3 weeks at 2–8°C. We recommend that fresh wash buffer be prepared before each assay. If the diluted buffer becomes visibly cloudy or develops a precipitate during storage, discard it and prepared fresh buffer.

NOTE: The 20X Wash Buffer normally develops a crystalline precipitate during storage at 2–8°C. This will dissolve upon warming at 37°C.

IV. Safety Guidelines for Working with Lentiviruses

A. Advisory

For your safety, and the safety of others around you, it is imperative to fully understand the potential hazards of working with recombinant lentiviruses and the necessary precautions for their use in the laboratory.

The National Institute of Health and Center for Disease Control have designated recombinant lentiviruses as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. The VSV-G pseudotyped lentiviruses packaged from the HIV-1-based vectors described here are capable of infecting human cells. The viral supernatants produced by these lentiviral systems could, depending on your insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy 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 lentivirus. **The user is strongly advised not to create VSV-G pseudotyped lentiviruses capable of expressing known oncogenes.**

For more information on Biosafety Level 2 agents and practices, download the following reference:

Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition (February 2007) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH. Available on the web at http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm

B. Biosafety Level 2

The following is a brief description of Biosafety Level 2. It is neither detailed nor complete. Details of the practices, safety equipment, and facilities that combine to produce a 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 lentiviruses.

Important Features of Biosafety Level 2:

Practices:

- Standard microbiological practices
- Limited access to work area
- Biohazard warning signs posted
- Minimize production of aerosols
- Decontaminate potentially infectious wastes before disposal
- Use precautions with sharps (e.g., syringes, blades)
- Biosafety manual defining any needed waste decontamination or medical surveillance policies

Safety equipment:

- Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is unrecirculated
- PPE: protective laboratory coats, gloves, face protection as needed posted

Facilities:

- Autoclave for waste decontamination
- Chemical disinfectants for spills

V. Additional Materials Required

The following materials, or their equivalents, are required to perform the Lenti-X p24 Rapid Titer Kit assay:

- Micropipettes for delivering volumes of 5 µl, 10 µl, 25 µl, 100 µl, and 200 µl. A multichannel pipette is preferred for dispensing reagents into microtiter plates.
- Distilled or deionized water
- A 37°C ± 1°C incubator
- Disposable plastic or glass test tubes; 5 ml and 10 ml capacities
- Laboratory glassware: 15 ml and 100 ml beakers; 1 L graduated cylinder; 1 ml, 5 ml, and 10 ml glass pipettes
- Absorbent paper towels
- Automatic microtiter plate washer or laboratory wash bottle
- Microtiter plate reader with 450 nm filter
- Latex gloves, safety glasses, and other appropriate protective garments

- Biohazard infectious waste containers
- Safety pipeting devices for 1 ml or larger pipettes
- Timer

VI. Assay Techniques & Microtiter Plate Washing

Efficient washing of the microtiter wells is a fundamental requirement of ELISA procedures, as thorough rinsing removes uncomplexed assay components and minimizes assay background. Using an automatic plate washer is recommended to enhance speed, efficiency, and well-to-well consistency. Manual plate washing can yield equivalent results if performed carefully. Each Lenti-X p24 assay requires performing three, six-rinse cycles.

IMPORTANT:

- The Lenti-X p24 Assay contains reagent systems which are optimized and balanced for each kit lot. Do not interchange reagents from kits with different lot numbers. Do not interchange vial caps or stoppers either within or between kits.
- Allow foil bags to warm to room temperature before opening. This avoids condensation on the inner surface of the bag, which may contribute to a deterioration of coated strips intended for future use.
- Reagents should be dispensed with the tip of the micropipettes touching the side of the well at a point about mid-section. Follow manufacturer's recommendations for automatic processors.
- Always keep the upper surface of the microtiter strips free from excess fluid droplets. Reagents and buffer over-spill should be blotted dry on completion of the manipulation.
- Do not allow the wells to completely dry during an assay.

A. Automatic Plate Washing

Each rinse cycle must consist of six consecutive washes. On completion of a rinse cycle, invert the plate or strips onto absorbent paper towels and tap firmly. Check for any residual wash buffer in the wells and blot the upper surface of the wells with a dry paper towel. In addition, automatic plate washers should meet the following criteria:

- All wells are completely aspirated.
- All wells are completely filled (350 µl) during each rinse cycle.
- Wash buffer is dispensed at a good flow rate.
- The apparatus must be well maintained to prevent contamination from previous use. Perform cleaning procedures regularly, according to the manufacturer's instructions.

