



Retroviral Expression Systems

pRetro-E1 Vector	E514
pRetro-E2 Vector	E515
pRetro-HA Vector	E516
pRetro-His Vector	E517
Retro-E1 Expression Kit	E512
Retro-E2 Expression Kit	E513
Retro-HA Expression Kit	E518
Retro-His Expression Kit	E519

Table of Contents

Notice to the Purchaser	1
Technical Support	1
Biosafety	2
Protocol at a Glance	3
Retroviral Expression System	6
Materials	7
Additional Materials Required	7
Storage	7
Protocol	8
Expression Vector Construction	8
Culturing Pack-Easy Cells	8
Subculturing Pack-Easy Cells	9
293T Cells	9
Viral Production	10
Concentrating Virus	11
Determining Viral Titre	12
Alternative Viral Titre Method	13
Target Cell Transduction	14
Troubleshooting	16
References	17
Appendix A: Titration of Antibiotic Selection	18
Notes	19
Contact Information	20

Notice to the Purchaser

The products are for research use only. Use in and/or for diagnostics or therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The components in this kit may not be distributed, resold, or modified for resale without prior written approval from Applied Biological Materials (abm) Inc. If you do not agree to the above conditions, please return the UNOPENED product to abm Inc. within ten (10) days of receipt for a full refund. The information in this document is subject to change without notice and should not be construed as a commitment by abm Inc. or its affiliated corporations. In no event shall abm Inc. or its affiliated corporations be liable for incidental or consequential damages in connection with or arising from the use of this manual and product.

abm Inc. products are warranted to meet our QC testing standards at the time of shipment. Notice of problematic products must be made to abm Inc. within 15 days of receipt of the product. This product warranty limits abm Inc.'s liability to the replacement of the product only.

Technical Support

For more information on abm Inc. products, please visit our website:

<http://www.abmGood.com>

For additional information or technical assistance, please call or email us at:

Applied Biological Materials, Inc.

Phone: (604) 247-2416

1-866-757-2414

Fax: (604) 247-2414

E-mail: technical@abmGood.com

Biosafety

Our Retro-Easy™ expression vector has been specifically designed to have no genetic overlap with any of the packaging plasmids, thus eliminates the possibility of generating replication-competent retroviruses. However, as the protocols presented here involve producing, handling, and storing recombinant retroviruses, proficient understanding of safe laboratory practices and potential retroviral hazards is essential. Wild-type MMLV does not naturally infect human cells; however, recombinant retroviral vectors packaged with VSV-G envelope are capable of infecting human cells. In addition, depending on your gene insert, the viral supernatants produced by co-transient transfection could be potentially hazardous. For these reasons, extra precautions should be observed when working with recombinant retroviruses expressing known oncogenes or other toxic proteins.

General guidelines in most jurisdictions require that retroviral production and transduction be performed in a Biosafety Level 2 (BL2) facility. For more information about the BL-2 guidelines and retrovirus handling, refer to “Biosafety in Microbiological and Biomedical Laboratories,” 5th Edition, published by the Center for Disease Control (CDC). This document may be downloaded at the following address:

<http://www.cdc.gov/biosafety/publications/bmbl5/index.htm>

Additional information is also available at:

<http://bmbl.od.nih.gov/contents.htm>

All published BL-2 guidelines for proper waste decontamination should be strictly followed.

It is also important to consult with the health and safety officer(s) at your institution for guidelines regarding the use of retroviruses, and to always follow standard microbiological practices, which include:

- Wear gloves and a lab coat at all times.
- Always work with pseudoviral particles in a Class II culture facility and that all procedures are performed carefully to minimize splashes and aerosols.
- Work surfaces are decontaminated at least once a day and after any spills of viable material.
- All cultures, stocks and other regulated wastes are decontaminated before disposal by an approved decontamination method, like autoclaving.

