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Massachusetts Institute of Technology

# Production of glycoprotein-deleted rabies viruses for monosynaptic tracing and high-level gene expression in neurons

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**Recombinant rabies viruses rendered replication-deficient by the deletion of their envelope glycoprotein gene are useful tools for neuroscientists, permitting (1) extraordinarily high transgene expression levels within neurons, (2) retrograde infection of projection neurons through their axon terminals, (3) targeted infection of genetically specified neurons and (4) monosynaptic tracing of neuronal inputs. Here we present a detailed protocol for the production of high-titer and high-purity viral stocks, from initial generation of infectious virus from cDNA through amplification on complementing cell lines, pseudotyping if desired, purification by ultracentrifugation and titering. The procedure requires 3–4 weeks to complete.**

## INTRODUCTION

Wild-type rabies virus is already a highly effective tool for neuroscientists. A pathogen specialized for spread within and between mammalian nervous systems, it infects peripheral nerve terminals at the site of a bite and proceeds through the central nervous system from neuron to neuron. This spread appears to be specifically trans-synaptic, with no spread observed between nearby but unconnected cells, and exclusively retrograde, from postsynaptic to presynaptic cell[1–4]. Although rabies virus eventually causes neuronal dysfunction underlying the neurological symptoms that are essential to its life cycle, this is not accompanied by widespread cell death, and neuronal structures remain intact even at postmortem examination[5]. This contrasts with the marked cytopathicity of the other group of neurotropic viruses used for trans-synaptic tracing, the α-herpesviruses such as herpes simplex virus type 1 and pseudorabies virus[6–8]. This combination of transsynaptic specificity and low cytopathicity makes rabies virus in its unmodified form an already excellent tool for identifying circuits of synaptically connected neurons when inoculated into a muscle or directly into the nervous system[2,9,10]. However, working with wild-type virus is limiting in several ways. One of these is the poor anatomical resolution afforded by the only way of visualizing infected neurons: immunohistochemical staining for viral protein. A more serious problem is the intrinsic asynchrony of trans-synaptic spread. Tracing viruses, and trans-synaptic tracers more generally, cross stronger, more densely innervating connections faster than weaker and sparser ones[1,7,11,12]. Because they also continue spreading through any infected neuron, they can traverse strong polysynaptic connections faster than weak monosynaptic ones, so that the two situations cannot be distinguished[1,12].

Construction of custom recombinant forms of the virus became possible only in 1994, with the report by Schnell *et al.*[13] of the first successful 'rescue' of an infectious rabies virus from a cDNA copy of its genome. This surprisingly late entry into the world of genetic technology is a result of the problematic nature of the genome of rabies virus (genus *Lyssavirus*, family *Rhabdoviridae*) and of the nonsegmented, negative-strand RNA viruses (order *Mononegavirales*) more generally. Although the herpesviruses, for example, possess immediately manipulable (if inconveniently large) DNA genomes, and the RNA genomes of retroviruses naturally pass through an intermediate proviral DNA phase, the rabies virus genome remains in RNA form throughout its entire replication cycle. Generation of rabies virus from a DNA copy—the requirement for practically manipulating its genome—presented significant technical challenges, and its achievement was a major breakthrough. Most importantly, it enabled the immensely valuable reverse-genetic study of the virus itself, but a considerable benefit for neuroscientists is simply the ability to include marker genes in viruses that are used as tracers[14,15].

Recombinant technology enables not only adding genes but deleting them, too, for the purpose of creating replication-deficient vectors for transgene delivery. The single-stranded RNA rabies virus genome contains only five genes, termed *N*, *P*, *M*, *G* and *L*[16]. *N* encodes the nucleocapsid protein, which sheathes the genome along its entire length and is wound with it into a tight helix within the bullet-shaped viral particle. *P* and *L* encode two more viral proteins, the phosphoprotein and the 'large' protein, which are bundled with the nucleocapsid and together form the viral polymerase, transcribing its genes and replicating its genome cytoplasmically and independently of any host cell polymerase. A matrix protein (*M* gene), as well as playing roles regulating transcription and replication, interfaces between the nucleocapsid and the phospholipid bilayer membrane that surrounds the virion. This membrane, or envelope, is studded with trimeric 'spikes' of the fifth and final viral protein, the glycoprotein, encoded by G.

Deleting the glycoprotein gene transforms the virus into a single-round vector unable to spread beyond initially infected cells[4,14,17,18]. The glycoprotein is responsible for binding to receptors on the surface of a host cell, which results in the endocytosis of the virus and its retrograde transport along the axon, then fusing the membranes of the virus and the endocytic vesicle to release the viral core into the cytoplasm. In the absence of the glycoprotein gene, the few viral particles that are still produced are completely noninfectious[17]. However, if, as described in this protocol, a G-deleted virus is grown in cells that express the viral glycoprotein *in trans*, the resulting virions will bear the glycoprotein on their membranes

despite lacking its gene in their genomes. These particles can infect neurons but, with no means of synthesizing glycoprotein inside them, are unable to produce infectious progeny. Although a similar replication-deficient virus could also be created by deleting any of the other four viral genes, deleting the glycoprotein gene has several major advantages.

The first advantage of deleting G is that it produces a vector that expresses transgenes at extremely high levels[14]. This is presumably because this particular deletion leaves the gene transcription and replication machinery of the virus intact, so that, although the virus cannot spread beyond initially infected cells, it can still replicate within it, expressing its genes and any transgenes as it does so. In fact, as an unexpected bonus, deleting G not only allows unfettered gene expression by the intracellularly replication-competent virus but actually increases the level of transgene expression severalfold above that caused by an otherwise-identical non–G-deleted rabies virus[19]. The spectacular expression levels that ensue, combined with the ability to infect distant projection neurons by way of their axon terminals at an injection site, make G-deleted viruses ideally suited for retrogradely labeling neurons for anatomical study, with fluorophore expression vivid enough for visualizing and tracing the fine processes of dendrites and axons[14,20,21].

A second advantage of G deletion is that it removes the major source of cytotoxicity[5]. Despite the preserved intracellular replication of the virus, infected neurons remain alive and available for anatomical or physiological study for an extended time, with no widespread mortality found until ~16 d postinfection[14]. This is also partly due to the naturally 'stealthy' replication strategy of rabies virus, which depends on maintaining neuronal viability as it spreads through the brain[22]. This ability to conduct a full-scale intracellular infection without swiftly annihilating its host cell is a distinctive specialty of rabies virus: a G-deleted version of the closely related vesicular stomatitis virus, for example, has recently been shown to cause impressively fast and potent transgene expression in neurons, but the infected cells begin dying within 1–2 d (ref. 23).

