Chapter 19

Lentiviral Vectors to Analyze Cell Death Regulators

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Abstract

Ectopic expression of proteins involved in cell death pathways is an important tool to analyze their role during apoptosis or other forms of cell death. Lentiviral vectors offer the advantages of high rate of transduction and stable integration of donor DNA into the genome of the host cell, leading to reproducible and relevant readouts compared to classical overexpression by transfection of naked plasmid DNA.

Here, we describe the production and application of lentiviral vectors to express cell death proteins in eukaryotic cells. A packaging cell line, usually HEK293T cells, is transfected with viral packaging plasmids and your gene of interest, which is flanked by long terminal repeat sites with an internal ribosome entry site in the 5′UTR (*U*n *t*ranslated *r*egion). Virions are harvested from the supernatant and can be directly used to transduce target cells. Varied selection markers as well as a variety of promoters that regulate expression of the gene of interest make this system attractive for a wide range of application in many cell lines or in whole organisms.

Key words Lentivirus, Transduction, Inducible gene expression

1 Introduction

The analysis of single proteins, or mutants thereof, involved in signaling pathways is a powerful method to investigate molecular mechanisms of cell death. Overexpression using classical transfection of naked DNA is inefficient and often results in unspecific bystander effects, which are not reflective of the mechanisms that occur under endogenous conditions. Furthermore, transfection efficiency varies greatly between cell types. A particular problem in cell death research is that uncontrolled overexpression of effector proteins, such as caspases or proapoptotic members of the Bcl2 family, leads to cell death in the absence of a death stimulus [1–4]. Expression of such effectors therefore have to be tightly regulated and stable integration of donor DNA into the host genome, in combination with controlled gene expression using inducible promoters, offers the possibility to study cell death proteins under more physiological conditions.

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Retroviruses are a family of enveloped RNA viruses that replicate in their host cell through reverse transcription. Lentiviruses are a genus of the Retroviridae family and have the advantage over other retroviruses to infect nondividing cells. Lentiviruses integrate their DNA into the host cell and are able to deliver relatively large amounts of RNA compared to other retrovirus family members, making them ideal tools for in vitro research. Furthermore, for research purposes and safety, lentiviruses are rendered replication incompetent and, thus, are unable to replicate in the host cell.

Here, we describe a protocol using the second generation of lentiviral plasmids as deposited with Addgene by Weinberg and Trono labs. To produce lentiviral particles, packaging cells are transfected with three mammalian expression plasmids: a plasmid encoding the gene of interest; a plasmid containing the viral envelope protein; and a plasmid encoding the viral components Gag, Pol, Rev, and Tat (*see* Fig. 1). In contrast to the second generation system, the third generation uses two packaging plasmids and does not use the Tat protein. The third generation system therefore offers increased biosafety, but it is more cumbersome as it involves transfection of a total of four plasmids in the packaging cell line. Due to better transduction efficiency in our hands, we favor working with the second generation system.

The packaging cell line is an easy-to-transfect cell type, which is able to produce proteins in large amounts. HEK293T cells (HEK293 containing the SV40 Large T antigen allowing for plasmid replication and enhanced protein expression) are a popular choice as they are easy to grow in culture and produce large amounts of virions. The packaging plasmid, **pCMV delta R8.2** encodes essential virion proteins Gag (matrix, nucleoprotein), Pol (transcriptase), Rev (reverse transcriptase), and Tat (transactivator of transcription). The Envelope plasmid, **pCMV VSV-G** [5], encodes for VSV-G, the glycoprotein G of the Vesicular stomatitis virus. VSV-G is an envelope glycoprotein and pseudotypes the lentiviral particles to allow transduction to all mammalian cell types. The gene of interest is transfected in a separate **transfer plasmid**, which harbors a *l*ong *t*erminal *r*epeat (**LTR**) sequence allowing integration into the host cell genome, an internal ribosome entry site (**IRES**) in the 5′UTR as well as a Ψ (**Psi**) sequence, which serves as a packaging sequence for the viral RNA genome. The viral genes on the packaging and envelope plasmids do not have a Ψ sequence or LTRs, therefore RNA transcripts are not packed into the virions and consequently target cells will not produce further lentiviruses. In addition to the packaging, envelope and transfer plasmid we recommend cotransfection with a **Marker plasmid** (e.g. **pEGFP-N1**) to monitor successful transfection of the packaging cell line.