Protocol at a Glance

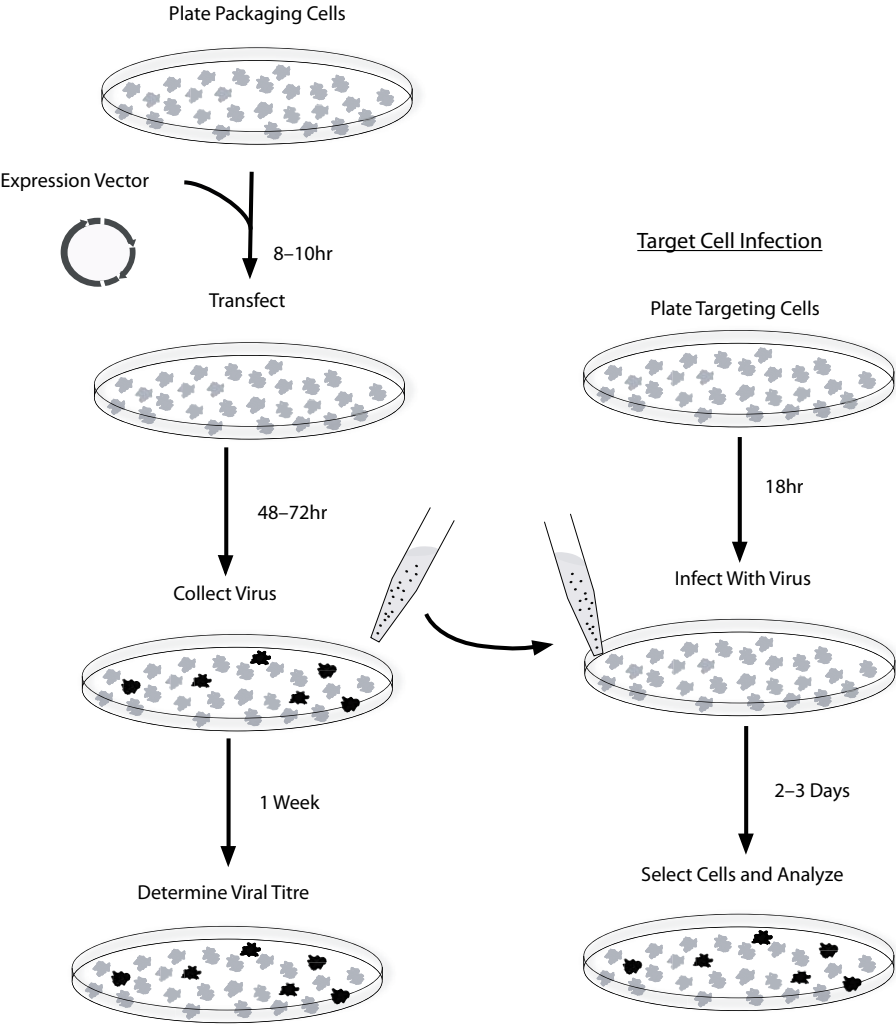


Figure 1: Overview of transient retrovirus production. To produce recombinant retrovirus, both retroviral expression vector and packaging mix are co-transfected into packaging Pack-Easy cells. After 48-72 hours, viral supernatant is collected for either titre determination or transduction of target cells.