A final advantage of deleting the glycoprotein gene is that it gives the investigator the ability to genetically target infection to particular neurons. Because the virus no longer produces its own glycoprotein, one from a different virus can be substituted on its envelope: so-called pseudotyping. To do this, the virus is simply grown in cells that express the glycoprotein from another virus, with the glycoprotein's cytoplasmic domain suitably modified for compatibility with the other rabies virus proteins. The resulting virus bears the foreign glycoprotein on its envelope[4,24], conferring the tropism of the donor virus upon the rabies virus. Rabies virus pseudotyped with EnvA, the envelope protein of the avian retrovirus ASLV-A, has virtually no ability to infect mammalian neurons unless they have been specifically engineered to express its receptor, the avian cell-surface molecule TVA[4]. This allows specific infection of particular target neuronal populations of interest.

In summary, G-deleted rabies viruses are gene-delivery vectors that: (1) cause transgene expression at extremely high levels while (2) allowing host neuron viability for up to several weeks and that (3) can be flexibly targeted to infect only neurons of interest to the investigator. Whether delivered by retrograde infection or by genetic targeting, the G-deleted virus is a replication-deficient vector that will not spread beyond the initially infected neurons; that is, unless the glycoprotein is supplied *in trans* within them. If so, with all its genes present, the virus can once again produce infectious progeny, which will spread from these complementing cells to the neurons directly presynaptic to them. Because the virus will not spread beyond these cells if they do not themselves express G, the result is a monosynaptic tracer, unambiguously labeling directly connected neurons[4].

This protocol describes the production of G-deleted rabies virus, with its membrane bearing either its endogenous glycoprotein for retrograde infection or the ASLV-A glycoprotein EnvA for genetic targeting, at high titers for neuroscientific investigations *in vivo*. High titers are crucial for the success of most experiments, not because of increased expression levels—infected neurons will inevitably become fully infected owing to the intact intracellular replication of G-deleted virus—but because the titer will determine the number of infected cells. For virus bearing its native glycoprotein (referred to below as 'unpseudotyped' virus), higher titers translate directly into greater numbers of neurons retrogradely infected by an injection of a given volume. For EnvA-pseudotyped virus, the higher the titer, the greater the chance of infecting target TVA-expressing neurons. This protocol differs considerably from previously published ones for making G-deleted rabies virus and results in titers higher than those that have previously been reported. This is primarily because of the steps from *Amplifying stocks* (Step 13) onward rather than the initial rescue steps, which vary relatively little from those in the method described by Faul *et al.*[25] but are included here for the sake of completeness. In the case of EnvA-pseudotyped rabies virus, no protocol for making concentrated stocks has been published previously.

### Experimental design

**Figure 1** is a flowchart of the sections of the protocol, proceeding from *Rescue from cDNA* to *Amplifying stocks*, then *Producing supernatant for ultracentrifugation*, with two options depending on whether unpseudotyped or EnvA-pseudotyped virus is being produced, followed by *Concentrating by ultracentrifugation*. *Titering*, as described in **Box 1** (see also **Fig. 2**), is carried out at several

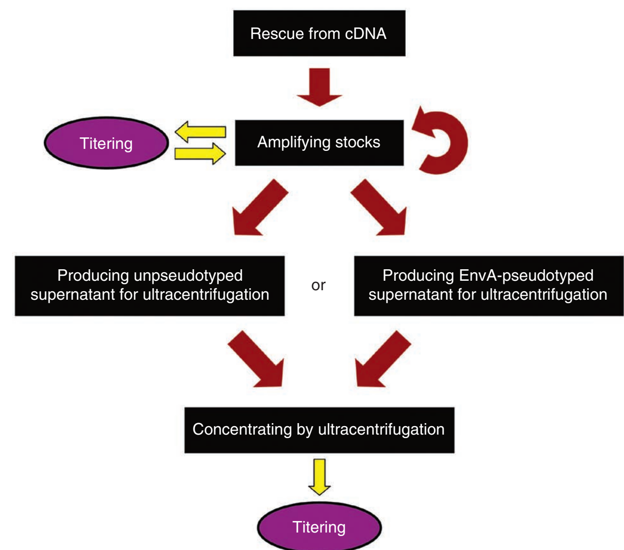


**Figure 1 |** Flow diagram of the protocol. *Amplifying stocks* can be repeated as needed. The steps described in **Box 1** are repeated several times throughout the protocol.

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## BOX 1 | TITERING

Any of several titering techniques could be used for viruses discussed in this protocol. The focus-forming unit assay usually used to titer replication-competent viruses without fluorophores[36] could be adapted for use here if complementing cells such as B19G2 cells are used. The method described below uses flow cytometry ('FACSing' below) to count fluorescent cells, although manually counting fluorescent cells is an obvious alternative. Flow cytometry requires the additional investment of resuspending the cells and is more expensive but provides greater accuracy and makes the counting itself trivial, saving time on balance when analyzing more than a small number of samples. We include details such as fluid volumes for the serial dilutions that would work for either technique and that in any case can safely be varied appropriately.

We use 293T cells for titering as (a) routinely titering on neurons would be impractical and (b) the titers for B19G-pseudotyped virus do not differ much between 293T cells and BHK cells, although the fluorescence level of infected 293T cells, within the first few days at least, is higher than that of infected BHK cells, so that infected cells are more easily distinguished from the uninfected ones.

(1) Day I: Plate cells in 24-well plates at $3\times10^5$ cells per well, 0.5 ml medium each, 5 wells per sample.

If titering unpseudotyped virus, use 293T cells for all wells. Plate two extra wells, one for counting at the time of virus application (day 2) and one for a negative control when carrying out flow cytometry.

If titering an EnvA-pseudotyped virus, use 293T cells for the 'zeroth order' well and 293T-TVA800 cells, which express the EnvA receptor TVA, for all other wells. Plate two extra wells of TVA-expressing cells (one for counting at the time of virus application and one for a flow cytometry blank). Also plate three extra wells of 293T cells: one to count at the time of virus application, one for a possible flow cytometry blank (if any of the wells have sufficiently numerous fluorescent cells to warrant carrying out flow cytometry) and one to count at the time of the manual count of fluorescent cells (done at the same time as carrying out flow cytometry on the other wells).