Upon transfection with the lentiviral expression plasmids, the packaging cell line produces viral proteins as well as mRNA of the transfer plasmid, which is packed into the virions due to its Ψ sequence.

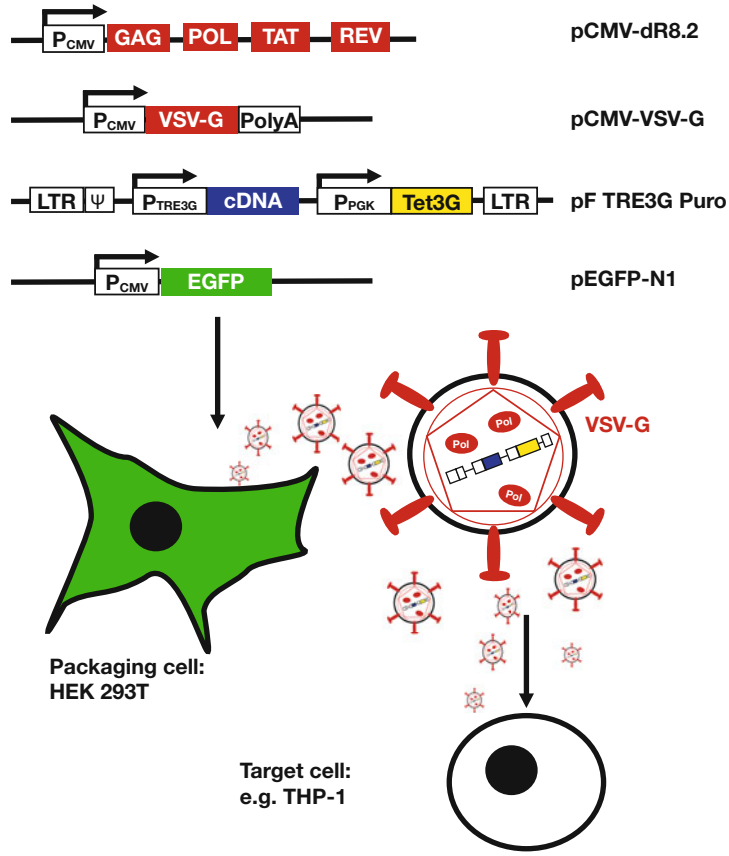

Fig. 1 Generation of lentiviral particles. Plasmids used to generate lentiviral particles: The virus packaging plasmid (pCMV-dR8.2), the envelope plasmid (pCMV-VSV-G), the transfer plasmid (pF TRE3G Puro) containing the cDNA of choice and a puromycin resistance marker, and the marker plasmid (pEGFP-N1) are transfected into HEK 293T cells in a ratio of 3:1:5:1. The packaging cells (HEK 293T) will express GFP and produce lentiviral particles containing the transfer DNA and reverse polymerase to infect the target cells. The envelope protein VSV-G ensures infection in all mammalian cells. Alternatively, plasmid containg the envelope protein ENV can be used for the infection of rodent cells only, providing a safer approach and tool for transduction of cells. The transfer DNA will be integrated in the host cell genome upon infection

Along with the lentiviral RNA, the packaging cell equips the virions with viral reverse transcriptase to transcribe the virion RNA genome into DNA, once it has been delivered to the host cell.

Upon infection of the target cell, the LTR sequences in the virion genome allow the reversely transcribed DNA to integrate into the host genome. The site of insertion is unpredictable and was considered to occur randomly. However, recent studies show that the DNA integrates preferentially in active sites of the genome, which are more accessible for integration [6]. Due to the unpredictable nature of insertion into the host genome, the level of gene expression between different cells can vary considerably.

Therefore in many cases it is required that single cell clones are selected with moderate expression of the gene of interest. Selection of individual clones is particularly encouraged if the gene of interest is driven by an inducible promoter. If the gene has been inserted in a hyperactive region of the genome or downstream of an active promoter, leaky expression (i.e., expression in the absence of the inducing agent) could ensue. Therefore, isolation of clonal lines may be warranted.