B. Manual Plate Washing

For manual plate washing, perform the following steps for each rinse cycle:

1. Aspirate well contents using a vacuum line fitted with a trap to collect liquid.
2. Fill all wells to the brim with wash buffer dispensed from either a multichannel pipettor or a squeeze-type laboratory wash bottle.
3. Aspirate all wells.
4. Repeat steps 2 and 3, five times.
5. Invert the microtiter plate and tap firmly on absorbent paper towels.

VII. Protocols: Lenti-X p24 Assay Procedures

IMPORTANT: Please read the entire protocol before starting. Detailed instructions are provided for the quantitative assay of p24.

A. Protocol: Specimen Collection and Storage

The Lenti-X p24 Assay is intended for use with tissue culture supernatants. Specimens should be tested as soon as possible, but can also be stored frozen at –80°C, if necessary. Thoroughly mix thawed samples before testing.

B. Protocol: Wash Buffer Preparation

Prepare 1X wash buffer by diluting 1 part Wash Buffer (20X) with 19 parts distilled or deionized water. If the kit will be utilized over a period greater than 4 weeks, then prepare only enough working strength wash buffer for immediate needs. Each strip of 8-wells can be adequately washed with ~60 ml of working strength wash buffer.

C. Protocol: Preparing Dilutions for the p24 Standard Curve (0.5 hr)

NOTE: This note pertains to samples with High to Very High Levels of p24 (e.g., lentiviral supernatants generated using Lenti-X Packaging Single Shots). Samples containing high levels of p24 (i.e. >200 pg/ml) must be diluted prior to assay in order to obtain accurate p24 values. Such samples may include lentiviral supernatants produced using Lenti-X Packaging Single Shots, which often require diluting 10–10,000-fold. We recommend making several serial 10-fold dilutions to generate at least one dilution in the range of the standard curve. Mix dilutions thoroughly before assaying or diluting them further, assay each sample in duplicate, and be sure to multiply each result by its dilution factor to determine the correct p24 value in the original sample.

To test samples quantitatively and determine accurate virus titers, you will need to prepare a p24 standard curve (0–200 pg/ml). Examples of data collected for a typical standard curve and for test samples are shown in Table 1 and Figure 3. To prepare dilutions for the standard curve:

1. Prepare a working strength p24 positive control stock solution by diluting 20 µl of the p24 Control (10 ng/ml) into 980 µl of fresh complete tissue culture medium (e.g., DMEM containing 10% FBS), for a 1:50 dilution. This will produce a 200 pg/ml stock solution.
2. Using the 200 pg/ml stock and complete tissue culture medium as the diluent, make a series of four additional standard dilutions of 100, 50, 25, and 12.5 pg/ml. Dispense 500 µl of media into each of four labeled tubes. Add 500 µl of the 200 pg/ml stock into the 100 pg/ml tube, mix, and using a fresh pipet tip, transfer 500 µl of this 100 pg/ml solution into the 50 pg/ml tube and mix. Repeat similar transfers for the 25 and 12.5 pg/ml tubes.

D. Protocol: Assaying Your Lentiviral Supernatants (~4 hr)

1. Allow all reagents to reach room temperature (18–25°C).
2. Select a sufficient number of 8-well strips to accommodate all standards, test specimens, controls, and complete culture medium blanks (negative controls) in duplicate. Fit the strips into the holding frame. Label wells according to specimen identity using the letter/number cross reference system molded into the plastic frame.
3. Dispense 20 µl of lysis buffer into each well.
4. Dispense 200 µl of each standard curve dilution, supernatant sample, and culture medium into appropriately labeled duplicate wells.
5. Incubate at 37°C ± 1°C for 60 ± 5 min.
6. Aspirate the contents of the wells, and wash the microtiter plate as described in Section VI.
7. Dispense 100 µl of Anti-p24 (Biotin conjugate) detector antibody into each well.

8. Incubate at 37°C ± 1°C for 60 ± 5 min.
9. Aspirate the detector antibody from the wells, and wash the microtiter plate as described in Section VI.
10. Dispense 100 µl of Streptavidin-HRP conjugate into each well.
11. Incubate at room temperature (18–25°C) for 30 ± 5 min.
12. Aspirate the conjugate from the wells, and wash the microtiter plate as described in Section VI.
13. Without delay, dispense 100 µl of Substrate Solution into each well. A multichannel pipet should be used for best results.
14. Protect the plate from direct light/sunlight, and incubate at room temperature (18–25°C) for 30 ± 2 min.
15. Stop the reaction by adding 100 µl of Stop Solution to each well including the culture medium blanks. The blue solution should change to a uniform yellow color. Ensure that the undersides of the wells are dry and that there are no air bubbles in the well contents.
16. Immediately after adding the Stop Solution, read the absorbance values at 450 nm using a microtiter plate reader blanked on the negative control well.