(2) Day II: Infect titer wells:

Make up serial tenfold dilutions in 1.5 ml tubes (or similar), with the following suggested volumes:

For unconcentrated supernatants:

| Log dilution | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Initial medium volume (µl) | 165 | 297 | 297 | 297 | 297 |

Then transfer into the tubes, sequentially from left to right and mixing each tube thoroughly:

165 µl sup, 33 µl '0', 33 µl '1', 33 µl '2' and 33 µl '3'.

Then count the cells in one of the extra wells using a hemocytometer and apply 250 µl of each dilution to the corresponding titer well, giving applied virus volumes of:

125 µl, 12.5 µl, 1.25 µl, 0.125 µl and 0.0125 µl.

For stocks that have been ultracentrifugated:

| Log dilution | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Initial medium volume (µl) | 328.35 | 297 | 297 | 297 | 297 |

Then transfer sequentially from left to right, mixing thoroughly:

1.65 µl stock, 33 µl '0', 33 µl '1', 33 µl '2' and 33 µl '3'.

Then count the cells in one of the extra wells and apply 250 µl of each dilution into the corresponding titer wells, giving applied virus volumes of:

1.25 µl, 0.125 µl, 0.0125 µl, 0.00125 µl and 0.000125 µl.

Again, in the case of an EnvA-pseudotyped virus, the zeroth-order wells will be a cell type (293T) that is different from those in the other dilution orders.

(3) Day III: Aspirate wells from right to left (i.e., from higher- to lower-order dilutions; in the case of an EnvA-pseudotyped virus, do not contaminate the zeroth-order 293T wells with a tip used in the higher-order 293T-TVA800 wells). Replace all supernatants with 0.5 ml of medium. The interval between virus application and medium change should ideally be kept consistent—e.g., at 20 h.

(4) Day IV or V: Prepare cells for fluorescence-activated cell sorting (FACS): for each sample, prepare a labeled 5-ml polystyrene tube containing 0.5 ml 2% paraformaldehyde (see REAGENT SETUP). Proceeding again from higher- to lower-order dilutions to conserve tips and time, aspirate medium, wash with ~0.25 ml Dulbecco's phosphate-buffered saline (DPBS) (this can even be omitted as 293T cells adhere loosely) and apply ~0.2 ml trypsin per well, followed by ~0.3 ml medium to stop the reaction. The exact volumes of these fluids are not important. However, it is essential not to let the trypsinization proceed too long (more than ~1.5 min), as this will cause the cells to clump and clog the cytometer. After addition of the medium, triturate the cells well with a 1,000-µl tip and squirt them into the appropriate 5-ml tube containing fixative. Once the tubes are prepared for FACS analysis, cap tightly and invert the rack holding the tubes several times to ensure that the fixative is thoroughly mixed with the resuspension.

■ **PAUSE POINT** Tubes can be stored at 4 °C for up to 3 d before FACS analysis.

Carry out FACS analysis on the cells and determine the percentage of fluorescent cells for each tube, as illustrated in **Figure 2**.

In the case of an EnvA-pseudotyped virus, only FACS the 293T-TVA800 wells. The 293T (i.e., zeroth order) wells should have too few fluorescent cells to be reliably detected by FACS. For these wells, manually count the number of fluorescent cells using the inverted

## BOX 1 | CONTINUED

fluorescence microscope. At the same time, determine the total number of cells present in the 293T wells by dissociating the cells in the extra well and counting them using a hemocytometer.

The titer (in infectious units, or IU) can be determined using the following formula:

$$\text{Titer (IU ml}^{-1}) = -\ln(P_{\text{uninfected}}) \times n / v,$$

where $P_{\text{uninfected}}$ is the fraction of uninfected cells, $n$ is the number of cells in each well at the time when the virus was added to the wells and $v$ is the volume in milliliters of virus stock that was added to the well. The Google search box is a convenient means of evaluating this expression. As an example, assume a concentrated stock of EnvA-pseudotyped virus is titered on 293T-TVA800 cells. The number of cells at the time of virus application was $3.0\times10^5$, and 4.03% of cells in the second-order well (which had received 0.0125 µl of virus) were fluorescent. Type into the Google search box (to avoid typos, paste in the following line and modify appropriately):

$$-\ln((100-4.03)/100)\times 3.0E5/(1.25E-2/1{,}000)$$

to give a titer of $9.9\times10^8$ IU ml$^{-1}$.

The natural logarithm is meant to account for multiple infections of the same cell but is not perfect. The titer calculated from the different dilution wells should be in approximate agreement at low dilution orders. As a standard procedure, we take the value for the well with between 1% and 10% fluorescent cells as the titer for the virus.

For manually counted cells, e.g., 293T cells infected by an EnvA-pseudotyped virus, $P_{\text{uninfected}}$ is given by $P_{\text{uninfected}} = 1 - (n_{\text{fluorescent}}/n_{\text{total}})$, where $n_{\text{total}}$ is the hemocytometer-counted number of cells per well at the time of the fluorescence count. However, the $n$ that is used elsewhere in the formula remains the number of cells per well at the time of the virus application. As an example, consider the zeroth-order well of the above example virus, consisting of 293T cells. The number of cells at the time of virus application was determined to be $3.2\times10^5$, and the well in question received 1.25 µl of virus. After 3 d, the total number of cells in a second control well was found to be $1.7\times10^6$, and the number of fluorescent cells was found to be 127. The titer on 293T cells would therefore be computed as:

$$-\ln(1-(127/1.7E6))\times 3.2E5/(125E-2/1{,}000)$$

or $1.9\times10^4$ IU ml$^{-1}$.

stages. Completing the protocol in minimal time entails overlapping the stages, so that the first steps of *Amplifying stocks*, for example, take place before the last steps of *Rescue from cDNA*. Throughout the protocol we have suggested days on which to carry out the various steps and indicated where they can be postponed if necessary.