The choice of using either inducible or cell type-specific promoters to study your gene of interest is a major advantage of the lentiviral transduction method. In cell death research, inducible Doxycycline or Tamoxifen-inducible promoters are often used, as expression of the gene of interest can be regulated by titrating these inducing agents.

Here, we describe virus production in 293T cells and the transduction of both an adherent cell line, e.g., mouse embryonic fibroblasts (MEFs), and a suspension cell line, human monocytic THP-1 cells. The lentivirus produced contains the protein of choice as well as a puromycin selection marker. In adherent cells, selection of single clones can be achieved by seeding a small number of cells in normal tissue culture plates. For cells in solution, single cell colonies are generated using limiting dilution in 96-well plates or by culturing cells in 0.3 % agar.

2 Materials

2.1 Plasmids

1. Lentiviral packaging plasmid: pCMV delta R8.2 (Addgene, deposited by the Trono lab).
2. Lentiviral envelope plasmid: pCMV-VSV-G (Addgene, deposited by the Weinberg lab).
3. Transfer plasmid: Any plasmid which includes LTRs, IRES, a Ψ sequence, and your gene of interest. We routinely use pF TRE3G Puro, a derivate of the pRetroX vector (Clonetech) [7] (*see* **Note 1**).
4. Marker plasmid: pEGFP-N1 (Clonetech) or similar.

2.2 Cell Culture and Transfection

1. Packaging cell line: Human embryonic kidney (HEK) 293T cells, sourced from ATCC.
2. Target cell lines: Here, we use human monocyte cell line THP-1 (ATCC) and mouse embryonic fibroblast MEFs immortalized with SV40 large T antigen [8], generated as described before [9] (*see* **Note 2**).
3. Cell culture medium: 293T and MEFs are cultured in *D*ulbecco's *M*odified *E*agle *M*edium (DMEM) supplemented with 10 % fetal calf serum, 2 mM l-glutamine, and (optional)

antibiotic solution containing 100 IU/ml Penicillin and 100 µg/ml Streptomycin. THP-1 cells are cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum, 2 mM l-glutamine, and antibiotic solution. All cell lines are cultured at 37 °C in a humified incubator at 10 % CO_2.

4. *P*hosphate *b*uffered *s*aline (PBS).
5. Trypsin-EDTA.
6. Transfection reagent: This protocol uses the calcium phosphate coprecipitation protocol for transfection of viral vectors. The solutions required are: (1) 2× HBSS (50 mM HEPES, pH 7.05, 280 mM NaCl, 1.5 mM Na_2HPO_4); (2) 2.5 M $CaCl_2$. Both solutions must be sterile-filtered and stored at −20 °C.
7. Sterile water.
8. Vortex.
9. Hemocytometer for counting cells.

2.3 Lentivirus Production and Harvest

1. 10 cm tissue culture plates.
2. 20 ml syringe.
3. 0.4 µm syringe filter.
4. 5 ml Cryotubes for storage of virion containing supernatants.
5. Fluorescent microscope.

2.4 Transduction of Target Cells

1. 6-well tissue culture plates.
2. Polybrene (*see* **Note 3**).
3. Temperature regulated bench top centrifuge with inserts for tissue culture plates.
4. 10 ml Falcon tubes.

2.5 Selection of Infected Cells

1. 6-well cell culture plates.
2. Selection marker: Here, we use 10 mg/ml Puromycin Dihydrochloride in 20 mM HEPES.

2.6 Selection of Single Cell Clones

1. Two water baths, set to 37 °C and 50 °C, respectively.
2. 10 cm tissue culture dishes and 48-well tissue culture plates (for adherent cells); 6-well tissue culture plates or 96-well U-bottom cell culture plates (for suspension cells).
3. Trypsin solution.
4. 3 % agar in sterile water.
5. Dissection microscope for picking of single clones.

2.7 Testing of Single Cell Clones (Inducible Gene Expression)

1. 1 mg/ml Doxycycline stock (1000×).