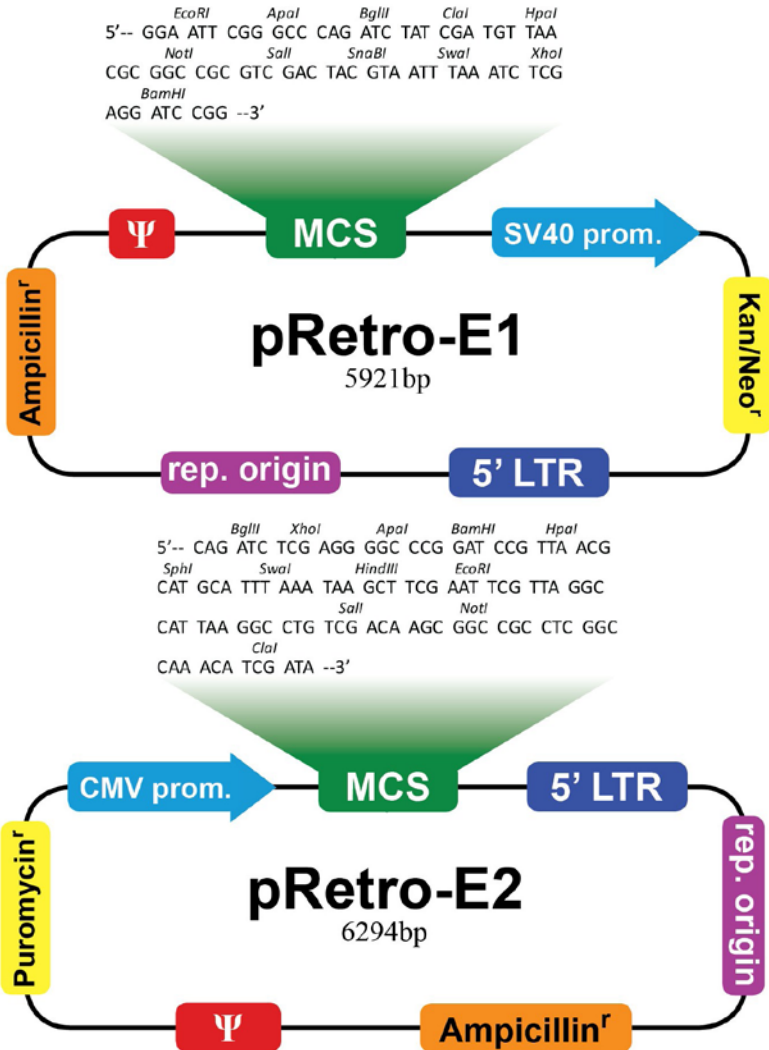


Figure 2: Map of pRetro-E1 & pRetro-E2

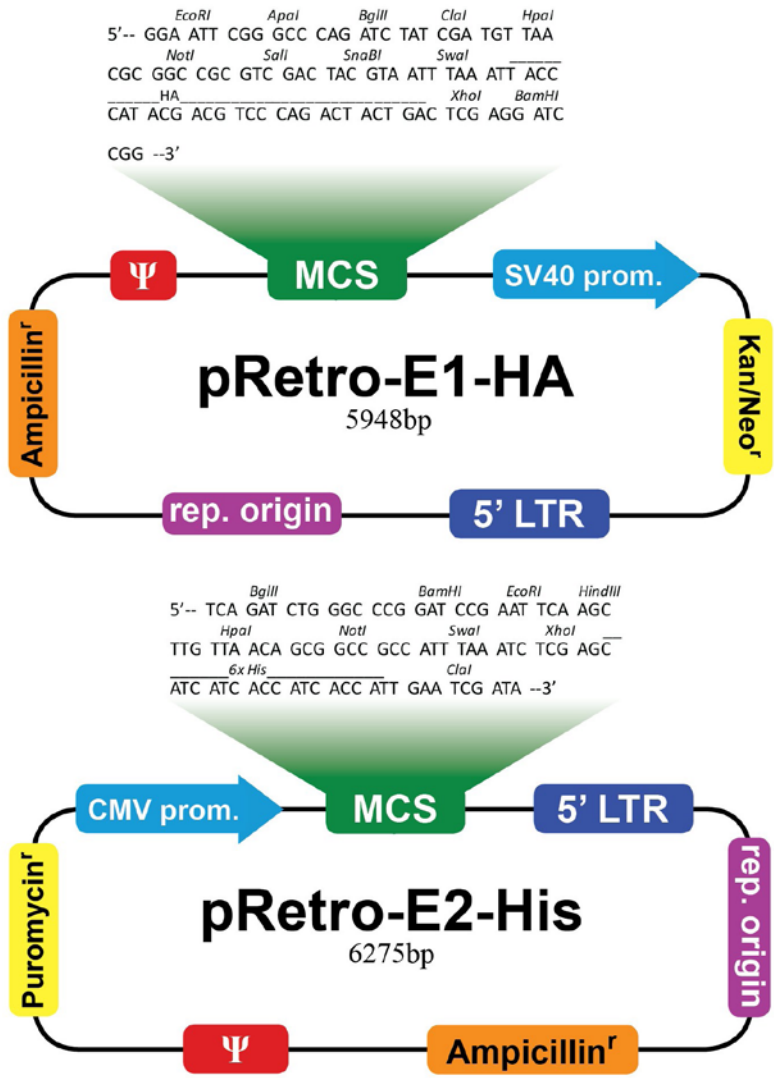


Figure 3: Map of pRetro-HA & pRetro-His

Recombinant Retrovirus is a widely used vessel for successful, stable expression of any transduced dividing cell type (Mann et al., 1983; Miller & Buttimore 1986). During cell division, the nuclear membrane is disintegrated and the viral DNA can access the host genome. Retroviruses can integrate into the host genome efficiently, giving rise to permanent and stable gene expression. Because recombinant retroviral vectors cannot actively pass through the nuclear membrane, the transduction efficiency of target cells with retroviral vectors is low, especially in slow dividing primary cells.

One of the most significant developments in recombinant retro- viral technology is the optimization of high-titre retrovirus production by transient transfection using 293-derived packaging cell lines (Pearl W. S. et al., 1993). This eliminates the time-consuming process of generating retrovirus producer cell lines. In addition, the procedure significantly increases the safety of the recombinant retrovirus production as it eliminates the culturing of stable virus-producing cell lines.

abm's retroviral expression systems provide a simple and effi- cient means for producing high-titre recombinant retroviruses. All of our retroviral vectors contain user-friendly multiple cloning sites (MCS), allowing for easy cloning of your gene of interest into our vectors. In addition, our pRetro-HA and pRetro-His vectors offer the flexibility of adding either HA or His tags in frame to your gene of interest.

Our Retro-Combo™ Mix contains all the genetic elements re- quired for the production of high-titre retroviruses using the transient transfection approach. The Gag/Poly and VSV-G Envelope plasmids of the Retro-Combo™ Mix have been meticulously optimized for high-titre retroviral production. The Retro-Combo™ Mix is compatible with retroviral vectors derived from the Murine Stem Cell Virus (MSCV), Moloney murine leukemia virus (MoMuLV) and the Moloney murine sarcoma virus (MoMuSV). With Retro-Combo™ packaging mix, virions are pseudotyped with the envelope glycoprotein from the vesicular stomatitis virus (VSV-G). VSV-G mediates viral entry through lipid binding and plasma membrane fusion, giving rise to higher transduction efficiency (Burns et al., 1993; Emi et al., 1991). Figure 1 shows a schematic overview of retrovirus production by transient transfection in the Pack-Easy packaging cell line.