### Rescue from cDNA

The rescue protocol described here derives from that of Faul *et al.*[25], which is descended with several improvements from the original rescue technique described by Schnell *et al.*[13]. Briefly, a genome vector containing a cDNA version of the intended viral genome is transfected into cells along with expression vectors for the viral genes N, P and L (and G, in the case of a G-deleted genome). The phage T7 RNA polymerase is used both to generate RNA transcripts of the genome (in antisense orientation, to prevent hybridization with the mRNA of the helper plasmids' genes) and also to drive expression of the helper genes. In the original version, the T7 polymerase was provided by using a constitutively expressing cell line; Faul *et al.*[25] simply transfect a T7 expression vector along with the other plasmids, as we do here. The original helper plasmids were soon replaced[26,27] by versions containing an internal ribosome entry site (IRES) for increased expression levels; these are the pTIT plasmids described below. A significant improvement to the genome vector, first used in rabies virus rescue by Inoue *et al.*[28], was the inclusion of a self-cleaving RNA sequence, the hammerhead ribozyme, between the T7 promoter and the start of the genome. The genome vector of Faul *et al.*[25] and, by extension, the G-deleted versions we describe here incorporate this improvement.

The rescue steps of this protocol deviate from Faul *et al.*[25] only in providing more detail and using (1) the widely available and easily transfectable 293T cells instead of the BSR cell line, (2) Lipofectamine 2000 instead of FuGene, (3) a slightly different T7 polymerase expression vector and (4) a much larger plate, 15 cm

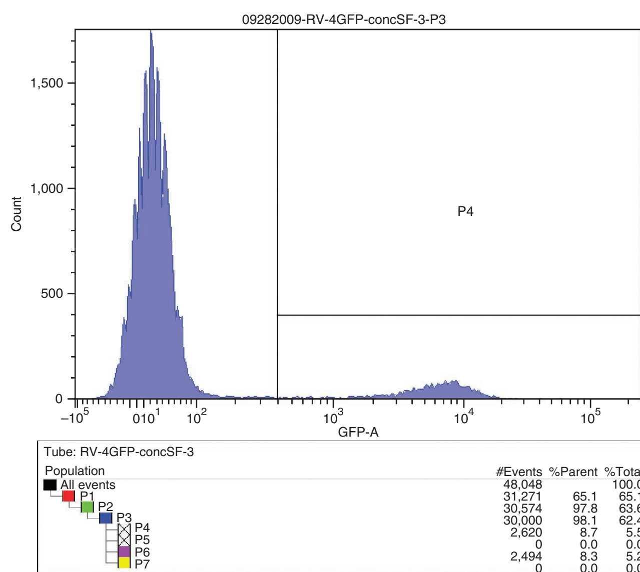


**Figure 2 |** Flow cytometry screen snapshot. In total, 8.7% of the cells in this third-order well were fluorescent, with 0.00125 µl of virus having been applied to $4.975\times10^5$ cells. The virus titer was therefore calculated to be $3.6\times10^{10}$ IU ml$^{-1}$ (for further details, see **Box 1**).

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versus 3.5 cm, to increase the likelihood of success. If reagents are limiting, one can use a smaller plate. Investigators introducing other transgenes into the viral genome could use the genome vector cSPBN-4GFP as a control.

**Amplifying stocks**

Despite the improvements described above, the unnatural process of rescuing rabies virus from cDNA remains an extremely low-efficiency endeavor in which only a tiny minority of transfected cells will produce infectious virus. The initial stocks obtained must therefore be amplified by passaging on a complementing cell line (BHK-B19G2 or similar) until titers and quantities are high enough for subsequent use. The immediate goal is to prepare a large number of stock tubes to be stored in the freezer and used as starting stock for each large-scale prep. This is necessary when starting either with the transfection supernatants from the rescue steps or with a tube of virus obtained from another investigator. As with cell lines, viruses accumulate mutations with repeated passaging, so this should be kept to a minimum and the passage number of stocks should be recorded. Another consideration unique to viruses is the accumulation of defective interfering particles: mutant virions with truncated genomes that are unable to replicate in the absence of nondefective virus but that have a large replication advantage in co-infected cells[29,30]. The classical approach to preventing their accumulation is to passage only at very low multiplicity of infection (MOI, or the number of infectious units per cell) to avoid co-infection. However, this results in titers impractically low for our purposes. In our hands, defective interfering particles do not appear to be a significant problem in stocks generated in the few passages after rescue as described in this protocol, even at the relatively high MOI of 1. However, investigators should keep in mind that this might not be appropriate for repeatedly passaged virus passed between laboratories and that beginning with the lowest-passage virus rescued from cDNA is preferable. For the final passage to generate virus to be concentrated by ultracentrifugation, ensuring that the majority of cells are infected is a paramount consideration, so we use the slightly higher MOI values of 3–5 (for making unpseudotyped virus) and 2 (for EnvA-pseudotyped virus), as described below.

**Producing supernatant for ultracentrifugation**

This consists of a final passage on complementing cells to produce a final supernatant of just over 150 ml for ultracentrifugation. A higher MOI than on previous passages is used to increase titers in this supernatant. For making EnvA-pseudotyped virus, the BHK-EnvARGCD2 line ('EnvA2 cells') is used instead of the G-expressing line BHK-B19G2 ('B19G2 cells'), and several steps are taken to minimize the remnants of the unpseudotyped virus used to infect them initially. For yet-higher titers, twice as many plates can be used and the supernatants ultracentrifugated consecutively in the same tubes.

**Concentrating by ultracentrifugation**

The ultracentrifugation procedure used here evolved from a lentiviral protocol[31] that works well with rabies virus. Modifications that we have introduced include (1) combining the two spins into one, so that the supernatants are layered onto sucrose directly, and (2) dispensing with trituration in favor of the gentler procedure of overnight shaking at 4 °C to resuspend the pelleted virus.

## MATERIALS

### REAGENTS

- Cell lines:
  - HEK-293T/17 cells (ATCC, cat. no. CRL-11268)
  - BHK-B19G2
  - BHK-EnvARGCD2
  - 293T-TVA800 cells. The 293T-TVA800 (ref. 32) cell line can be requested from Dr John Young (jyoung@salk.edu). The BHK-B19G2 and BHK-EnvARGCD2 lines can be requested from the authors.
- Plasmids (if rescuing virus from cDNA):
  - Genome vector, such as cSPBN-4GFP (GenBank, accession number GU299211)
  - pTIT-N (GenBank, accession number GU299212)
  - pTIT-P (GenBank, accession number GU299213)
  - pTIT-G (GenBank, accession number GU299214)
  - pTIT-L (GenBank, accession number GU299215)
  - pCAGGS-T7 (GenBank, accession number GU299216)