2. 24-well plates (for nonadherent cells) or 6-well plates (for adherent cells).
3. NP40 buffer (150 mM sodium chloride 1.0 % NP-40 (Triton X-100 can be substituted for NP-40) 50 mM Tris pH 8.0) with protease inhibitors (*see* **Note 4**).
4. RIPA buffer (150 mM sodium chloride 1.0 % NP-40 or Triton X-100 0.5 % sodium deoxycholate 0.1 % SDS (sodium dodecyl sulfate) 50 mM Tris, pH 8.0) containing protease inhibitors (*see* **Note 4**).
5. Western blot reagents and apparatus.
6. Antibodies for detecting your protein of interest.

3 Methods

Carry out all procedures in sterile conditions in a tissue culture cabinet. Cell culture media are warmed to 37 °C in a water bath (*see* **Notes 5** and **6**).

3.1 Lentiviral Production

1. The day before transfection, seed 1×10^6 HEK 293T cells in a 10 cm tissue culture plate for each transfection. Let adhere for a minimum of 16 h.
2. The following day, aspirate cell culture medium and replace with 9 ml fresh medium per well.
3. Label two 1.5 ml microfuge tubes for each transfection:

Tube A: 64 µl 2 M $CaCl_2$	Tube B: 500 µl 2×HBSS
3 µg pCMV delta R8.2	
1 µg pCMV-VSV-G	
5 µg pFTRE3G	
1 µg pF-EGFP	
Top up to 500 µl with sterile water	

4. Add contents of tube A to tube B in a drop wise manner while gently vortexing intermittently.
5. Incubate at room temperature for 30 min. A small precipitate may form.
6. Using a 1000 µl pipette, add the transfection mix drop wise to the cells in a 10 cm dish.
7. Incubate cells over night at 37 °C in a humified CO_2 incubator.
8. The next day, check under a fluorescent microscope for transfection efficiency (GFP positive cells).
9. Carefully aspirate supernatant. Add 6 ml of fresh medium to cells. Incubate for 24 h for maximum virion production.

10. After 24 h, recover the virion containing supernatant using a 20 ml syringe and remove cells and cellular debris using a 0.4 μm syringe top filter.
11. The virion containing supernatant can either be used immediately or stored at −80 °C for up to 6 months. We recommend to store the virion supernatant in small aliquots (1–2 ml) to avoid multiple freeze thawing.

3.2 Transduction of the Target Cell Line

3.2.1 Using Adherent Cells, e.g., MEFs

1. The day before infection, seed 5×10^5 MEFs into each well of a 6-well plate. Culture for 14–16 h at 37 °C in a humified CO_2 incubator.
2. The following day, add polybrene at 4 μg/ml to the virion containing supernatant (*see* **Note 7**).
3. Remove medium from MEFs and replace with 2 ml virion containing supernatant per well.
4. Spin the plates in a cell culture centrifuge at $1000 \times g$ for 45 min at 30 °C. Keep cells at room temperature for minimum 30 min after spin. Alternatively, MEF cells may be infected by culturing cells with virion supernatant for 2 h in the cell culture cabinet, no spin required (*see* **Note 8**).
5. Incubate the plates at 37 °C in a humified incubator with 10 % CO_2 for 6–14 h.
6. Aspirate and discard the supernatant. Replace with 2 ml fresh medium per well. *Critical*: Some cell lines do not tolerate polybrene for more than 6 h!
7. Split the cells when they reach 90 % confluence.

3.2.2 Using Suspended Cells, e.g., THP-1 Cells

1. Seed 2.5×10^5 cells in a volume of 1 ml into each well of a 6-well plate.
2. Add polybrene at 4 μg/ml to the virion containing supernatant. Add 1 ml of virion containing supernatant to 1 ml of cell suspension in each well.
3. Spin the plates in a cell culture centrifuge at $2000 \times g$ for 90 min at 30 °C. Keep cells for a minimum 30 min in the cell culture cabinet after spin.
4. Transfer cells to a 37 °C in a humified CO_2 incubator (10 %) and leave for 6–14 h.
5. We recommend a double infection for suspension cells: Spin cells at $300 \times g$ for 5 min at 30 °C, aspirate, and discard supernatant. Add 1 ml of fresh culture media and 1 ml of virion supernatant with polybrene. Repeat procedure described in **step 3**.
6. Incubate the plates for another 6–14 h at 37 °C in a humified incubator with 10 % CO_2. Collect cells from all wells in a 10 ml Falcon tube. Spin at $300 \times g$ for 5 min at room temperature.