Materials

Table I. Retroviral Vectors and Kits

Component	Cat. No.	Quantity	E512	Kit Cat. No.			E519
				E513	E518	E519	
pRetro-E1 Vector	E514	10ug	✓				
pRetro-E2 Vector	E515	10ug		✓			
pRetro-HA Vector	E516	10ug			✓		
pRetro-His Vector	E517	10ug					✓
Retro-Combo Mix	E510	10 Rxns	✓	✓	✓		✓
Pack-Easy Cells	E511	1 Flask	✓	✓	✓		✓

Additional Materials Required

The following materials and reagents are required but not provided:

- Dulbecco's Modified Eagle's Medium (Invitrogen Cat: 11995)
- Fetal bovine serum (FBS) (Cat. No. TM999-500) Note: does not need to be heat-inactivated.
- 200 mM L-Glutamine (Sigma Cat. No. G7513)
- Solution of 10,000 units/ml Penicillin G sodium and 10,000 µg/ml Streptomycin sulphate (Sigma Cat. No. P0781)
- Complete Medium: DMEM supplemented with 100 units/ml penicillin G sodium, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS)
- G418 (Cat. No. C020) Note: Make a 10 mg/ml active stock solution by dissolving 1g of powder in approximately 70ml of complete medium without supplements. Sterile filter and store at 4°C.
- Puromycin (Cat. No. C021)
- Polybrene (Hexadimethrine Bromide; Cat. No. G062)
- Trypsin-EDTA (Trypsin; Sigma Cat. No. T3924)
- Dulbecco's phosphate buffered saline (DPBS; VWR Cat. No. 82020-066)
- BD Biocoat Collagen Type I 12-well plates (BD Biosciences Cat. Nos. 354500 & 356500)
- Cloning cylinders (PGC Scientific Cat. No. 62-6150-40, -45)
- NIH-3T3 cells (ATCC Cat. No. CRL-1658)
- Calciumfectin (abm Cat # G099)
- Chloroquine (Sigma Cat. # C6628)
- Tissue culture plates and flasks

Storage

- Pack-Easy cells in Liquid Nitrogen.
- All other components at -20°C.
- Spin briefly to recover contents and avoid repeated freeze-thaw cycles.

NOTE: The following protocol is broken into sections for convenience. However, time should be taken to read through the full procedure before attempting.

A. Expression Vector Construction

Use standard molecular biology techniques to subclone your gene of interest into any one of our retroviral expression vectors (Sambrook & Russell, 2001).

- 1. The gene insert should contain an ATG start codon. Also, adding a** Kozak consensus ribosome-binding site may improve expression levels in mammalian cells (Kozak, 1987). Please note that all genes subcloned into a retroviral vector must not interfere with the retroviral life cycle and allow complete transcription of the full-length viral genome. Sequences such as poly-A signals must not be included, as all retroviral vectors already have a poly-A signal sequence following their 3'-LTR (Coffin et al., 1996).
- 2. Digest both the insert gene and the vector with the appropriate** restriction enzyme(s) followed by purification.
- 3. Ligate the insert gene fragment into the vector.**
- 4. Transform ligation products into *E. coli* cells.**
- 5. Screen for recombinant plasmids by restriction analysis and confirm** by sequencing.

B. Culturing Pack-Easy Cells

The Pack-Easy Cell Line is widely used for optimal retrovirus production due to its high transfection efficiency. The health of Pack-Easy cells at the time of transfection is a critical factor for the success of retrovirus production. The use of "unhealthy" cells will negatively affect the transfection efficiency, resulting in low titre retroviral stocks. For optimal retrovirus production, follow the guidelines below to culture Pack-Easy cells before transfection:

- **Make sure the cells possess greater than 90% viability.**
- **Subculture and maintain cells in complete medium containing** 0.1 mM MEM Non-Essential Amino Acids, 4 mM L-Glutamine, 1 mM sodium pyruvate, 500 µg/ml Geneticin® and 10% FBS.
- **Do not allow cells to overgrow before passaging.**

C. Subculturing Pack-Easy Cells

In general, Pack-Easy cells should be plated at 10^6 cells per 10cm plate and split every 2–3 days when they reach ~90% confluency.

Note: Pack-Easy cells do not adhere well to culture vessels and extra care should be taken to avoid dislodging the cells during media change over. When changing the medium during retrovirus production, add the medium slowly against the side of culture vessels.

Split the cells as follows:

1. **Aspirate and wash cells once with serum-free culture medium.**
2. **Add 4-6ml of trypsin-EDTA solution and incubate at room temperature for 2–5 minutes.**
3. **Add 3 ml of complete media to inhibit trypsinization.**
4. **Resuspend cells gently by pipetting. Transfer cells into a 15ml culture tube.**
5. **Spin at 1000g for 5 minutes to pellet cells.**
6. **Aspirate the supernatant, then add 2ml of complete media.**
7. **Resuspend cells by pipetting, then plate cells at a density of 80% for virus production on the following day.**

D. 293T Cells

The human 293T cell line is widely used for optimal retrovirus production (Naldini et al., 1996). The health of 293T cells at the time of transfection is a critical factor for the success of retrovirus production. The use of “unhealthy” cells will negatively affect the transfection efficiency, resulting in low titre retroviral stocks. For optimal retrovirus production, follow the guidelines below to culture 293T cells before use in transfection:

- Ensure cell viability is greater than 90%.
- Subculture and maintain cells in complete medium containing 0.1mM MEM, Non-Essential Amino Acids, 4mM L-Glutamine, 1mM sodium pyruvate.
- 500µg/ml Geneticin and 10% FBS.
- Do not allow cells to overgrow before passaging.
- Use cells that have been subcultured for less than 16 passages.

