

Chapter 4

Expansion and Retroviral Transduction of Primary Murine T Cells for CAR T-Cell Therapy

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Abstract

The development of chimeric antigen receptor (CAR) T cells has been a revolutionary technology for the treatment of relapsed and refractory leukemias and lymphomas. The synthetic CAR molecule redirects T cell function toward tumor surface-expressed antigens through a single-chain variable fragment (scFv) fused to CD3z and intracellular costimulatory domains. Here, we describe a protocol for the generation of CAR T cells using primary mouse T cells and a gammaretroviral vector encoding a CAR transgene. This protocol outlines several transduction and expansion methods based on the use of two transduction enhancers, RetroNectin® and Vectofusin®-1, and cell culture systems such as conventional plates or G-Rex® devices.

Key words Chimeric antigen receptor (CAR), T cell, Transfection, Transduction, Gamma-retroviral vector

1 Introduction

CAR T-cell therapy capitalizes on synthetic biology to enable T cells to recognize and eradicate cancer cells. This adoptive immunotherapy has reshaped the treatment of relapsed or refractory B-cell malignancies, but leveraging the full potential of CAR T cell therapy remains hampered by limitations in our current understanding of the underlying biological determinants of efficacy. Immune-competent mouse models of cancer allow for the characterization of CAR T cells from multiple tissues, across time points, in an immune-relevant context, and provide complementary information to that obtained from patient samples and in vitro and xenograft studies with human T cells. Herein, we focus on the generation of murine CAR T cells that can be used in widely available mouse models.

Although gammaretroviruses and lentiviruses are both used for human T cell transduction preclinically and clinically, retroviruses

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are the preferred modality for primary mouse T cells as they yield more efficient transduction [1–3]. Herein, we describe a transduction protocol based on the use of the MSGV1 gammaretrovirus transfer plasmid [4] and the pCL Eco packaging plasmid to provide the accessory gag, pol, and ecotropic envelope genes in trans [5], the latter of which is readily available through Addgene. We have used this protocol to generate CAR T cells with multiple specificities, including EGFRvIII and CD19, and have used the Thy1.1 marker expressed from an IRES to identify transduced T cells. These methods have enabled us to understand how to combine cell therapies with other types of immunotherapies that engage multiple innate and adaptive immune components, such as oncolytic viruses [6, 7].

Modification of primary cells with retroviral vectors often requires the presence of transduction-enhancing reagents. Here, we describe protocols based on the use of two different enhancers, RetroNectin® (Clontech) and Vectofusin®-1 (Miltenyi). RetroNectin® (also described as CH-296) is a recombinant fragment of human fibronectin with three functional domains: the RGDS motif-containing cell adhesion domain, the heparin-binding (H) domain, and the CS-1 cell adhesion domain [8]. The molecule acts as a bridge as virus particles binding the H-domain are brought into close proximity with cells interacting with the RGDS or CS-1 domains through VLA-5 and VLA-4 on target cells, respectively. Vectofusin®-1 is a synthetic cationic amphipathic peptide that induces aggregation of viral particles and facilitates the adhesion and fusion of viral and cellular membranes [9–11]. While RetroNectin® must be precoated onto cell culture surfaces to enhance transduction via spinoculation, Vectofusin®-1 is a soluble reagent that can enhance transduction under static conditions.

T cells can be efficiently spinoculated and expanded in 24-well plates; however, static transduction enhancers enable the use of G-Rex® devices to grow T cells and avoid splitting the cells during the expansion step. The G-Rex® has a silicone membrane at the base of the vessel and facilitates efficient gas exchange in a manner that is independent of the volume of media above the cells [12]. As such, the cells can grow to a greater density without media exchanges, and these vessels have been ubiquitously used to expand preclinical and GMP-grade materials, including T cells [13–17].

In this chapter, we have included methods for CAR T cell generation using a gammaretroviral vector that make use of different cell expansion and transduction enhancer products. We describe transduction by spinoculation using the transduction enhancer RetroNectin® in **Method A** and Vectofusin®-1 in **Method B**. Finally, in **Method C** we describe static transduction with the transduction enhancer Vectofusin®-1 using regular 24-well plates (**C1**) or the G-Rex® devices (**C2**). Each method has advantages and disadvantages which are outlined in Table 1.

