Protocol

# Lentiviral Vectors for Retrograde Delivery of Recombinases and Transactivators

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Lentiviral vectors pseudotyped with the rabies virus (RV) envelope glycoprotein efficiently infect via axon terminals to stably deliver transgenes to distant neurons projecting to an injection site, but the resulting expression levels are too low and variable for most neuroscientific applications. If used to deliver recombinases or transactivators, however, lentiviral vectors are excellent means of targeting projection neurons when used in reporter mice or in combination with a second virus to express "payload" transgenes at high levels. For retrograde infection of significant numbers of neurons, high virus titers are critical. Here we present reagents and a protocol for generating high-titer supernatants that can be concentrated 1000-fold for final titers in excess of $10^{10}$ infectious units per milliliter. We demonstrate the usefulness of these vectors by selectively targeting corticothalamic and corticotectal neurons for high-level expression of a fluorophore in knock-in reporter mice.



## MATERIALS

**It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.**

**RECIPE: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.**

### Reagents

Bleach (10%)

Cell culture medium for virus production <R>

Cell lines

- HEK 293T/17 (ATCC CRL-11268)
- 293T-Switch2, for titering Cre-expressing virus

  *This cell line strongly expresses the red fluorescent protein mRFP1 (Campbell et al. 2002) in the absence of Cre recombinase. Cre recombinase excises the mRFP1 gene so that the cell instead expresses enhanced green fluorescent protein (EGFP); see Discussion. This line can be requested from the authors.*

Dulbecco's phosphate-buffered saline (DPBS) (Life Technologies 14190-250)

Ethanol (70%)

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Lipofectamine 2000 transfection reagent (Life Technologies 11668-019)
Opti-MEM reduced serum medium (Life Technologies 11058-021)
Plasmid DNA, maxiprepped (see Discussion)

- pMDLg/pRRE (Addgene 12251) (expression vector for HIV-1 gag-pol [Dull et al. 1998])
- pRSV.Rev (Addgene 12253) (expression vector for HIV-1 Rev [Dull et al. 1998])
- pBOB-U-mCre or pBOB-CAG-SD-iCre

  *These are lentiviral transfer vectors with strong ubiquitous promoters (ubiquitin C [Schorpp et al. 1996] or CAG [Niwa et al. 1991]) driving expression of Cre recombinase. These previously unpublished plasmids may be requested from the authors of this protocol.*

- pCAG-B19GVSVGCD

  *This is an envelope protein expression vector with the strong CAG promoter (Niwa et al. 1991) driving expression of a fusion protein consisting of the ectodomain and transmembrane domain of the RV (SAD B19 strain [Conzelmann et al. 1990]) glycoprotein fused to the cytoplasmic domain of the vesicular stomatitis virus (VSV) (Indiana strain) glycoprotein (Rose et al. 1980). This previously unpublished plasmid may be requested from the authors of this protocol; alternatively, the similar pCAGGS-FuG-B may be requested from the authors of Kato et al. (2011).*

Poly-L-lysine (0.01%) (Sigma P4832)

*Add 100 mL of poly-L-lysine solution to a 500-mL bottle of DPBS and mix well. Store at 4°C until the expiration date of either component.*

Trypsin (0.05%) (Life Technologies 25300-120)



## Equipment

Biohazard disposal containers
Biosafety cabinet (e.g., NuAire NU-425-400)
Bottles (150-mL sterile, disposable; Thermo Scientific 455-0150)
Cell culture incubator (37°C, 5% $CO_2$) (e.g., Heracell 150, Thermo Scientific)
Cell culture plates (15-cm; Corning 353025)
Electronic pipette controller (e.g., Pipet-Aid XP; Drummond)
Filters (0.45-µm Stericup for 250 mL) (Millipore SCHVU02RE)

*We have found these to be much less prone to clogging than similar units from Nalgene, presumably because of a larger filter area.*

Filters (0.45-µm Steriflip for 50 mL) (Millipore SE1M003M00)

