Protocol to produce high-titer retrovirus for transduction of mouse bone marrow cells

Regulating gene expression through retroviral infection has been widely used in mouse bone marrow transplantation (BMT) to test the capacity of self-renewal, as well as multi-lineage differentiation of hematopoietic stem and progenitor cells (HSPCs). However, it remains challenging to achieve high transduction efficiency in bone marrow cells as transduction of these cells subsequently leads to transplantation failure. Here, we present a modified protocol to overcome this issue, enabling reproducible and high-efficient retroviral transduction of HSPCs for BMT.
Protocol to produce high-titer retrovirus for transduction of mouse bone marrow cells

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SUMMARY
Regulating gene expression through retroviral infection has been widely used in mouse bone marrow transplantation (BMT) to test the capacity of self-renewal, as well as multi-lineage differentiation of hematopoietic stem and progenitor cells (HSPCs). However, it remains challenging to achieve high transduction efficiency in bone marrow cells as transduction of these cells subsequently leads to transplantation failure. Here, we present a modified protocol to overcome this issue, enabling reproducible and high-efficient retroviral transduction of HSPCs for BMT.

For complete details on the use and execution of this protocol, please refer to Yang et al. (2019).

BEFORE YOU BEGIN

Timings: 1–2 weeks

1. To obtain high titer retrovirus supernatants, high quality, and endotoxin free packaging plasmids are essential. We amplify and isolate the plasmids from Top10 E. Coli cells using an endotoxin free Plasmid isolation Kit.

2. Low passage and good condition of HEK293T cells are critical for high yield production of retrovirus. We thaw and grow healthy HEK293T cells in DMEM media containing 10% fetal bovine serum for at least 3 passages before seeding for transfection.

3. Certified class II biological safety cabinets must be used and personal protective equipment (PPE) including double gloves, lab coats, and masks must be worn when working with retroviruses. Culture medium and other virus contaminated materials such as dishes, plates, and tubes should be autoclaved prior to disposal.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| Mouse recombinant SCF | PeproTech | 250-03 |
| Mouse recombinant Flt-3 | PeproTech | 250-31 |
| Mouse recombinant IL-6 | PeproTech | 216-16 |
| Mouse recombinant IL-3 | PeproTech | 213-13 |
| 5-Fluorouracil | Sigma-Aldrich | F6627 |

(Continued on next page)
Dissolve the Baytril in alkaline water (pH 9.0–10.5) to make 100 x stock solution. Dilute it into 1 x into drinking water when use. The stock solution can be saved at 4°C for 2–3 months. Sterilization is not necessary.

### MATERIALS AND EQUIPMENT
Dissolve the Baytril in alkaline water (pH 9.0–10.5) to make 100 x stock solution. Dilute it into 1 x into drinking water when use. The stock solution can be saved at 4°C for 2–3 months. Sterilization is not necessary.
**Bone marrow flushing and FACS buffer**

| Reagent            | Final concentration (%) | Amount |
|--------------------|-------------------------|--------|
| FBS (Heat inactive)| 2%                      | 10 mL  |
| PBS (pH 7.4)       | n/a                     | 490 mL |
| Total              | n/a                     | 500 mL |

Mix well and sterilize by filtration through 0.22 µm filter. Store the solution at 4°C for 1–2 months.

**ACK red blood cell lysing buffer**

| Reagent            | Final concentration (mM) | Amount |
|--------------------|--------------------------|--------|
| Ammonium chloride  | 155                      | 8.3 g  |
| Potassium bicarbonate | 10                     | 1 g    |
| EDTA (0.5M, pH 7.4)| 0.1                      | 0.1 mL |
| H2O                | n/a                      | 500 mL |
| Total              | n/a                      | 500 mL |

Autoclave and store the solution at 4°C for 4-6 months.

**5-FU solution**

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| 5-FU    | 10 g/L              | 0.5 g  |
| NaCl    | 0.9%                | 0.45 g |
| H2O     | n/a                 | 50 mL  |
| Total   | n/a                 | 50 mL  |

Dissolve the 5-FU in a 55°C water bath with vigorous vortexing as described [Daadi, 2019]. Sterilize by filtration through 0.22 µm filter and store aliquots at −70°C.