Table 1
Advantages and disadvantages of various transduction and cell expansion processes

	Processes/ format	Advantages	Disadvantages
Transduction method	Spinoculation	Yields the highest transduction rate when associated with transduction enhancers	Time consuming May induce cell stress
	Static	Time-saving	Lower transduction rate than spinoculation
Transduction enhancer	RetroNectin®	Yields very high transduction rates	Pre-coating the plate is time consuming Spinoculation may induce cell stress
	Vectofusin®-1	High-efficiency transduction with spinoculation Moderate transduction efficiency with static conditions	Requires serum-free media for the transduction step
Expansion format	24-well	Easy to source, low cost, amenable to small or large batch production	Scaling many 24-well plates can be cumbersome
	G-Rex®	No media changes during the process. Provides gas exchange to the cells to maximize expansion	Cannot visualize the cells under the microscope Cannot be spinoculated to enhance transduction Mouse T-cell culture is shorter than human T cell culture and may not maximize the long term advantage of the G-Rex®

2 Materials

2.1 293T Cell Culture

1. DMEM supplemented with 10% FBS (v/v), store at 4 °C.
2. 1× PBS: without Ca/Mg; store at room temperature.
3. 0.25% trypsin–EDTA; store at 4 °C.
4. Poly-D-lysine-coated plates (*see* **Note 1**); store at room temperature.
5. 50-mL tubes.

2.2 Transfection

1. DMEM supplemented with 10% FBS (v/v); store at 4 °C.
2. Lipofectamine® 2000 transfection agent; store at 4 °C.
3. Opti-MEM®; store at 4 °C.
4. MSGV1 1D3-28BBz IRES Thy1.1: retroviral transfer plasmid encoding the CAR cassette and the marker gene (anti-mouse CD19 CAR with CD3); store at −20 °C.
5. pCL Eco: retroviral packaging plasmid; store at −20 °C.

6. CAR T-cell media:
   (a) RPMI-based CAR T cell media (**Method A**).
      (i) RPMI 1640 with L-glutamine supplemented with:
         1. 100 U/mL penicillin/streptomycin.
         2. 1× MEM nonessential amino acid solution.
         3. 1 nM sodium pyruvate.
         4. 50 μM BME.
         5. 10% (v/v) FBS.
   (b) Serum-free media (**Methods B and C**) (*see* **Note 2**):
      (i) TexMACS™ media supplemented with:
         1. 100 U/mL penicillin/streptomycin
7. 50-mL tubes.

2.3 Splenocyte Collection

1. Mouse spleen (i.e., from C57BL/6 or Balb/c background) freshly collected (*see* **Note 3**).
2. RPMI; store at 4 °C.
3. ACK (ammonium–chloride–potassium) lysis buffer; store at room temperature.
4. 100-μM cell strainer.
5. 10-mL syringe.
6. 50-mL tubes.

2.4 T-Cell Activation and Culture

1. RPMI-based CAR T-cell media (*see* Subheading 2.2).
2. Concanavalin A (ConA) (2.5 mg/mL stock); store at −20 °C.
3. Murine recombinant cytokines: IL-7 (50 μg/mL stock) and IL-15 (50 μg/mL stock); store at −20 °C (*see* **Note 4**).
4. Tissue-culture-treated 24-well plates.
5. 50-mL tubes.

2.5 Transduction

Method A: Spinoculation Using Transduction Enhancer RetroNectin®

1. RetroNectin® (1 mg/mL stock); store at −20 °C.
2. Non-tissue-culture-treated 24-well plates.
3. 1× PBS: without Ca/Mg; store at room temperature.
4. Parafilm.
5. High-speed swinging-bucket centrifuge, with adapters for 24-well plates.
6. PBS supplemented with 2% bovine serum albumin (BSA) (w/v) and filter sterilized.
7. 50-mL tubes.

Method B: Spinoculation Using Transduction Enhancer Vectofusin®-1

1. Vectofusin®-1 (1 mg/mL stock); store at –80 °C.
2. Tissue-culture-treated 24-well plates.
3. 1× PBS: without Ca/Mg; store at room temperature.
4. Parafilm.
5. High-speed swing-bucket centrifuge, with plate adapters.
6. 50-mL tubes.

Method C: Static Using Transduction Enhancer Vectofusin®-1

C1 with regular tissue culture 24-well plates

1. Vectofusin®-1 (1 mg/mL stock); store at –80 °C.
2. Tissue-culture-treated 24-well plates.
3. 50 mL tubes.

C2 with G-Rex® tissue culture 6-wells

1. Vectofusin®-1 (1 mg/mL stock); store at –80 °C.
2. G-Rex® 6 multi-well cell culture (*see* **Note 5**).
3. 50-mL tubes.