*These are very convenient for filtering small volumes (≤50-mL) of supernatant or other fluids.*

Lab coats (full-front, waterproof; Myriad 3009-0050)
Microscope (inverted, capable of imaging fluorophore[s] encoded by virus, e.g., Olympus IX-70)
Pipette tips (200-µL, with filters; Eppendorf 022491296)
Pipetter (200-µL; Eppendorf 3120000054)
Pipettes, serological (2-mL; VWR 53283-704)
Pipettes, serological (5-mL; Greiner Bio-One 606 160)

*These measure volumes up to 7.5 mL, so are convenient for transfection.*

Pipettes, serological (10-mL; Greiner Bio-One 607 180)

*These measure volumes up to 12.5 mL, so are convenient for 12-mL medium changes and splitting cells resuspended in 12 mL of total volume.*

Pipettes, serological (25-mL; Greiner Bio-One 760 180)

*These measure volumes up to 35 mL.*

Pipettes, serological (50-mL; Greiner Bio-One 768 180)

*These measure volumes up to 60 mL, so are convenient for applying 12 mL of fresh medium to each of five plates.*

Tubes (50-mL conical; Corning 352098)

## METHOD

*Ensure that institutional biosafety procedures are followed in all of the steps below. Typically, all steps involving open plates or tubes take place in a biosafety cabinet with waterproof Tyvek lab coats and gloves worn by personnel (procedures that also reduce the risk of contaminating cell cultures and viral preparations) following Biosafety Level 2 (or greater) guidelines. All solid waste in contact with virus (plates, tips, etc.) should be decontaminated with 70% ethanol before disposal in biohazard containers for autoclaving. All liquid waste should be disinfected with 10% bleach (in the aspirator receptacle or otherwise) before disposal.*

1. Twenty-four hours before cell transfection, add 5 mL of diluted poly-L-lysine to each of the fourteen 15-cm plates, shaking each plate until the bottom is covered in solution. Let sit for at least 15 min at room temperature.
2. After 15 min, aspirate the poly-L-lysine solution thoroughly. Add 20 mL of cell culture medium to each plate.
3. Split five near-confluent 15-cm plates of HEK 293T cells into the 14 plates.

   *Low-passage 293T cells can be split at a ~1:3 ratio for confluency the following day. Growth slows at higher passages, necessitating a more conservative split ratio; thus, using lower-passage cells is advised for transfections.*
4. The following morning, inspect the plates for confluency. If cells are not 95% confluent, wait several hours for increased confluency (if the schedule permits).
5. Working in a biosafety cabinet, add 49 mL of OptiMEM to one 50-mL conical tube and one 150-mL sterile bottle.
6. To the conical tube containing OptiMEM, add the plasmid DNA in the following quantities:

| Plasmid | Quantity to add (µg) | Quantity per plate (µg) |
|---|---|---|
| pBOB-U-mCre | 403 | 28.8 |
| pMD-L.g/p | 262 | 18.7 |
| pRSV.Rev | 101 | 7.2 |
| pCAG-B19GVSVGCD | 141 | 10.1 |

   Cap and invert several times to mix.
7. Aspirate the medium from each plate and wash with 10 mL of DPBS per plate, tilting the plates to rinse all normal medium off the plate walls before aspirating the DPBS. Replace the DPBS with 12 mL of Opti-MEM per plate and return the plates to the incubator. To efficiently perform this step by batch processing in stacks of four or five, remove one stack at a time from the incubator, aspirate the medium from all plates in the stack, and add 10 mL of DPBS to each plate using a 50-mL serological pipette, and so on.