Protocol

E. Viral Production

Recombinant retroviruses are produced in Pack-Easy cells through transient transfection, which can be done by standard Calcium Phosphate reagents (Cat. No. G099). For reasons still unknown, retrovirus production with standard calcium phosphate transfections consistently produces higher titre (1-5x) than those with other types of transfection reagents like Lipofectamine. Transfections are generally performed in 10cm dishes. Higher titre retroviruses can be produced when Pack-Easy cells are at 85% to 95% confluency. As a guideline, subculturing a confluent 10cm culture plate in 1:2 ratio, or a confluent 15cm dish to five 10cm dishes 12-16 hours before transfection provide suitable cell density for transfection the following day.

- 1. One day before transfection, plate Pack-Easy cells in a 10cm** tissue culture plate so that they will be 90-95% confluent on the day of transfection (i.e. 5×10^6 cells in 10ml of growth medium with serum).
- 2. Prior to transfection, add 10ml media (DMEM with 10% FBS) containing** 25 μ M chloroquine (final concentration). **Note:** *Adding chloroquine prior to transfection may increase transfection efficiency 2-3 fold. Prepare a 25mM stock of chloroquine in distilled water and sterile filter. 1-2 hours before transfection, replace media with media containing 25 μ M of chloroquine (Pear et al., 1993).*
- 3. For each transfection, prepare Solution A and Solution B in separate** 5ml sterile tubes.

Solution A: Add components in the following order and mix well:

- 10 – 15 μ g retroviral vector DNA
- 7.5 μ g Retro-Combo™ Mix
- 64 μ l 2M CaCl₂
- X μ l H₂O Top up to 500 μ l (Total)

Solution B: 500 μ l 2X HBS

- a) Carefully and slowly vortex Solution B while adding Solution** A drop-wise. (Alternatively, blow bubbles into Solution B with an autopipettor while adding Solution A drop-wise.
- b) Incubate reaction at room temperature for 10-15 minutes.**
- c) Add mixture drop-wise to the culture plate, gently but quickly.**
- d) Incubate the plates at 37°C for 5-8 hours without disturbing.**
- e) Take out transfection complex and add 10ml of fresh media.**
- f) Incubate overnight at 37°C.**

Protocol

- 4. The next day, change media and incubate for another 24 hours.**
Plate target cells at 50–60% confluency in 6-well plates to prepare for transduction the following day.
- 5. Harvest Pack-Easy cell supernatant. Feed cells with another 10ml fresh media and incubate for another 24 hours.**
- 6. Filter viral supernatant through a 0.45µm filter to remove cell debris.** *Note: The 0.45µm filter should be cellulose acetate or polysulfonic filter (low protein binding). Do not use a nitrocellulose filter because it binds to proteins in the retroviral membrane and destroys the virus.*
- 7. Either infect target cells as shown in the Target Cell Transduction section or freeze down viral supernatant at -80°C for future applications.** *Note: Aliquot filtered supernatant into single-use tubes to avoid multiple freeze-thaw cycles. Multiple freeze-thaw cycles should be avoided since titre can drop as much as 20–40% with each cycle (Higashikawa & Chang, 2001; Kwon et al., 2003). Store tubes at -80°C and no cryoprotectant is required.*
- 8. Collect the second viral supernatant after the 24 hours. Filter through 0.45µm a filter and either infect target cells or freeze viral supernatant down at -80°C for future applications.**

F. Concentrating Virus

If high-titre virus is needed, the retroviruses can be concentrated by ultracentrifugation.

- 1. Remove cell debris and aggregated virus by centrifugation at 2000g for 5 minutes at 4°C.**
- 2. Pellet the virus at 50,000g for 90min at 4°C. Remove the supernatant.**
- 3. Resuspend the virus to 0.5–1% of the original volume in viral suspension buffer (50mM Tris-HCl [pH 7.8], 130mM NaCl, 1mM EDTA).**
- 4. Determine the viral titre of the pre- and post-concentrated viral supernatants using the protocol discussed in the following section. Alternatively, recombinant retrovirus can also be easily purified using an ion exchange-based filter (abm Cat. # G130).**

Protocol

G. Determining Viral Titre

It is useful to titre viral supernatants before proceeding with transduction experiments for the following reasons:

- **Ensure that the viral stock is viable.**
- **Determine the percentage of target cells that can be transduced with pseudoviral stock.**
- **Control the number of integrated viral constructs per target cell.**

The simplest protocol for measuring titres uses a positive control expression plasmid (i.e. GFP mixed with expression construct) as an internal control at a ratio of 1:100 and is packaged into pseudoviral particles. In an alternative approach, the GFP control plasmid can be packaged separately but in parallel with your construct, as an external control. In this scenario, the control plasmid can be used to check and optimize the transfection/packaging steps.