2.5.1 Validation of Transduction

1. Fluorescently labelled anti-mouse antibodies against Thy1.1, CD4, CD8, CD44, CD62L, and a fixable live/dead staining dye (*see* Table 2 for a sample panel).
2. FACS buffer (1% BSA [w/v], 2 mM ethylenediaminetetraacetic acid [EDTA], 0.05% NaN_3 [w/v]).
3. 50-mL tubes.
4. 5-mL round-bottom polystyrene tubes with caps.

Table 2
Example antibody panel with associated fluorochromes and clones used to evaluate the CAR expression on T cells by flow cytometry

Antigen	Clone	Conjugate	Source	Dilution
Fixable live/dead stain		NIR	Biolegend	1/1500
CD4	GK1.5	BV421	Biolegend	1/750
CD8β	53–5.8	PerCP-Cy5.5	Biolegend	1/500
Thy1.1	OX-7	PE	Biolegend	1/1000
CD44	IM7	BV786	Biolegend	1/500
CD62L	MEL-14	BV605	Biolegend	1/500

5. 96-well round-bottom plate.
6. High-speed centrifuge with a swinging-bucket rotor, with plate adapters.
7. Flow cytometer.

3 Methods

3.1 Retrovirus Production

The following protocol is for transfecting one 10-cm plate, which yields around 9–12 mL of viral supernatant. Scale as required. Thaw fresh 293T cells approximately 1 week prior to the date they are needed.

3.1.1 Day -1: Plate 293 T Cells for Transfection

1. Collect 293T cells: Aspirate the media and wash the cells with PBS. Use 0.25% trypsin–EDTA to detach the cells from the vessel. Neutralize the trypsin with DMEM supplemented with 10% FBS and resuspend the cells. Transfer the cells to a 50-mL tube.
2. Spin at 300×*g* for 5 min at room temperature. Aspirate the supernatant and resuspend the cells in 10 mL of DMEM supplemented with 10% FBS. Count the cells using a hemocytometer.
3. Dispense 3.5×10^6 cells per poly-D-lysine-coated 10-cm plate in approximately 10 mL of DMEM supplemented with 10% FBS. Rock the plate(s) in different directions to evenly distribute the cells and incubate at 37 °C overnight.

3.1.2 Day 0: 293T Cell Transfection

1. Aspirate the media on the 293T cells and add 10 mL of fresh DMEM supplemented with 10% FBS within 30 min of transfection.
2. Prepare two 50-mL tubes with 1.5 mL of Opti-MEM® in each (adjust for the number of plates as required):
   - Tube #1: Add 60 μL of Lipofectamine.
   - Tube #2: Add 14.1 μg of MSGV1 1D3-28BBz IRES Thy1.1 + 9.9 μg of pCL Eco.

   Incubate for 5 min at room temperature.
3. Add the contents of Tube#2 to Tube#1. Mix only very gently. Incubate for 20 min at room temperature.
4. Add 3 mL of the lipid/DNA complex dropwise to the 293T cells. Incubate the plate(s) at 37 °C overnight (*see* **Note 6**).

3.1.3 Day 0: Splenocyte Collection

1. Mash the spleen through a 100-μM filter with the syringe plunger onto a 50-mL tube and wash twice with 5 mL of RPMI.

2. Spin at 300×*g* for 5 min at room temperature. Resuspend in 1 mL of ACK buffer. Incubate for 1 min at room temperature and then add 9 mL of RPMI.
3. Spin at 300×*g* for 5 min at room temperature. Aspirate the supernatant and resuspend the cells in 20 mL of RPMI. Count the cells using the hemocytometer.
4. Spin at 300×*g* for 5 min at room temperature. Aspirate the supernatant and resuspend at 2×10^6 cells/mL in RPMI-based CAR T-cell media + ConA (2.5 μg/mL) + murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL).
5. Plate 2 mL per well (total 4×10^6 per well) and put the plate (s) into the 37 °C incubator overnight.