**100 × Baytril solution**

| Reagent | Final concentration (g/L) | Amount |
|---------|---------------------------|--------|
| Baytril | 20                        | 1.0 g  |
| H2O     | n/a                       | 50 mL  |
| Total   | n/a                       | 50 mL  |

**Cytokine solution (to make stock solution of cytokines)**

| Reagent            | Final concentration (%) | Amount |
|--------------------|-------------------------|--------|
| BSA                | 2                       | 0.2 g  |
| PBS (pH 7.4)       | n/a                     | 10 mL  |
| Total              | n/a                     | 10 mL  |

Mix well and sterile filter. Dissolve the cytokines in a proper volume to make 100 µM stock solution. Store the aliquots at −20°C freezer.

**1000 × Polybrene stock solution (8 g/L)**

| Reagent | Final concentration (g/L) | Amount |
|---------|---------------------------|--------|
| Polybrene | 8                        | 0.08 g |
| PBS (pH 7.4)| n/a                    | 10 mL  |
| Total   | n/a                       | 10 mL  |

Mix well and sterile filter by filtration through 0.22 µm filter. Store the solution at −20°C.
STEP-BY-STEP METHOD DETAILS

Retroviral particles production and concentration

⏱ Timing: 4 days

Package and concentrate retrovirus from HEK293T cells

1. Late afternoon, seed 6.0 × 10⁶ HEK293T (293T) cells per 150-mm dish containing 10% FBS in 20 mL high glucose and sodium pyruvate DMEM medium (293T medium). Gently mix the cells and culture medium before returning dish into incubator (37°C, 5% CO₂). Keep culturing for 16–20 h.

2. PEI transfection (Early in the morning)
   a. 293T cells should be 60%–70% confluent and well attached. To increase the transfection efficiency, add 5 mL fresh and warm 293T culture medium containing 25 μL 25 mM chloroquine (1000× stock, final concentration is 1 mM) per dish at 1 h before starting the transfection. Gently rock the dishes from side to side and return them to incubator.
   b. Take out the transfection plasmids and PEI aliquot from freezer. Per 150-mm dish, prepare transfection mixture A (25 μg retroviral plasmid and 20 μg pCL-Eco helper plasmid in 1 mL Opti-MEM) and mixture B (add 135 μL 1 mg/mL PEI into 1 mL Opti-MEM). Transfer the mixture B to A, vortex gently for a short period of time, and incubate the transfection mixture for 20 min at 20°C–25°C without disturbance.

   ° Note: Check plasmids integrity and isoforms by running agarose gel prior to starting transfection (Figure 1). The supercoiled plasmid has a higher transfection efficiency than other isoforms.

   c. Add 2 mL plasmids/PEI mixture to each 150-mm dish in a dropwise manner. Rock the dish gently from side to side before returning it to incubator.
   d. Twelve hours after transfection, carefully aspirate the old medium, pipet 20 mL fresh 293T medium slowly against the side wall of 150-mm dish and return plates to incubator.

   △ CRITICAL: The transfection medium should be replaced with fresh 293T medium 12–14 h post transfection because of the toxicity of PEI and chloroquine.
Note: PEI has a high transfection efficiency in 293T cells. More importantly, it costs much less than the transfection reagent that we used previously (Yang et al., 2019).

3. Collect and concentrate viral particles
   a. Twenty-four hours after changing medium (36 h post transfection), collect supernatant from each 150 mm dish into 50 mL tube.
   b. Centrifuge at 1000 × g for 10 min at 4°C to remove cells and debris. Carefully transfer the supernatant into a new 50 mL tube.
   c. Add 1 volume of Retro-X Concentrator for 3 volumes of supernatant. Gently mix by inverting the tubes every 10–20 min during the first hour of incubation at 4°C.
   d. Incubate mixture for 10–16 h at 4°C refrigerator.
   e. Centrifuge the samples at 1,500 × g for 45 min at 4°C. The white pellet at the bottom of 50 mL tube will be visible. Discard as much as possible of the supernatant.
   f. Add 1/10 to 1/50 of the original volume of RPMI medium supplied with 2% heat inactivated FBS. Gently shake the tube on ice until the pellet completely goes into suspension.

   Note: To avoid damaging virus, do not vortex white pellet. Because the viral pellet is difficult to resuspend if adding too little medium, we recommend resuspending the pellet with more than 1/50 of the original volume medium.
   g. Titrate the concentrated virus immediately or freeze the aliquots at −80°C.