   *Note that these 50-mL serological pipettes hold 60 mL, so a single pipette can be used to add 12 mL of medium to each of five plates. Despite the efficiency of such batch processing, for this many plates we recommend completing (or at least starting) the PBS washes and medium changes before beginning time-sensitive work with Lipofectamine (see Step 9).*
8. To the 150-mL bottle containing Opti-MEM, add 2.268 mL of Lipofectamine 2000 (162 µL/plate) and invert several times to mix. Incubate for 5 min at room temperature.
9. Following the 5-min incubation, add the contents of the DNA tube to the bottle and invert several times to mix. Incubate for 20 min at room temperature. Complete the DPBS washes and medium changes in the plates during this time, if not done already.
10. When the 20-min incubation is almost over, transfer all the plates from the incubator to the hood.
11. Following the 20-min incubation, add 7.1 mL of the transfection mixture to each plate using a 5-mL serological pipette. Distribute drops as evenly as possible across each plate. Agitate each plate gently in orthogonal directions immediately after adding the transfection mixture. After adding 7.1 mL to each plate, distribute any remaining mixture as evenly as possible between the plates.
12. Return the plates to the incubator for 5 h.





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13. Following the 5-h incubation, aspirate the Opti-MEM from each plate and replace with 12 mL of cell culture medium per plate.
14. The following morning, aspirate the medium from all plates and replace with 12 mL of cell culture medium per plate.

    *This step should be performed at the same time of day as is planned for collecting the supernatants on the following days.*

15. Twenty-four hours later (2 d after transfection), collect the supernatants from all plates and replace with 12 mL of cell culture medium per plate. Filter the pooled supernatant using a 0.45-µm Stericup filter and store at 4°C.
16. Twenty-four hours later (3 d after transfection), collect, pool, and filter the supernatants from all plates and store at 4°C. Discard plates.
17. Concentrate and purify the virus by ultracentrifugation (see Protocol: **Concentration and Purification of Rabies Viral and Lentiviral Vectors** [Sullivan and Wickersham 2014]). Resuspend the viral stock in 50 µL of DPBS per tube, for a final total volume of 300 µL (1/1000 the original volume of supernatant), or 60 × 5-µL aliquots. Titer the resulting concentrated stocks as described, using 293T-Switch2 cells, or other Cre-reporter cell line, instead of 293Ts.

## DISCUSSION

Retrograde delivery of Cre or other recombinases using lentiviral vectors can be a powerful method of selectively targeting projection neurons for transgene expression at high levels with minimal toxicity when used with either a reporter mouse line (e.g., Madisen et al. 2010) or a Cre-dependent reporter virus (e.g., Atasoy et al. 2008). Two examples of such retrograde targeting are shown in Figure 1. A vector made with the transfer vector pBOB-U-Cre and the envelope plasmid pCAG-B19GVSVGCD (both described above), with a titer of 1.2E10 infectious units per milliliter, was injected into either somatosensory thalamus (Fig. 1B; 50-nL injection) or superior colliculus (Fig. 1D; 100-nL injection) of mice of the Ai6 line (in which the fluorophore ZsGreen is expressed from the strong CAG promoter following Cre excision of a stop cassette). Seven days after the injections, numerous layer 6 corticothalamic cells in somatosensory cortex (Fig. 1A) were brilliantly labeled with ZsGreen; layer 5 corticotectal neurons were also brightly labeled in the case of the collicular injections, but in fewer numbers (Fig. 1C).

Even though expression levels resulting from retrograde infection by lentiviral vectors are too low for most neuroscientific applications, this approach is successful because low-expression levels of Cre recombinase are perfectly adequate for catalyzing recombination of lox sites for applications such as removal of stop cassettes from reporter alleles in knock-in mice. Retrograde infection with the lentivirus allows selective targeting of the class of projection neuron of interest, while the high expression of a fluorophore, calcium indicator, or opsin is provided by the Cre-dependent reporter allele or by a Cre-dependent virus injected in the region of the targeted neurons' somata. A similar combination of Cre-expressing lentivirus and Cre-dependent reporter AAV can be used to achieve high level but sparse expression (Franks et al. 2011); in that case the lentivirus is not rabies virus glycoprotein (RVG)-enveloped for retrograde targeting of projection neurons but instead simply packaged with the VSV envelope protein for local infection, with the two viruses coinjected in the region of interest. Adeno-associated virus (AAV) can also be used for retrograde delivery of Cre in reporter mice (Aronoff et al. 2010); the success of that approach is likely to depend on the choice of serotype. The self-deleting strategy of mitigating Cre toxicity (see the description of pBOB-CAG-iCre-SD below) would not be possible with AAV, however, as it depends fundamentally on the uniquely retroviral behavior of replacing the "long terminal repeat" (LTR) region at the 5′ end of the viral genome with a copy of the corresponding region at the 3′ end.