3.1.4 Day 1: Replace Media on 293T Cells

1. Aspirate the media from the transfected plate(s) (*see* **step 4**, Subheading 3.1.2).
2. Add 9–12 mL of RPMI-based CAR T-cell media (**Method A**) or TexMACS™-based CAR T-cell media (**Methods B** and **C**) (*see* **Note 7**) on the 293T cell plate along the vessel wall. Be careful not to disturb and detach the cells. Repeat for all other plates (*see* **Note 8**).
3. Incubate the plate(s) at 37 °C overnight.

3.2 Transduction

3.2.1 Method A: Spinoculation Using Transduction Enhancer RetroNectin®

Day 1: RetroNectin® Coating

1. Plate 400 μL of PBS with 25 μg/mL of RetroNectin® in each well of a non-tissue-culture-treated 24-well plate.
2. Put the plate at 4 °C overnight.

Day 2: Harvest Retrovirus Supernatant

1. Collect the retrovirus supernatant with a pipette in a 50-mL tube. Spin at 500×*g* for 10 min at room temperature.
2. Transfer the supernatant to a new 50-mL tube and leave at least 1 mL behind so as to not disturb the cell pellet.

Day 2: T-Cell Transduction

1. Remove the PBS/RetroNectin® solution from the 24-well plate by aspirating. Add 0.5 mL of PBS supplemented with 2% BSA per well and incubate for 10–30 min at room temperature.
2. Remove PBS/BSA solution and wash each well with 1–2 mL of PBS.
3. Harvest T cells by gentle pipetting and pool them in a 50-mL tube. Count the cells using a hemocytometer. Set aside some cells to use as an untransduced control.

4. Spin the desired number of T cells to be transduced at 300×*g* for 5 min at room temperature. Resuspend the cells in viral supernatant at 1×10^6 cells/mL and add murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) (*see* **Note 7**).
5. Aspirate the PBS from the 24-well plate and transfer 1 mL of T cells in retrovirus supernatant per well.
6. Use parafilm to seal the plate. Spin the plate(s) for 90 min at 800×*g*; acceleration 4, brake 0; at 32 °C using biohazard rotor covers.
7. After the spin, add 1 mL of CAR media with murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) to each well.

Day 3: Expand T Cells

1. Split cells by gently resuspending and pipetting half the volume of each well (1 mL of cells) into a new tissue-culture-treated 24-well plate. Add 1 mL of CAR media with murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) to all wells.

3.2.2 Method B: Spinoculation Using Transduction Enhancer Vectofusin®-1

1. Collect the retrovirus supernatant with a pipette in a 50-mL tube. Spin at 500×*g* for 10 min at room temperature.
2. Transfer the supernatant to a new 50-mL tube and leave at least 1 mL behind so as to not disturb the pellet.

Day 2: Harvest Retrovirus Supernatant

Day 2: T-Cell Transduction

1. Harvest T cells by pipetting and pool them in a 50-mL tube. Count the cells using a hemocytometer. Set aside some cells to use as an untransduced control. Spin the desired number of cells to be transduced at 300×*g* for 5 min at room temperature. Resuspend the T cells in PBS to remove any residual serum, and spin again at 300×*g* for 5 min at room temperature.
2. Add 10 μg/mL of Vectofusin®-1 in viral supernatant and incubate for up to 5 min at room temperature. Resuspend the washed T cells in viral supernatant/Vectofusin®-1 at 1×10^6 cells/mL and add murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL).
3. Transfer 1 mL per well in a tissue-culture-treated 24-well plate.
4. Use parafilm to seal the plate. Spin for 90 min at 800×*g*; acceleration 4, brake 0; at 32 °C using biohazard rotor covers.
5. After the spin, add an additional 1 mL of RPMI-based media (with FBS) with murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) to each well in the 24-well plate.

Day 3: Expand T Cells

1. Split cells by gently resuspending and pipetting half the volume of each well (1 mL of cells) into a new 24-well plate. Add 1 mL of RPMI-based CAR media with murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) to all wells.

3.2.3 Method C: Static Transduction Using Vectofusin®-1

1. Collect the retrovirus supernatant with a pipette in a 50-mL tube. Spin at 500×*g* for 10 min at room temperature.
2. Transfer the supernatant to a new 50-mL tube and leave at least 1 mL behind so as to not disturb the pellet.