Table 1. Sample data for standard curve.

Standard (pg/ml)	Absorbance (450 nm) A	Absorbance (450 nm) B	Mean	Sample (pg/ml)
0	0.030	0.034	0.032	–
12.5	0.145	0.155	0.150	–
25	0.259	0.283	0.271	–
50	0.501	0.531	0.516	–
100	0.981	1.031	1.006	–
200	1.800	1.820	1.810	–
Test Sample 1	0.260	0.274	0.267	24.6
Test Sample 2	0.611	0.637	0.624	61.0

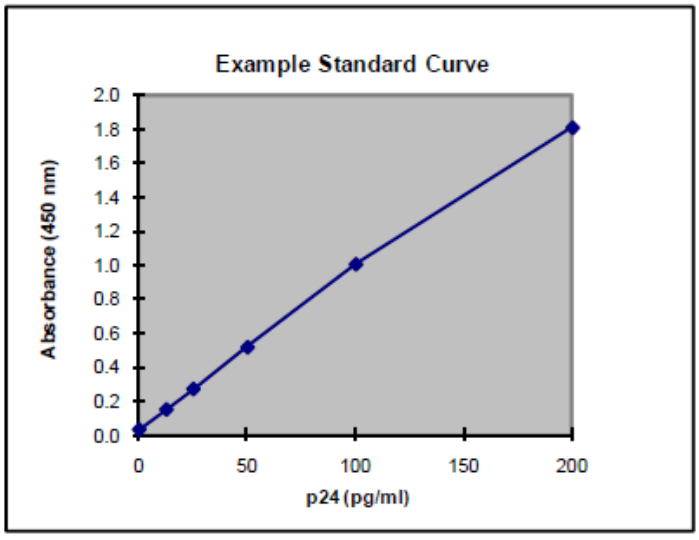

Figure 3. Lenti-X p24 Rapid Titer Kit standard curve. The curve was constructed using the p24 standards and results listed in Table 1. Samples were prepared and assayed as described.

VIII. Interpretation of Results

A. Assay Validation

The following criteria are required for a valid assay:

- A value of $\leq$0.10 for the negative control
- A value of $\geq$0.60 for the 100 pg/ml standard

B. Determining Your Virus Titer

Your p24 values can be used to determine the relative virus titers of your packaging cell supernatants. To calibrate your virus production system and determine a relationship between p24 levels and infectivity, it may be useful to determine the p24 levels of supernatants for which you have already measured the virus titer using an alternative method (i.e. determining infectious units based on expression of a fluorescent protein or drug-selective marker).

The following values and calculations may also be used to determine approximate titers, and are based on the observation that each lentiviral particle (LP) contains approximately 2,000 molecules of p24:

- 1 LP contains 8 x 10^{-5} pg of p24 (derived from (2000) x (24 x 10^{3}Da)/(6 x 10^{23})
- 1 ng p24 is equivalent to ~1.25 x 10^{7} LPs
- For a typical lentivirus vector, there is 1 IFU for every 100–1,000 LPs
- Therefore, a supernatant titer of 10^{7} IFU/ml $\approx$ 10^{9}–10^{10} LP/ml or 80–800 ng p24/ml

Appendix A: Troubleshooting Guide

1. p24 values determined using assays from different manufacturers or different methods may not be used interchangeably.
2. The assay cannot be used to quantitate samples having p24 values greater than the highest value on the p24 standard curve, unless the samples are diluted sufficiently. See Section VII for more information.
3. The performance characteristics have not been established for any matrices other than tissue culture media.
4. Signs of reagent deterioration are as follows:
   - The kit fails to meet the required criteria for a valid test (see Section VIII.A).
   - Reagents become visibly cloudy or contain a precipitate.

     NOTE: The 20X Wash Buffer normally develops a crystalline precipitate during storage at 2–8°C. This will dissolve upon warming at 37°C.

   - The TMB Substrate solution becomes dark blue in color. This is likely caused by chemical contamination of the substrate solution.

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