Because high titers are critical for retrogradely infecting neurons in appreciable numbers, several features of the current protocol should be emphasized. A key aspect is the use of a chimeric envelope

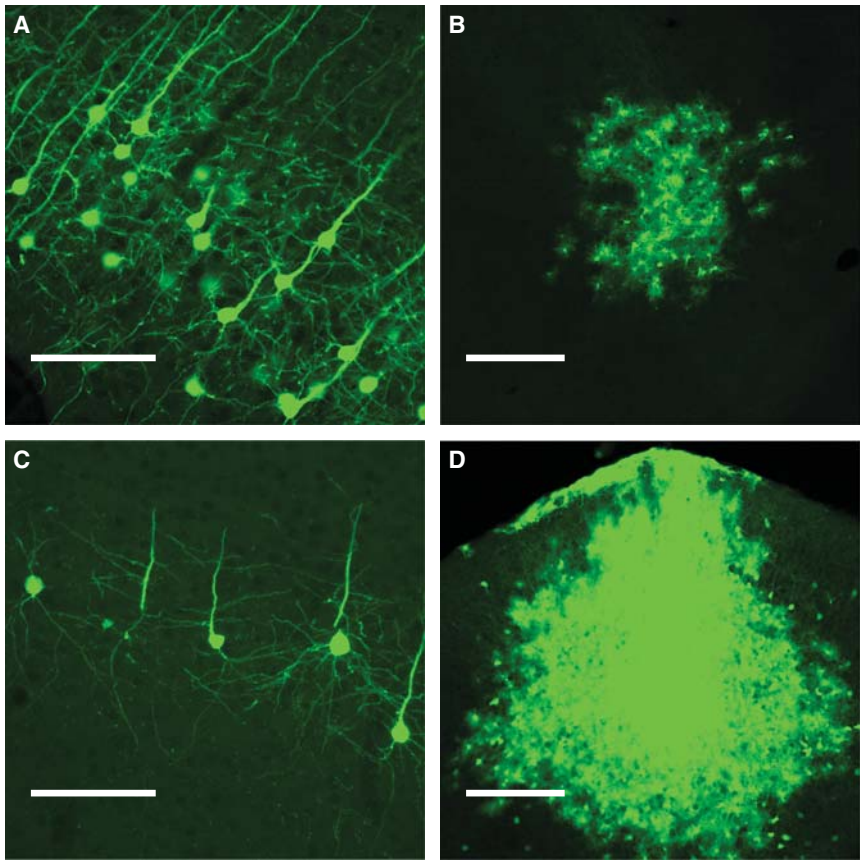


**FIGURE 1.** Retrograde infection of cortical projection neurons by LV-U-mCre in reporter mice. (*A*) Corticothalamic neurons in layer 6 of somatosensory cortex retrogradely infected from thalamus. (*B*) Thalamic injection site in the same animal as *A*. (*C*) Corticotectal neurons in layer 5 of primary visual cortex retrogradely infected from the superior colliculus. (*D*) Collicular injection site in the same animal as *C*. Scale bars: (*A*,*C*) 100 µm; (*B*,*D*) 200 µm.



glycoprotein consisting of the ectodomain and transmembrane domain of the RV glycoprotein but the cytoplasmic domain of the VSV glycoprotein (see below for more details). More pedestrian aspects are the use of low-passage cells, which increases transfection efficiency, and Lipofectamine 2000, which is expensive but highly reliable. Significant cost savings can be obtained by instead using calcium phosphate transfection (Tiscornia et al. 2006), but results in that case are sensitive to pH and therefore require careful optimization (Chen and Okayama 1987).