Day 2: Harvest Retrovirus Supernatant

Day 2: T-Cell Transduction

1. Harvest the T cells by pipetting gently and pool them in a 50-mL tube. Count the cells using a hemocytometer. Spin the desired number of cells to be transduced at 300×*g* for 5 min at room temperature. Resuspend the T cells in PBS to remove any residual serum, and spin again at 300×*g* for 5 min at room temperature. In parallel, set aside some T cells to use as an untransduced control.
2. Add 10 μg/mL of Vectofusin®-1 to the retrovirus supernatant and incubate for 5 min at room temperature. Resuspend the washed T cells in the retrovirus supernatant at 1×10^6 cells/mL and add murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL).
3. Aliquot 1 mL (1×10^6 cells) per well of a tissue culture 24-well plate (**Method C1**) or 5×10^6 to 1×10^7 cells in at least a volume of 5–10 mL in each well of a G-Rex® 6-well cell culture plates (**Method C2**) (*see* **Note 5**).
4. Incubate for 4 h at 37 °C. Add an additional 1 mL of CAR media (with FBS) with cytokines to the 24-well plate (**Method C1**) and an additional 10–15 mL to each G-Rex® well (**Method C2**).

Day 3: Maintain T-Cell Activation (Only for **Method C1**)

1. Split cells by gently resuspending and pipetting half the volume of each well (1 mL of cells) into a new 24-well tissue-culture-treated plate. Add 1 mL of CAR media with murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) to all wells.

3.3 Evaluation of CAR Transduction

3.3.1 Day 4 or 5: Harvest CAR T Cells

1. Gently resuspend cells by pipetting. Collect all the cells in 50-mL tubes and count using a hemocytometer. Prepare aliquots of 1×10^6 cells for flow staining and transfer to a 96-well round-bottom plate.
2. Spin the plate at 800×*g* for 2 min and resuspend cells in 100 μL of a fixable live dead stain diluted in PBS. Incubate for 10–20 min at room temperature.