To determine the relative viral titre, transduce a target cell line such as MDA-MB-468 in the presence of Polybrene (2µg/ml) for 12-16 hours. Count the number of GFP-expressing cells by either fluorescence microscopy or FACS.

- 1. For each viral stock, plate MDA-MB-468 cells one day prior to viral infection in a 24-well plate at a density of 0.6×10^5 cells per well.** Add 1ml of complete DMEM (with serum and antibiotics) and incubate cells at 37°C with 5% CO₂ overnight. **Note:** *It is possible to use bigger culture dishes for transduction, especially when a large number of cells is required for FACS analysis. In this case, the amount of cells should be adjusted depending on the growth area of the well/plate.*
- 2. On the following day, prepare complete DMEM media with 10% FBS and Polybrene to a final concentration of 2µg/ml.** **Note:** *Polybrene is a polycation that neutralizes charge interactions to increase binding between the pseudoviral capsid and the cellular membrane. The optimal concentration of Polybrene depends on cell type and may need to be empirically determined (usually in the range of 2-10µg/ml). Excessive exposure to Polybrene (>12 hr) can be toxic to some cells.*
- 3. Remove culture media and replace with 0.5ml of complete DMEM with 10% serum and Polybrene (from Step 2).** For each viral stock, use three wells. Infect MDA-MB-468 cells with 100µl, 500µl, 1.0ml and 2.0ml of viral supernatant in the presence of 2µg/ml Polybrene in the early morning. Additional 1.9ml, 1.5ml, and 1.0ml of complete media should be added to viral supernatant that are less than 2.0ml to bring the total infection volume to 2.0ml. For control wells, add 2ml of DMEM medium with Polybrene. Incubate cells at 37°C with 5% CO₂ for 6-8 hours.

Protocol

- 4. Six to eight hours later, repeat the infection for overnight infection (second hit).**
- 5. The following day, split the cells 1:3 to 1:5 if necessary, depending on the growth rate of cells.** Incubate in complete DMEM for an additional 24-48 hours.
- 6. Count the fraction of fluorescent cells using FACS analysis.** You may also count the GFP positive cells under a fluorescent microscope, but the results may be less accurate due to inconsistencies in counting. Use the average of the fraction of GFP+ cells in 5-10 random fields to estimate the overall percentage of GFP+ cells on the plate. Calculate viral titre based on the percentage of GFP positive cells over total number of cells analyzed by either microscopy or FACS.

H. Alternative Viral Titre Method

Traditionally, recombinant retroviral titre has been assayed using the number of selection marker resistant colonies following transduction of NIH3T3 cells.

- 1. Plate NIH3T3 cells 24 hours prior to target cell transduction in 6-well plates at a density of $0.5-1 \times 10^5$ cells per well.**
- 2. Prepare 20ml of complete media and add 300 μ l of 0.8mg/ml Polybrene.**
- 3. Collect virus-containing media from packaging cells.**
- 4. Filter media through a 0.45 μ m cellulose acetate or polysulfonic (low protein binding) filter.**
- 5. Prepare six 10-fold serial dilutions as follows:**
 - a) Add 1.35ml of the media to six 1.5ml microcentrifuge tubes.**
 - b) Add 150 μ l of virus-containing media to the first tube. Mix.**
 - c) Transfer 150 μ l of viral stock dilution from tube 1 to tube 2.** Continue serial dilutions by transferring 150 μ l of each successive dilution to the next prepared tube.
- 6. Transduce NIH3T3 cells by adding 1ml of the diluted virus medium (from Step 5) to the wells.** Final polybrene concentration will be 4 μ g/ml in 3ml.

Protocol

- 7. The following day, change the media.**
- 8. Start antibiotic selection 48 hours after infection, using appropriate antibiotic concentration based on the killing curve information (see Appendix A).**

I. Target Cell Transduction

The following protocols are general guidelines for the transduction of adherent cells, such as NIH3T3 or HeLa. It is recommended that users optimize for efficient gene transduction of suspension cells using the same principle as optimizing the transduction of adherent cells. **Note:** *Multiple rounds of infection can improve your results by increasing the number of infected cells as well as increasing the copy number per cell.*