We introduce a number of previously unpublished reagents here, all of which are available from the authors by request.

## pCAG-B19GVSVGCD

This is a "reverse-engineered" version of a similar expression vector described in Kato et al. (2011), with the strong CAG promoter driving expression of a chimeric glycoprotein consisting of the extracellular and transmembrane domains of the RV glycoprotein and the cytoplasmic domain of the VSV glycoprotein. The authors' trick of replacing the cytoplasmic tail of the RV envelope glycoprotein gene (G) with that of G from the VSV results in markedly increased titers—in our hands, by a factor of 10 above those obtained with either an otherwise-identical plasmid encoding the unmodified RV glycoprotein ("pCAG-B19G," also available from the authors on request) or another expression vector with the same unmodified RV glycoprotein gene driven by the cytomegalovirus (CMV) promoter (Sena-Esteves et al. 2004). Our version uses the sequences from the Street Alabama Dufferin (SAD) B19 strain of RV (Conzelmann et al. 1990) and the Indiana strain of VSV (Rose and Gallione 1981).


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### pBOB-U-mCre

This lentiviral transfer vector (derived, like the other transfer vectors described here, from pCSC-SP-PW-GFP [Marr et al. 2004; Addgene 12337]) uses the mouse ubiquitin C promoter (Schorpp et al. 1996) to drive expression of a mouse codon-optimized version of the Cre recombinase gene (Koresawa et al. 2000).

### pBOB-CAG-iCre-SD

Although toxicity due to Cre overexpression seems unlikely in retrogradely infected neurons, it is at least a theoretical possibility in cells at the injection site, which could in turn mediate plasticity in the neurons that project to it. This "self-deleting" lentiviral transfer vector is designed to address any such concerns by causing expression of Cre only transiently. Because of an loxP site introduced into the 3′ long terminal repeat, which is copied onto the 5′ LTR in the early stages of infection, the provirus that integrates into the host cell genome has loxP sites flanking the expression cassette. The initial burst of Cre expression therefore results in excision of the expression cassette itself. This vector is improved in several ways with respect to the previously published LV-Cre-SD (Pfeifer et al. 2001; Addgene 12105): it contains a mammalian codon-optimized version of the Cre gene (Shimshek et al. 2002), and the CAG promoter is substituted for the CMV promoter, which allows the plasmid to be better grown in bacteria and also results in higher expression in mammalian neurons (Matsuda and Cepko 2004). On the other hand, matched preparations in pilot studies yielded titers (on 293T-Switch2 cells) sevenfold lower with pBOB-CAG-iCre-SD than with pBOB-U-mCre.

### pBOB-CAG-Switch

This lentiviral transfer vector contains the CAG promoter followed by two transgenes, the first encoding the red fluorescent protein mRFP1 (Campbell et al. 2002) and flanked by tandem loxP sites, and the second encoding EGFP (Yang et al. 1996), which is only expressed upon Cre-mediated deletion of the preceding floxed mRFP1 cassette. Lentivirus made with this transfer vector was used to create the cell line 293T-Switch2.

### 293T-Switch2

This cell line expresses the red fluorescent protein mRFP1 (Campbell et al. 2002) in the absence of Cre recombinase. Cells infected by a virus expressing Cre cease mRFP1 expression and instead brightly express EGFP. This line was designed for titering Cre-expressing viruses.

## RECIPE

#### *Cell Culture Medium for Virus Production*

Prepare 10-mL aliquots of antibiotic-antimycotic (100×) (Life Technologies 15240-062) in 15-mL conical tubes and store at −20°C. To prepare cell culture medium, add 100 mL of fetal bovine serum (characterized; Thermo Scientific SH30071.02) and 10 mL of antibiotic–antimycotic to a 1000-mL bottle of Dulbecco's modified Eagle medium (DMEM) (high glucose; Life Technologies 11995-081) and mix well. Store at 4°C for up to several months.

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