Discard supernatant (to remove polybrene) and resuspend the pellet in 3 ml fresh medium.

7. Split the cells when they reach a density of 2×10^6 cells/ml.

3.3 Selection of Infected Cells

To test for infection efficiency, transduced cells can be selected with a marker that is included in the transfer plasmid. This can be either, as described here, resistance to a antibiotic such as puromycin or geneticin (G418), or a fluorescent marker. If a fluorescent marker is used, infected cells can be identified using a fluorescent microscope and cells can be sorted by Flow cytometry. Here, we describe a protocol using puromycin as the selection agent.

3.3.1 Using Adherent Cells, e.g., MEFs

1. Seed four wells of a 6-well plate with 2.5×10^5 transduced cells per well, let adhere for 14–16 h. As a control, seed four wells of nontransduced cells. If it is unknown what concentration of antibiotic efficiently kills the cell line used, it is recommended that a titration treatment of the cells to be carried out with the selected antibiotic. The optimal concentration kills 100 % of nontransduced cells in 3–4 days.
2. The next day, remove supernatant and add 2 ml fresh medium to each well.
3. Add puromycin at increasing concentrations (1 μg/ml, 2 μg/ml, 4 μg) to the cells. Leave one well of transduced and nontransduced cells as control.
4. After 48–72 h, check for cell death using microscopy. For numerical assessment, a FACS based cell death assay can be performed.
5. Choose the concentration where you observe near 100 % cell death in nontransduced cells.
6. Transduced cells from the corresponding concentration can be further cultured as a polyclonal population.
7. To maintain selection pressure on the cells, keep the cells culturing using 0.5–2 μg/ml of puromycin in the cell culture medium for the first week.

3.3.2 Using Suspended Cells, e.g., THP-1 Cells

1. Seed 1×10^6 cells in at least four wells of a 6-well plate in a volume of 2 ml/well. Also seed four wells of nontransduced cells as control.
2. Add puromycin at increasing concentrations (1 μg/ml, 2 μg/ml, 4 μg/ml) to the cells. Leave one well of transduced and nontransduced cells as control.
3. The next day, check for cell death using FACS.
4. Choose the concentration in which you observe near 100 % cell death in nontransduced cells.
5. Transduced cells from the corresponding concentration can be further cultured as a polyclonal population.

6. To selection pressure on the cells, keep the cells culturing using 0.5–2 μg/ml puromycin in the cell culture medium for the first week.

3.4 Isolation of Single Cell Clones

Selection of single cell clones is not essential, but can be favorable for consistent results. It is essential, that **at least three independent clones are selected and analyzed**. In particular when using inducible promoters, we encourage selection of single cell clones with minimal expression in the uninduced stage.

3.4.1 Using Adherent Cells, e.g., MEFs

1. Trypsinize and harvest the transduced, polyclonal cell population. Centrifuge at 300×*g* for 5 min at room temperature. Resuspend in 1 ml medium and count the cells using a hemocytometer.
2. Seed 1000, 100, or 10 cells each in a 10 cm plate. Let colonies form over the next few days by incubating at 37 °C in a humified incubator with 10 % CO_2. Check daily for colony formation.
3. Once the colonies are distinguishable, aspirate supernatant. Wash the cells with 10 ml PBS. Aspirate PBS and add 5 ml Trypsin solution.
4. Prepare ten wells of a 48-well plate with 200 μl medium per well.
5. Check the cells under a microscope. Once the cells start to lift from the plate, use a 200 μl pipette and sterile pipette tips to aspirate cells from individual clones. Hold the plate at an angle and start picking colonies from bottom end of the plate to top to avoid mixing cells. *Critical*: Colonies have to be picked as quickly as possible or they dry out! Resuspend each clone in one well of the prepared 48-well plate.
6. Expand the clones and test basal expression and induction of your gene of interest.