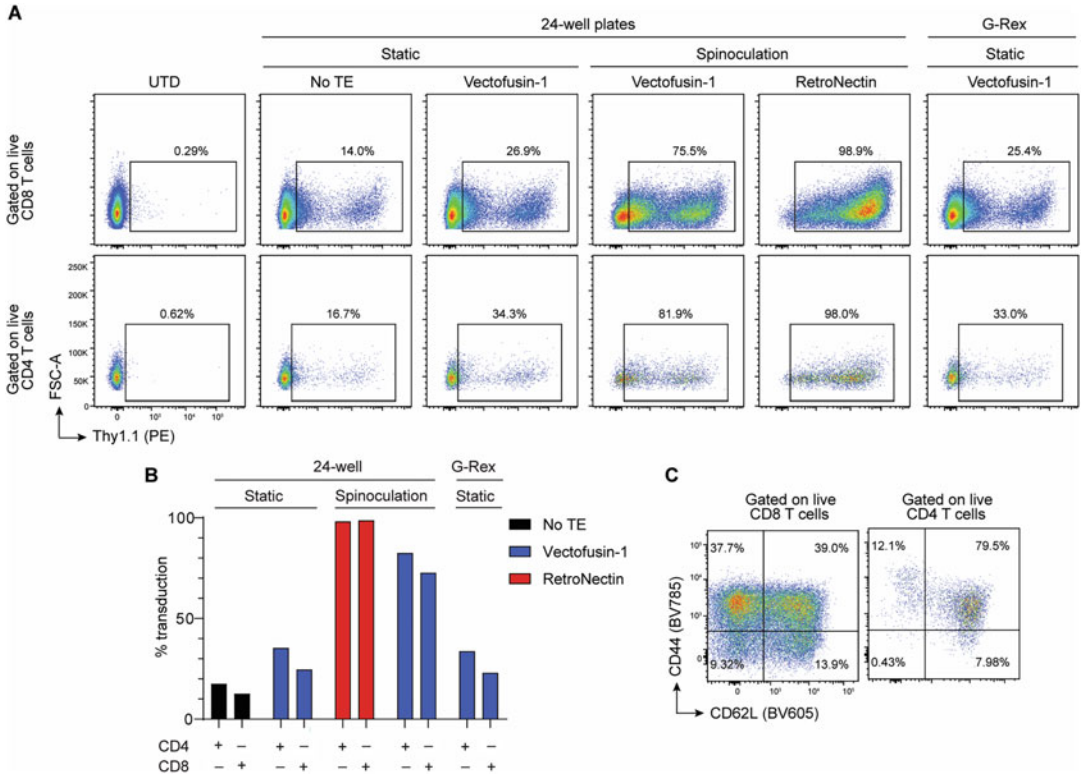


Fig. 1 On day 1, 9 mL of media was used to replace the transfection media. T cells were transduced in the presence of the indicated transduction enhancers and expanded in the indicated culture systems. Samples were run on a BD FACSymphony™ and analyzed using FlowJo 10 software. (**a**) Representative flow plots gated on live CD8 or CD4 T cells showing the expression of the CAR marker transgene Thy1.1 on day 4. (**b**) Transduction rates in CD4 and CD8 T cells from indicated transduction conditions. (**c**) Representative flow plots of the expression of CD44 and CD62L in CD8 and CD4 T cells following transduction by spinoculation in the presence of RetroNectin® (**Method A**). *TE* transduction enhancer; *UTD* untransduced

3. Spin the plate at 800×*g* and resuspend the cells in 100 µL of the antibody mastermix diluted in FACS buffer. *See* Table 2 for a sample antibody panel. Incubate for 20 min at 4 °C.
4. Spin the plate 800×*g* and resuspend cells in 150 µL of FACS buffer to wash the cells.
5. Spin the plate 800×*g* and transfer the cells to the 5-mL round-bottom tubes. Bring the volume up to ~400 µL and run on an appropriate flow cytometer. Refer to Fig. 1 and **Notes 9–11** for expected results.

4 Notes

1. Poly-D-lysine promotes 293T cell attachment and thus increases viral yield from the producer cells. Either pre-coated plates may be used or a poly-D-lysine solution may be used to directly coat the plates following the manufacturer's instructions.
2. The efficacy of Vectofusin®-1 is affected by the presence of serum in the media. Serum-free culture media such as TexMACS™ should be used to replace the transfection media on the 293 T cells on day 1 instead of RPMI-based media containing FBS.
3. While human T cells can be readily frozen and thawed, mouse T cells are less resilient to freezing and we have always used fresh T cells to maximize their viability and functionality.
4. Various cytokines can be used to expand the T cells. We have successfully used human IL-2 alone as well as combinations of mouse IL-7 (10 ng/mL) and mouse IL-15 (5 ng/mL) with or without mouse IL-21 (30 ng/mL) on day 0 only. IL-7, IL-15, and IL-21 are well documented to limit terminal differentiation of T cells and, in our hands, yield a better viability compared to human IL-2.
5. G-Rex® devices cannot be spun due to the silicone membrane at the bottom of the vessel.
6. From this point on, this supernatant has infectious retrovirus in it. All materials that come in contact with the retrovirus should be disinfected in 10% bleach.
7. The volume of media used to replace the transfection mix on day 1 can be adjusted to alter the viral titer. For example, for retroviruses with a small gene insert, 12 mL can be used, and the volume can even be diluted further (2/3 retrovirus supernatant +1/3 media). For instances where the titer needs to be maximized (i.e., the transgene size approaches the maximum retrovirus coding capacity, or when using Vectofusin®-1), the volume can be reduced to enhance the transduction efficiency.
8. In contrast to lentivirus which can readily be made as a large batch and stored at −80°C, the ecotropic retrovirus cannot be frozen without a significant loss of titer. Therefore, we prepare fresh retrovirus supernatant for each transduction.
9. As the T-cell expansion and transduction are extremely efficient, the yield on day 4 is >90% T cells and a sorting step is not typically performed. If desired, a magnetic bead-based sort for Thy1.1 or untouched T cells can be performed.

10. If 9 mL of media is used to replace the media on the 293T cells on day 1, the following transduction rates can be obtained:
    - **Method A:** >90% transduction
    - **Method B:** 70–85% transduction
    - **Method C:** 20–35% transduction
11. To illustrate a typical expansion and yield for **Method A**, the following example is provided:

    To treat three groups of six mice with 10^7 CAR T cells → 18 doses = 1.8×10^8 cells + excess for injection.

    Day 0: Harvest spleens from four donor mice.

    Transfect 8 × 10 cm plates of 293 T cells.

    Day 1: Coat 5 × 24-well plates with RetroNectin®.

    Day 2: Harvest T cells and expect to collect 70–90% of the total number of cells plated on day 0. Bring the retrovirus supernatant volume up to 120 mL. Spinoculate 5 × 24-well plates.

    Day 3: Split cells ½ into 10 × 24-well plates.

    Day 4: Harvest T cells and expect to collect approximately three times more cells than plated on day 2. Overall expected yield is ~4×10^8.

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