  At present, these plasmids are still only available by request from individual labs. The rabies virus protein expression vectors pTIT-N, -P and -L[26], pTIT-G[27] and the G-deleted genome vector pSADΔG-Ppu[14] can be requested from Dr Karl-Klaus Conzelmann (conzelma@lmb.uni-muenchen.de). The genome vector cSPBN[33]—with convenient restriction sites flanking the glycoprotein gene, but not itself a glycoprotein deletion mutant—can be requested from Dr Matthias Schnell (matthias.schnell@jefferson.edu). The G-deleted version cSPBN-4GFP, derived from cSPBN by replacement of the glycoprotein gene with that of the enhanced green fluorescent protein[34], can be requested from the authors. The T7 RNA polymerase expression vector pCAGGS-T7 (ref. 35) can be requested from Dr Robert Lamb (ralamb@northwestern.edu), or the nearly identical pC-T7 can be requested from Matthias Schnell.
- Starting stock of unpseudotyped G-deleted rabies virus (if not rescuing virus from cDNA but only propagating from existing stock)
- Dulbecco's phosphate-buffered saline (DPBS) (Gibco, cat. no. 14190)
- 10× Dulbecco's phosphate-buffered saline (DPBS) (Gibco, cat. no. 70011)
- Trypsin 0.05% (Gibco, cat. no. 25300)
- Dulbecco's modified Eagle's medium (DMEM), high glucose (GIBCO, cat. no. 11995)
- Fetal bovine serum (FBS) (Hyclone, cat. no. SH30071.02)
- 100× antibiotic–antimycotic (Gibco, cat. no. 15240)
- 95% ethanol (e.g., Pharmco-AAPER, cat. no. 111000190) **! CAUTION** Ethanol is flammable. Avoid exposure to ignition
- Poly-L-lysine 0.01% (Sigma, cat. no. P4832)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668-027) if rescuing virus from cDNA
- OptiMEM I (Gibco, cat. no. 11058) dilution medium for Lipofectamine 2000
- Dimethyl sulfoxide (DMSO) (EMD, cat. no. B10323-74), for freezing cells **! CAUTION** DMSO is toxic, carcinogenic, teratogenic, volatile and readily absorbed through the skin.
- 16% Paraformaldehyde (PFA), 10 ml ampoules (Ted Pella, cat. no. 18505) **! CAUTION** Causes skin burns and eye damage. Avoid dust generation by evaporation.
- Sucrose (Sigma, cat. no. S9378)

### EQUIPMENT

- Biosafety cabinet (e.g., NuAire, cat. no. NU-425-400) **! CAUTION** When handling laboratory strains of rabies virus, the use of a class II biosafety cabinet and waterproof infectious disease gowns as well as the usual biosafety level 2 personal protective equipment is typically mandated—see **Supplementary Note 1**.
- Incubator, cell culture
- Microscope, inverted (e.g., Olympus, IX-70) with fluorescence lamp and appropriate filters for the fluorophore being used (e.g., EGFP)

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- Gloves, disposable
- Water bath, set to 37 °C, for thawing frozen cells and virus
- Tabletop centrifuge for pelleting cells when freezing or thawing
- Pipette gun
- Measuring pipettes (sterile, individually wrapped)
  - 2 ml (VWR, cat. no. 53283-704) (Note: All VWR catalog numbers are North American versions.)
  - 5 ml (VWR, cat. no. 53283-706)
  - 10 ml (VWR, cat. no. 53283-708)
  - 25 ml (VWR, cat. no. 53283-710)
- 15 ml conical-bottom tubes (BD Falcon, cat. no. 352196)
- 50 ml conical-bottom tubes (BD Falcon, cat. no. 352098)
- 24-well tissue culture plates (BD Falcon, cat. no. 353047)
- 10-cm tissue culture plates (BD Falcon, cat. no. 353003)
- 15-cm tissue culture plates (BD Falcon, cat. no. 353025)
- Cryogenic tubes, 1.2 ml (sterile) (VWR, cat. no. 16001-100) for freezing cells
- Liquid nitrogen refrigerator (tank) (e.g., Taylor-Wharton, cat. no. HC34) for cell storage
- CryoCanes (Nalgene, cat. no. 5015-0002), holders for cryotubes in liquid nitrogen refrigerator
- CryoCoders (Nalgene, cat. no. 5020), labels for CryoCanes
- Spray bottles, for 70% ethanol
- Gowns, full-front polypropylene infectious disease grade (Myriad 3009-0050; DuPont PP 701 FWH 00 005000) **! CAUTION** For further safety advice, see **Supplementary Note 1**.
- Filtration units:
  - 0.45 µm, 50 ml Steriflip (Millipore, cat. no. SE1M003M00)
  - 0.45 µm, 250 ml Stericup (Millipore, cat. no. SCHVU02RE) cryogenic tubes, 5 ml (sterile) (VWR, cat. no. 87003-414) for unconcentrated supernatants **! CAUTION** Note that these '5-ml' tubes will not hold 5 ml of medium once contents are frozen—fill with 4.5 ml at most.
- Labels for cryogenic tubes (Electron Microscopy Sciences, cat. no. 77560-Y)
- Storage boxes for 0.5 or 1.2 ml cryogenic tubes (VWR, cat. no. 82007-162)
- Storage boxes for 5 ml cryogenic tubes (VWR, cat. no. 16001-156)
- Pipetters (2–10 µl, 20 µl, 200 µl and 1,000 µl)
  - 0.1–2.5 µl (e.g., Eppendorf, cat. no. 022470001)
  - 2–20 µl (e.g., Eppendorf, cat. no. 022470108)
  - 10–100 µl (e.g., Eppendorf, cat. no. 022470205)
  - 100–1,000 µl (e.g., Eppendorf, cat. no. 022470302)
- Pipetter, 5,000 µl (e.g., Eppendorf, cat. no. 022472151) not essential, but very handy for preparing stock aliquots
- Tips, sterile with filters
  - 10 µl (e.g., Eppendorf, cat. no. 022491211)
  - 20 µl (e.g., Eppendorf, cat. no. 022491270)
  - 200 µl (e.g., Eppendorf, cat. no. 022491296)
  - 1,000 µl (e.g., Eppendorf, cat. no. 022491253)
  - 5,000 µl (e.g., Eppendorf, cat. no. 022491261)
- 150-cm$^2$ tissue culture flasks, vent-cap (e.g., Corning, cat. no. 430825) referred to below as 'T-150 flasks'
- Hemocytometer (e.g., Hausser Scientific, cat. no. 1490)
- 1.5 ml tubes (e.g., Eppendorf, cat. no. 022363328), autoclaved
- 5-ml polystyrene tubes (BD Falcon, cat. no. 352058) for flow cytometry (fluorescence-activated cell sorting (FACS))
- Ampoule crackers (Electron Microscopy Sciences, cat. no. 60605-10) for opening paraformaldehyde ampoules
- Flow cytometer, usually in a core facility
- Ultracentrifuge (e.g., Beckman or Sorvall)
- Ultracentrifuge rotor(s) with 30-ml buckets (e.g., Beckman, cat. no. SW 28 or Sorvall, cat. no. AH-627)
- Adapters for conical-bottom ultracentrifuge tubes (Beckman, cat. no. 358156), work with both Beckman and Sorvall rotors
- Ultracentrifuge tubes, 30-ml conical-bottom (Beckman, cat. no. 358126), work with both Beckman and Sorvall rotors
- Forceps, fine, for removing ultracentrifuge tubes from buckets
- Balance, battery-powered, for weighing ultracentrifuge buckets when filled
- Vortex (for vortexing resuspended virus after ultracentrifugation)
- Cryogenic tubes, 0.5 ml with O-ring (sterile) (VWR, cat. no. 89004-282), for aliquoting concentrated virus
- Micro-centrifuge, for spinning down virus aliquots before freezing
- –80 °C freezer, for virus storage