3.4.2 Using Suspended Cells, e.g., THP-1 Cells

For suspension cells, single clones can be isolated using either limiting dilution or culture in soft agar (*see* **Note 9**).

Limiting dilution

1. Harvest and count cells (from Subheading 2.6).
2. Dilute cells to 100 cells/10 ml and 200 cells/10 ml in fresh medium.
3. Seed 200 μl of the low density cell dilutions (100 cells/10 ml) in a U-bottom 96-well plate. Incubate at 37 °C in a humified incubator with 10 % CO_2. Check daily for cell growth.
4. After 1 week, determine how many wells are harboring cells. Select the cell dilution in which less than 50 % of the wells have a cell clone. Most of these clones are likely to originate from a single cell.
5. Prepare ten wells of a 48-well plate with 200 μl medium per well.

6. Select ten clones and transfer them to the 48-well plate.
7. Expand the clones and test basal expression and induction of your gene of interest.

Colony formation in soft agar

1. Prepare 50 ml of culture media and keep it in a 37 °C water bath.
2. Setup a water bath to 50 °C to keep agar solution.
3. Slowly heat a 3 % agar solution in a microwave. Stopping every 30 s to avoid spilling. Make sure all the agar is dissolved and solution is smooth and liquid. Equilibrate it in the 50 °C water bath until used.
4. Harvest cells (from Subheading 2.6), spin at 300×*g* for 5 min at room. Resuspend cells in RPMI/20 % FCS and count.
5. Dilute cells in media with 20 % FCS to a concentration of 2000 cells/ml.
6. Seed cells at 1000 (500 μl), 250 (125 μl), and 62 (30 μl) cells/well.
7. Add 5 ml of agar to 50 ml of warm RPMI to obtain a 0.3 % agar solution.
8. Quickly add 3 ml of the agar mix per well, swirling to mix cells and media. Avoid creating bubbles.
9. Let the agar set in the plate in the tissue culture hood with the lid open to avoid condensation on the lid.
10. Once set, place 7 ml of sterile water in the gaps between the wells of the plate. This maintains the plate humid and avoids agar from drying out.
11. Place plates back into the incubator and incubate for 10–15 days.
12. To culture single colonies, add 100 μl of fresh culture media containing 10 % FCS and necessary supplements in a U-bottom 96-well plate.
13. Collect single colonies with a 100 μl tip attached to a pipette to reduce the risk of contamination. Place tip in the 100 μl in a 96-well plate, pipetting gently up and down.
14. Place the 96-well plate in the incubator and culture for 5–7 days. Once colonies grow, expand to larger culture dishes for further analysis.

3.5 Testing of Single Cell Clones (Inducible Gene Expression)

1. For nonadherent cells, seed each clone in at least two wells of a 24-well plate (250,000 cells; 0.5 ml medium/well). Also seed nontransduced cells as a control.
2. Add 5–20 ng/ml doxycycline in one of the wells per clone; leave the other well untreated (*see* **Note 10**).

3. 4 h after addition of doxycycline, lyse the cells in 100 μl lysis buffer of choice (*see* **Note 11**).
4. Test expression of your protein of choice by Western blot analysis.
5. For adherent cells, these same steps are followed except 6-well plates are used instead of 24-well plates.

4 Notes

1. Transfer plasmids may contain markers themselves. They may have fluorescent markers such as GFP or Texas-Red or protein-tags such as HA or FLAG. The expression of those markers may be used to identify cells that have been successfully infected. Infected cells can be identified by Western blotting or flow cytometry and selection may be done by single cell sorting. HA and FLAG tags may also be useful for immunoprecipitation assays. Those markers may be dependent or independent of expression of the gene of interest.
2. Lentiviruses are very effective in infecting primary cells and this protocol is therefore highly suitable for immortalization of primary cells. Examples are immortalization of primary MEFs with the SV 40 large T antigen [9] or the generation of factor dependent myeloid cells from fetal liver progenitor cells using HoxB8 or HoxA9 [10, 11].
3. Addition of the cationic polymer polybrene has been shown to increase virion absorption in target cells [12]. Additionally we recommend centrifugation of supernatants onto cultured cells (spin infection) to maximize transduction rates.
4. For cell lysis, the buffer of choice may depend on whether the protein is cytosolic or nuclear. For cytosolic protein extraction, NP40 buffer (or equivalent) containing protease inhibitors and for nuclear protein extraction, RIPA buffer may be used.
5. All materials are to be kept sterile and only cell culture grade reagents and plastic ware are recommended. Media and solutions are stored at 4 °C and warmed to 37 °C before usage. Avoid freeze thawing of chemicals and reagents.
6. Appropriate waste management for any solutions and plastic ware containing, or in contact with lentiviral particles needs to be in place. In particular, solutions containing virions need to be inactivated using 4 % hypochlorite solution. All work is carried out in PC2 facilities or according to local regulations.
7. Thaw virion supernatant in a 37 °C water bath immediately before use.
8. Lentiviruses are temperature sensitive and should be used for infections at the optimal temperature of 30–32 °C. Higher