- 1. Plate the target cells 12–18 hr before infection, at a cell density of 5×10^4 per 6 well plate.** **Note:** *Retroviral particles can only passively enter the nuclei of actively dividing cells, not non-dividing cells.*
- 2. Add virus to target cells. Unless you have determined the viral titre, use as much virus-containing medium as possible for the infection.** Store the remaining viral supernatant at -80°C . As a general guideline, use 2ml of viral supernatant for a 6-well size culture vessel in the presence of 2-20 $\mu\text{g/ml}$ Polybrene (first hit for 8-10 hours, and then second hit for overnight). Make Polybrene stock at 0.8mg/ml. Use 20 $\mu\text{l/well}$ in a 6-well plate and 100 $\mu\text{l/10cm}$ dish. **Notes:** *Titre will decrease 20-40% per freeze-thaw cycle based on our in-house data. The optimal final concentration of polybrene may need to be empirically determined, but it is generally within a range of 2-10 $\mu\text{g/ml}$. Excessive exposure to polybrene (>24hr) can be toxic to cells.*
- 3. Replace medium with fresh complete medium the following day.** Some target cells (especially primary cells) are very sensitive to conditioned viral supernatant from packaging cells, partly due to nutrient starvation. This can be alleviated by the following steps:
 - a) Dilute virus-containing supernatant at least 2-fold with fresh media.**
 - b) Expose target cells to the virus for 4-6 hours and then replace with fresh medium, though this will significantly decrease transduction efficiency.**

Protocol

- 4. The following day, depending on the growth rate of cells, split** the cells 1:3 to 1:5 in the presence of the appropriate selection marker (G418 or Puromycin) as determined by the killing curve (see Appendix A). Change medium every 3-4 days to maintain consistent selection drug concentration. The selection should be complete in 2 weeks for most target cell types.
- 5. Pick up 10-20 colonies for expansion and screening for stable** clones with high level of transgene expression.

Troubleshooting

Problem	Possible Cause	Solution
No Colony Growth	Wrong antibiotic or antibiotic concentration too high.	Use kanamycin at 50ug/ml of LB agar media.
	Poor transformation efficiency.	Check transformation efficiency using control plasmid like a parental plasmid.
Low Plasmid Yield	Retroviral constructs use a low-copy pBR322 ori.	Grow more liquid culture and purify using low-copy purification procedures.
Plasmid Difficult to Grow	Plasmid may rearrange due to presence of LTR's.	Switch to alternate <i>E. coli</i> strain for unstable DNA selection.
Poor Viability	Improper thawing procedures.	Follow thawing procedures in Culturing Pack-Easy Cells section.
	Improper culture media.	Grow Pack-Easy cells in DMEM with 10% FBS; use high glucose DMEM media only.
Poor Transfection Efficiency	Cell density not optimal; a cell density of 85-90% is best for high titre virus production.	Optimize DNA and transfection reagent amounts and exposure time.
Low Titre (<10 ⁵ cfu/ml)	Too many freeze/thaw cycles.	Avoid freeze/thaw viral supernatant; each cycle decreases titre approx. 20-40%.
	Truncated viral RNA.	Check for presence of poly(A) between LTR's.
	Poor transfection efficiency.	Optimize transfection.
	Virus harvested too early.	Harvest virus 48-72hrs after transfection.
	Vector too large.	The limit of the vector is 8.3kb from LTR to LTR.
	Polybrene not used.	Add Polybrene during titration & transduction.
	Improper selection during titration.	Perform an antibiotic kill curve on titration targets prior to titration.
Poor Infection Efficiency	Target cells not dividing.	Plate cells at lower confluency, activate with mitogen, or use another method to induce cell division.
	Low titre.	See previous section.
Target Cell Viability Poor	Virus supernatant may be affecting cell growth.	Dilute viral media or shorten exposure time to viral supernatant.
	Improper Polybrene concentration.	Titrate Polybrene or shorten exposure time to viral supernatant.
Low Expression Level	Possible promoter inactivation.	Split cells, activate with mitogen, treat cells with 5-azacytidine or choose a tissue-specific promoter.
	Poor cell viability.	Check growth parameters.

References

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. M., Seidman, J. G., Smith, J. A. & Struhl, K., Eds.** (1995) *Current Protocols in Molecular Biology*. (John Wiley & Sons, NY).
- Burns, J. C., Friedmann, T., Driever, W., Burrascano, M. & Yee, J.-K.** (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* 90:8033–8037.
- Coffin, J. M. & Varmus, H. E., Ed.** (1996) *Retroviruses* (Cold Spring Harbor Laboratory Press, NY). Devroe, E. & Silver, P. A. (2002) Retrovirus-delivered siRNA. *BMC Biotechnol.* 2(1):15.
- Emi, N., Friedmann, T. & Yee, J.-K.** (1991) Pseudotyped formation of murine leukemia virus with G protein of vesicular stomatitis virus. *J. Virol.* 65:1202–1207.
- Freshney, R. I.** (2000) *Culture of Animal Cells, Fourth Edition* (Wiley-Liss, NY).
- Kozak, M.** (1987) At least six nucleotides preceding the AUG initiator codon enhances translation in mammalian cells. *J. Mol. Biol.* 196:947–950.
- Mann, R., Mulligan, R. C. & Baltimore, D.** (1983) Construction of a retrovirus packaging mutant and Miller, A. D. & Baltimore, C. (1986) Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* 6(8):2895–2902.
- Pear, W. S., Nolan, G. P., Scott, M. L. & Baltimore, D.** (1993) Production of high titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* 90(18):8392–8396.
- Sambrook, J. & Russell, D.W.** (2001) *Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Appendix A: Titration of Antibiotic Selection

Prior to using G418, or Puromycin to generate stable cell lines of transduced target cells, it is important to titrate selection antibiotics to determine the optimal concentration used for the selection of transduced target cells. For optimal selection, we recommend that you perform two experiments for each drug: (1) a titration to determine the optimal drug concentration, and (2) an experiment to determine the optimal plating density.