**REAGENT SETUP**

**Normal cell culture medium** 1,000 ml DMEM + 100 ml FBS + 10 ml antibiotic–antimycotic. Where not otherwise specified, 'medium' refers to normal cel culture medium throughout the protocol. This can be stored at 4 °C for several months.

**Cell freezing medium** 10% DMSO + 90% normal medium (see above). As it is used in small quantities, this is most efficiently prepared immediately before use in the quantity desired.

**Plasmid DNA (if generating virus from cDNA)** Prepare using standard molecular biological techniques in the quantities listed below in Step 2, with results verified by restriction digestion and gel electrophoresis. This can be stored indefinitely at −20 °C.

**1:6 Poly-L-lysine** 100 ml Poly-L-lysine + 500 ml DPBS. Can be stored at 4 °C for several months.

**70% Ethanol** For 1 liter of 70% ethanol: 734 ml 95% ethanol + 263 ml deionized water. Can be stored in sealed glass bottles indefinitely.

**20% sucrose in DPBS (30 ml needed per prep)** For 50 ml, 10 g sucrose + DPBS to a total of 50 ml. Filter-sterilize with 0.45 µm filter (e.g., Steriflip) before use or storage. After filter sterilization, the solution can be stored at 4 °C indefinitely.

**2% Paraformaldehyde in PBS** To prepare, e.g., 80 ml of the solution, mix 10 ml of 16% PFA, 8 ml 10× DPBS and 62 ml distilled water. Store at 4 °C for up to 1 month.

## PROCEDURE

### Rescue from cDNA: Day 1

**1|** Apply 5 ml diluted poly-L-lysine (PLL, see REAGENT SETUP) to a T-150 flask or 15-cm plate and shake until liquid completely covers the bottom. Let stand for at least 15 min before aspirating completely. Split HEK-293T cells 1/3:1 (if splitting from a near-confluent 15-cm plate) into this treated flask and add medium to a total of 22.5 ml. As their surface areas are almost the same, either T-150 flasks or 15-cm plates can be used. The flasks improve biosafety by greatly reducing opportunities for spillage, although they are more expensive.

### Day 2

**2|** Combine DNA in the following quantities in a 15-ml tube: genome vector (e.g., cSPBN-4GFP) 45.9 µg, pTIT-N 23.0 µg, pTIT-P 11.6 µg, pTIT-G 9.2 µg, pTIT-L 11.6 µg, pCAGGS-T7 or pC-T7 13.8 µg. Add 3.375 ml OptiMEM and mix. In a separate tube, combine 135 µl Lipofectamine 2000 with 3.375 ml OptiMEM and mix. After 5 min, combine the contents of the DNA and Lipofectamine tubes and then proceed to Step 3 while allowing the mixture to incubate for 20 min.

**3|** While incubating, aspirate medium from cells and wash gently with 10 ml DPBS, then aspirate and add 15.75 ml OptiMEM.

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**4|** At the end of the 20 min incubation, add DNA–Lipofectamine mixture to the plate, agitate to distribute and return the plate into the incubator.

**5|** After 6 h, aspirate OptiMEM and replace with 22.5 ml normal cell culture medium.

**Day 3**
**6|** Aspirate medium and replace with 22.5 ml fresh medium.

**Day 5**
**7|** If proceeding immediately to *Amplifying stocks*, thaw cells (see Step 13) and begin following the subsequent steps.

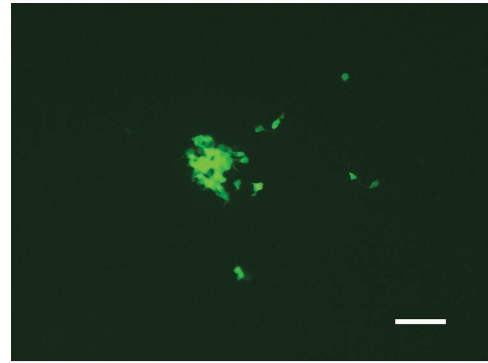

**Figure 3 |** Isolated cluster of cells infected by spreading virus in transfection plate, 4 d after transfection. Scale bar 100 μm.

**Day 6**
**8|** Inspect plates for fluorescence. Numerous isolated fluorescent cells should be present as well as less-widespread clusters of cells infected by spreading virus outbreaks, as seen in **Figure 3**.
**? TROUBLESHOOTING**

**9|** Collect supernatant (this is termed supernatant I) and replace with 11.25 ml fresh medium. As with all supernatants collected throughout this protocol, filter sterilize with a 0.45 μm filter (e.g., Steriflip for <50 ml, Stericup for larger quantities) and store at 4 °C until the supernatant is used (see Step 12).

**Day 7**
**10|** Collect the second supernatant (supernatant II) and replace with 11.25 ml medium.

**Day 8**
**11|** Collect the third supernatant (supernatant III) and replace with 11.25 ml fresh medium. The procedure *Amplifying stocks* can be started at this point (see Step 13).

**Day 9**
**12|** Collect the final supernatant (supernatant IV) and discard plates.
■ **PAUSE POINT** Supernatants can be stored at 4 °C for several days if necessary, with a titer drop of ~4–5% per day.