temperature may inactivate the virion and decreases efficiency of infection.

9. The limiting dilution method is relatively simple, but it is difficult to identify whether the clones originate from a single cell or from a small number of cells. Colony forming assay in soft agar is more elaborate but identifies single cell clones with higher accuracy.
10. If the gene of interest induces cell death, a cell death inhibitor (e.g., Q-VD-OPh for caspase-dependent apoptosis) can be added to the cell to avoid loss of protein due to cell death.
11. If the protein of interest is tagged with a fluorescent marker, protein expression should be tested by flow cytometry.

References

1. Boyd JM, Gallo GJ, Elangovan B, Houghton AB, Malstrom S, Avery BJ, Ebb RG, Subramanian T, Chittenden T, Lutz RJ et al (1995) Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. Oncogene 11(9):1921–1928
2. Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ (1996) BID: a novel BH3 domain-only death agonist. Genes Dev 10(22):2859–2869
3. Inohara N, Ding L, Chen S, Nunez G (1997) harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X(L). EMBO J 16(7):1686–1694
4. Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB (2001) BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev 15(12):1481–1486
5. Stewart SA, Dykxhoorn DM, Palliser D, Mizuno H, Yu EY, An DS, Sabatini DM, Chen IS, Hahn WC, Sharp PA, Weinberg RA, Novina CD (2003) Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA 9(4):493–501
6. Marini B, Kertesz-Farkas A, Ali H, Lucic B, Lisek K, Manganaro L, Pongor S, Luzzati R, Recchia A, Mavilio F, Giacca M, Lusic M (2015) Nuclear architecture dictates HIV-1 integration site selection. Nature 521(7551):227–231
7. Moujalled DM, Cook WD, Murphy JM, Vaux DL (2014) Necroptosis induced by RIPK3 requires MLKL but not Drp1. Cell Death Dis 5:e1086
8. Tevethia MJ (1984) Immortalization of primary mouse embryo fibroblasts with SV40 virions, viral DNA, and a subgenomic DNA fragment in a quantitative assay. Virology 137(2):414–421
9. Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, Benetatos CA, Chunduru SK, Condon SM, McKinlay M, Brink R, Leverkus M, Tergaonkar V, Schneider P, Callus BA, Koentgen F, Vaux DL, Silke J (2007) IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 131(4):682–693
10. Brumatti G, Salmanidis M, Kok CH, Bilardi RA, Sandow JJ, Silke N, Mason K, Visser J, Jabbour AM, Glaser SP, Okamoto T, Bouillet P, D'Andrea RJ, Ekert PG (2013) HoxA9 regulated Bcl-2 expression mediates survival of myeloid progenitors and the severity of HoxA9-dependent leukemia. Oncotarget 4(11):1933–1947
11. Salmanidis M, Brumatti G, Narayan N, Green BD, van den Bergen JA, Sandow JJ, Bert AG, Silke N, Sladic R, Puthalakath H, Rohrbeck L, Okamoto T, Bouillet P, Herold MJ, Goodall GJ, Jabbour AM, Ekert PG (2013) Hoxb8 regulates expression of microRNAs to control cell death and differentiation. Cell Death Differ 20(10):1370–1380
12. Davis HE, Morgan JR, Yarmush ML (2002) Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes. Biophys Chem 97(2–3):159–172