1. Titrate at fixed cell density that is going to be used during transduction of target cells interested.

a) Plate 2×10^5 cells in each of six 10cm tissue culture dishes containing 10ml of the appropriate complete medium plus varying amounts (0, 50, 100, 200, 400, 800, 100 μ g/ml). For Puromycin, add the drug at 0, 0.5, 2.4, 6, 8, and 10 μ g/ml.

b) Incubate the cells for 10-14 days, replacing the selection media every four days (or more often if necessary).

c) Examine the dishes for viable cells every two days. In general, use the lowest concentration that begins to give massive cell death in 7 days and kills all the cells within two weeks.

2. Once you have determined the optimal drug concentration, determine the optimal plating density by plating cells at several different densities in the presence of a constant amount of drug. If cells are plated at too high a density, they will reach confluency before the selection takes effect. Optimal plating density is dependent on population doubling time and cell surface area. For example, cells that double rapidly have a lower optimal plating density than cells that double slowly.

a) Plate cells at several different densities in each of six 10cm tissue culture dishes containing 10ml of the appropriate selective medium. Suggested densities (cells/10-cm dish): 5×10^6 , 1×10^6 , 5×10^5 , 2×10^5 , 1×10^5 , and 5×10^4 .

b) Incubate the cells for 5-14 days, replacing the selection media every four days.

c) Examine the dishes for viable cells every two days.

For selecting stable transfectants, use a plating density that allows the cells to reach 80% confluency before massive cell death begins (at about day 7). This is the cell density at which cells should be plated for selection of transduced target cells.

Contact Information

Applied Biological Materials Inc.

Website:

www.abmGood.com

Phone:

(8:30am-4:30pm PST M-F)

Toll Free: 1-866-757-2414

Local: (604) 247-2416

Fax: (604) 247-2414 (24Hr.)

Address:

Suite #8-13520 Crestwood Place

Richmond, BC

Canada

V6V 2G2

Email:

General Information:

info@abmGood.com

Order Products:

order@abmGood.com

Technical Support:

technical@abmGood.com

siRNA:

siRNA@abmGood.com

Business Development:

bd@abmGood.com

Distributors

North America

Canada

Applied Biological Materials Inc.

Tel: (604) 247-2416 / 1-866-757-2414

Fax: (604) 247-2414

www.abmGood.com

United States

Applied Biological Materials Inc.

Tel: (604) 247-2416 / 1-866-757-2414

Fax: (604) 247-2414

www.abmGood.com

Mexico

Quimica Lavoisier S.A. de C.V.

Tel: 52-333-848-8484

Email: informes@lavoisier.com.mx

www.lavoisier.com.mx

International

Australia

Biosensis Pty Ltd.

Tel: +61 43 166 5519

Email: sales@biosensis.com

www.biosensis.com

Belgium

Gentaur

Tel: 32 2 732 5688

Email: ea@gentaur.com

www.gentaur.com

France

Gentaur

Tel: 01 43 25 01 50

Email: ea@gentaur.com

www.gentaur.com

Germany

BioCat GmbH

Tel: +49 (0) 6221-714-1516

Email: info@biocat.com

www.biocat.com

India

G Biosciences

Tel: 0120-432-3330

Email: rohit@gbiosciences.com

www.GBiosciences.com

Israel

BioConsult

Tel: 972-(0)2-566-7043

Email: sales@bioconsult.co.il

www.bioconsult.co.il

Italy

MICROTECH s.r.l.

Tel: +39-0816107435

Email: microtech@microtech.eu

www.microtech.eu

Japan

Cosmo Bio Co. Ltd.

Tel: 03-5632-9610/9620

Email: mail@cosmobio.co.jp

www.cosmobio.co.jp

Singapore

Bio-REV PTE

Tel: (65) 6273-3022

Email: allan@bio-rev.com

bio-rev.com

South Korea

CMI Biotech

Tel: 02 444 7101

Email: cmibio@cmibio.com

www.cmibio.com

Taiwan

Interlab Co. Ltd.

Tel: +886-2-2736-7100

Email: service@interlab.com.tw

www.interlab.com.tw

United Kingdom

NBS Biologicals Ltd.

Tel: +44 (0)1480 433875

Email: info@nbsbio.co.uk

www.nbsbio.co.uk

The Source for

any Antibody, siRNA, and Viral Vector

The Depot for

PCR, qPCR, and Transfection Reagents