**Amplifying stocks: Day 5**
**13|** Thaw BHK-B19G2 cells ('B19G2 cells') if they are not already growing.

**Day 8**
**14|** Plate B19G2 cells in a PLL-coated T-150 flask to achieve near-confluency within 24 h (e.g., splitting a near-confluent 15-cm plate 1/3.5:1). Such a plate will contain ~1.5 × $10^7$ cells. Using PLL, as described in Step 1, before plating B19G2 cells increases titers. For further details on cell culture, see **Supplementary Note 2**.

**Day 9**
**15|** Aspirate medium from flask and apply the pooled supernatants I–IV. As with all future passaging steps, keep track of viral passage number (here 'P1').

**Day 11**
**16|** Collect supernatant I and replace with 11.25 ml fresh medium.

**17|** Plate 293T cells for titering (see **Box 1**).

**18|** Expand B19G2 cells to provide enough for plating on day 13.

**Day 12**
**19|** Collect supernatant II.
■ **PAUSE POINT** Supernatants should be stored at 4 °C while being titered and can be stored for longer if immediate titering is not possible.

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**20|** Infect titer wells (see **Box 1**).

**21|** If proceeding immediately to *Producing supernatant for ultracentrifugation*, thaw cells if necessary (see Step 33A(i) or 33B(i)) and begin following the subsequent steps.

**Day 13**
**22|** Change medium in titer wells (see **Box 1**).

**23|** Plate B19G2 cells in 20 or more PLL-coated T-150 flasks to achieve near-confluency within 24 h, as in Step 13, along with an uncoated 15-cm plate without PLL for counting cells. If the titer has already been determined at this point, prepare as many plates as the available virus stock will permit infecting at the desired MOI (see Step 24); otherwise, plate a large number to allow infecting as many as possible. The uncoated 15-cm plate is used as an imperfect proxy for estimating the number of cells present in the coated flasks.

**Day 14**
**24|** Carry out FACS and calculate the titer (see **Box 1**) to determine the total amount of virus available for infecting the B19G2 cells.

**25|** Resuspend the B19G2 cells in the 15-cm plate and calculate the total number using a hemocytometer, maintaining some cells in a new plate for subsequent use.

**26|** Using the calculated titer and number of cells per flask or plate, infect B19G2 cells at an MOI of 0.1. Assuming sufficient virus is available, using a higher MOI will increase the titers of the resulting supernatant but also its defective interfering particle content, as discussed in the Experimental design section. In practice, using an MOI of 1 or even higher for the low numbers of passages involved here does not appear to incur dire consequences. The issue is left to the discretion of the investigator.

**27|** If stocks are to be titered as soon as possible, plate cells for titering (see **Box 1**).

**Day 16**
**28|** Collect, pool and filter supernatants, discarding plates.

**29|** Aliquot in 5-ml cryotubes, 4.5 ml per tube. Decontaminate, label with cryogenic labels and store at −80 °C.
**! CAUTION** '5 ml' Cryotubes will not hold 5 ml of medium after they are frozen. Do not exceed 4.5 ml per tube.
**■ PAUSE POINT** Stocks can be maintained at −80 °C indefinitely.
**! CAUTION** Care required when decontaminating; see also **Supplementary Notes 1** and **3**.

**30|** If titering immediately, wait for several hours so that the tubes will have frozen completely, then thaw one in a 37 °C water bath, prepare dilutions and apply to titer wells (see **Box 1**).
**▲ CRITICAL STEP** Always thaw frozen virus in a 37 °C bath.

**Day 17**
**31|** Change medium in the titer wells (see **Box 1**).

**32|** Carry out FACS in titer wells and calculate the titer of the frozen stock (see **Box 1**). Steps 14–32 can be repeated as necessary if (a) the quantity of virus obtained so far is insufficient for proceeding with the subsequent steps, (b) a greater quantity is desired so as to allow a greater number of high-titer preps in future without later amplification or (c) the stocks generated on a previous date have been depleted.
**? TROUBLESHOOTING**

**Producing supernatant for ultracentrifugation: Days 12–21**
**33|** To prepare unpseudotyped virus for retrograde tracing, follow option A below. To make EnvA-pseudotyped virus for selective infection of TVA-expressing neurons, follow option B.
**(A) Producing unpseudotyped supernatant for ultracentrifugation: Day 12**
(i) Thaw B19G2 cells if not done previously.

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**Day 15**

(ii) Expand B19G2 cells for plating on day 17.

**Day 17**

(iii) Plate B19G2 cells for infection on day 18 (5 × T-150 flasks + 1 × 15-cm plate).
▲ CRITICAL STEP As the two supernatant collections will together yield somewhat less than 22.5 ml + 11.25 ml per flask, the number of producer plates used should be 5. A 15-cm plate is used for counting as resuspending cells growing in a flask is difficult, whereas the surface areas of a 15-cm plate and a T-150 flask are almost the same.

**Day 18**

(iv) Count cells in the 15-cm plate and infect the flasks at an MOI of 3–5 in a total volume of 22.5 ml. The amount of virus stock needed will be at least $2.25\times10^{8}$ IU (5 plates × ~$1.5\times10^{7}$ cells per plate × 3 IU $ml^{-1}$).

**Day 20**

(v) Collect and filter supernatant I. Replace with 11.25 ml per flask.

**Day 21**

(vi) Collect and filter supernatant II. This can be pooled with supernatant I or kept separate for titering separately. Discard flasks and proceed to *Concentrating by ultracentrifugation* (Step 34).

**(B) Producing EnvA-pseudotyped supernatant for ultracentrifugation: Day 12**

(i) Thaw BHK-EnvA2 cells (EnvA2 cells) into a 15-cm plate.

**Day 15**

(ii) Expand EnvA2 cells for plating on day 17.

**Day 17**

(iii) Plate EnvA2s for infection on day 18 (16 × 15-cm plates). As the first supernatant will be discarded in this case, more producer plates are needed to provide enough for ultracentrifugation (150 ml). As the first supernatant is replaced with only 11.25 ml medium for each plate, and not all of this will be recovered following collection and filtration of the second supernatant, 15 producer plates are used. Do not use PLL when plating EnvA2 cells, as it actually decreases titers somewhat. We cannot recommend using flasks instead of plates at this step, as it significantly complicates the washing and medium changes that follow and again results in lower titers.

**Day 18**

(iv) Count cells in one plate and infect the other 15 at an MOI of 2 in a total volume of 22.5 ml. Approximately $4.5\times10^{8}$ IU of unpseudotyped virus is needed (15 plates × ~$1.5\times10^{7}$ cells per plate × 2 IU per cell).
▲ CRITICAL STEP Here the MOI is kept lower than with the B19G2 line to minimize remnants of this starting stock in the final product.

**Day 19**

(v) Taking great care to disturb the cells as little as possible, aspirate the supernatant from each plate and wash twice with 10 ml DPBS, rocking the plate gently to rinse the walls. Replace medium with 11.25 ml normal medium.
▲ CRITICAL STEP The goal here is to remove as much of the initially applied virus as possible.

**Day 20**

(vi) Aspirate supernatant I, discarding it. Replace with 11.25 ml per plate. Despite the washes, the first supernatant is typically unduly contaminated with the initially applied virus and should be discarded.

**Day 21**

(vii) Collect and filter supernatant II. Discard plates and proceed to *Concentrating by ultracentrifugation* (Step 34).
(viii) Plate cells for titering (see **Box 1**).

**Concentrating by ultracentrifugation: Day 21**

**34|** Insert conical adapters and tubes into rotor buckets and add 5 ml 20% sucrose in DPBS (see REAGENT SETUP) to each.

**35|** Remove one tube at a time from the buckets and carefully layer 25 ml of filtered supernatant on top of the sucrose solution in each tube using a measuring pipette. Reserve ~0.5 ml of supernatant in a 0.5-ml tube for titering.

**36|** Using a balance within the biosafety cabinet, balance opposing buckets and their caps to within the tolerance of the ultracentrifuge used.

**37|** Cap buckets, decontaminate before removing from hood, place on rotor and spin at ~50,000*g* for 2 h at 4 °C. (The speed of 19,400 RPM, inherited from an effective lentiviral protocol[31], works well with either Beckman SW 28 or Sorvall AH-627 rotors despite their slightly different radii.)
**! CAUTION** Care required, see **Supplementary Note 1**.

**38|** If desired, cells can be plated on this day (21) for titering (see **Box 1**).

**39|** Again in the biosafety cabinet, aspirate liquid from each tube and replace with 100 μl DPBS. Aspiration can be safely achieved by following the receding surface down with the aspirator tip until reaching the beginning of the conical taper, then keeping the aspirator tip held against the tube wall at the cylinder–cone junction and tilting the tube to pour the remaining fluid into it. Add 100 μl DPBS to each tube immediately after aspiration so as not to allow the virus to desiccate.

**40|** Cap and decontaminate buckets and place on a shaker at ~1 Hz overnight at 4 °C.

**Day 22**

**41|** Pool resuspended virus in a 1.5-ml tube.

**42|** Vortex briefly to mix, spin down briefly on microcentrifuge and aliquot into 0.5-ml cryotubes in 20-μl aliquots.

**43|** Decontaminate tubes thoroughly (e.g., with 70% ethanol) and spin briefly on microcentrifuge to dry and to minimize fluid surface area.

**44|** Label with cryogenic labels and store at −80 °C in freezer until used. Proceed with titering (**Box 1**) thawed concentrated stocks as well as supernatants, concluding on day 24.
**? TROUBLESHOOTING**

### ● TIMING

The stages of the protocol can be overlapped as indicated in the PROCEDURE section for a total duration of 24 d.
Steps 1–12, rescue from cDNA: 9 d
Steps 13–32, amplifying stocks: 13 d
Step 33, producing supernatant for ultracentrifugation: 10 d
Steps 34–44, concentrating by ultracentrifugation: 4 d

### ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

**TABLE 1 |** Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 8 | No virus apparent in transfection plate | Problems with plasmid DNA | Check plasmids with restriction digest and gel electrophoresis; check 260/280 absorbance ratio with spectrophotometer |
| | | Low transfection efficiency | Transfect a control plate with a fluorophore expression vector. If modifying the genome vector to include a different transgene, consider conducting a control rescue using green fluorescent protein (GFP) construct cSPBN–4GFP in parallel |

(continued)



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**TABLE 1** | Troubleshooting table (continued).

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 32, 44 | Low titers in supernatants | Passage number of cells is too high | Use cells of as low a passage number as possible. Cells obtained from their laboratory of origination should be immediately expanded into a large number of low-passage aliquots that can be stored on liquid nitrogen and thawed for each new prep |
| | | Damage to monolayers during PBS washing (EnvA2 cells) and medium changes | Be extremely gentle when washing cells and changing medium. Aspirate and apply solutions onto the wall of the plates, at the same 'clock-face' position for all steps so that the damage that does occur will be restricted to a small area |
| | | Low MOI when infecting plate (for supernatants for ultracentrifugation) | Titer virus and count cells before infecting plates; infect at MOI values of 3–5 (unpseudotyped) or 2 (pseudotyped). Monitor fluorescence after infection to ensure that most cells are infected |
| 44 | Low titers in concentrated stocks | Damage to virus by mechanical resuspension | Resuspend by gentle overnight shaking at 4 °C without trituration |
| | Excessive contamination of pseudotyped stocks with unpseudotyped virus | Insufficiently rinsed plate walls during the wash step | Tilt plates gently during Dulbecco's phosphate-buffered saline (DPBS) washes to remove medium traces from walls |

## ANTICIPATED RESULTS

For unconcentrated stocks of unpseudotyped virus, titers on the order of $1\times10^8$ IU ml$^{-1}$ can routinely be attained. EnvA-pseudotyped titers are typically lower by an order of magnitude. Concentration with the provided ultracentrifugation protocol—reduction of volume by a factor of 250—produces expected titers over two orders of magnitude higher, on the order of $1\times10^{10}$ IU ml$^{-1}$ for unpseudotyped (see, e.g., **Fig. 2**) and $1\times10^9$ IU ml$^{-1}$ for pseudotyped virus. Titers of EnvA-pseudotyped virus on 293T cells should be ~50,000 times lower than on 293T-TVA800 cells. For comparison, an EnvA-pseudotyped lentivirus produced by transient transfection[31], for which infectivity of 293T cells must be entirely due to a nonzero intrinsic tropism of EnvA for mammalian cells, gives ~200,000-fold lower titers on 293T versus 293T-TVA800 cells. This suggests that the technique described here for removing contaminating unpseudotyped virus, although not perfect, gives results close to the theoretical limit.

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*Note: Supplementary information is available via the HTML version of this article.*

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**AUTHOR CONTRIBUTIONS** I.R.W. and H.A.S. carried out all experiments; H.S.S. supervised all experiments.

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