Title: Titration of retrovirus

© Timing: 4 days

4. Plate 50,000 healthy NIH 3T3 cells in 500 μL of complete DMEM (DMEM+10% FBS) per well of a 24 well plate. To ensure that each well has same number of cells as much as possible, we resuspend 1.3 × 10^6 cells in 13 mL complete DMEM and then aliquot 500 μL into each well. Return the plate into incubator and keep culturing for 16–20 h.

5. Trypsinize 3 wells from the plate and count cell number individually. Record average number as (#) cell plated.
   a. Aspirate old medium from each well and add 450 μL complete DMEM containing 8 μg/mL polybrene (for boosting infection efficiency).
   b. Thaw a vial of frozen retrovirus (or fresh viral supernatant) in 37°C water bath and prepare a series of 10 fold dilution of the tested virus as follow:
      i. 100 μL non-diluted viral supernatant.
      ii. 10⁻¹: 18 μL of non-diluted viral supernatant + 172 μL complete DMEM.
      iii. 10⁻²: 18 μL of 10⁻¹ dilution + 172 μL complete DMEM.
      iv. 10⁻³: 18 μL of 10⁻² dilution + 172 μL complete DMEM.
      v. 10⁻⁴: 18 μL of 10⁻³ dilution + 172 μL complete DMEM.
   c. Distribute 50 μL/well of each virus supernatant dilution in triplicate. Leave 3 wells as a non-viral infection control (add 50 μL/well complete DMEM).

   Note: Be sure to keep virus and cells warm, as a cold shock will decrease infection efficiency.
   d. Gently pipet up and down 3 times using a 1 mL pipette. Return the plate into incubator.

6. Twenty-four hours after infection, replace medium containing the viral supernatant with 500 μL fresh complete DMEM.

7. Forty-eight hours after infection, trypsinize and resuspend the 3T3 cells from each well in FACS buffer individually. Analyze the percentage (%) of GFP⁺ cells using Flow Cytometer (Figure 2).

For a well with 1%–10% of GFP⁺ cells, calculate virus titer as follow:
Virus infectious units per mL (IFU/mL) = (%) GFP+ cell plated (right before infection) x dilution factor/ 0.05 mL. The average value of triplicate wells represents the viral titer.

Note: The method provided above is suitable for fluorescent labeled and non-transmission (replication defective) virus. For the non-fluorescent retrovirus, please switch to other retroviral titration methods or commercial kit such as Retro-X qRT-PCR Titration Kit (Takara, Cat#: 631453).

Isolation of murine HSPCs

© Timing: 4 days

8. In order to enrich hematopoietic and progenitor stem cells (HSPCs), we intravenously inject (iv) 300 μL 10 mg/mL 5-Fluorouracil (5-FU) for a 20 g adult mouse (150 mg/kg) at 4 days prior to viral infection (Yang et al., 2019).

9. Euthanatize the mice through cervical dislocation at 4 days after administration of 5-FU. Rinse shortly with 70% ethanol, and then place the mouse on paper towel and then transfer it into a biosafety cabinet.

10. Cut the hind and front limbs (Figure 3A) into a 100-mm dish containing 10 mL PBS. Disconnect the joint and remove the muscles as much as possible. Place the bones in a new 100-mm dish (Figure 3B).

11. Flush out bone marrow into a new 50 mL tube with bone marrow flushing buffer using a 10–20 mL syringe and 25G 1 1/2 inch needle.

12. To dissociate the clumps of cells, pipet up and down twice by the same syringe and needle. Spin down the cells at 400 × g for 5 min at 4°C. Aspirate supernatant.

13. To lyse the red blood cells, add 5 mL ACK lysis buffer and resuspend the cell pellet into a new 15 mL tube. Keep the tube on ice until the lysis becomes clear.

14. Add 8 mL more flushing buffer into the tube to stop the lysis. Inverse the tube 3–5 times and then centrifuge at 400 × g for 5 min at 4°C.

15. Resuspend the bone marrow with 10 mL flushing buffer. To remove tissue and cell cluster, pass the suspension through a 40 μm strainer.
16. Enrich c-Kit+ HSPCs using CD117 magnetic beads and LS column following manufacturer’s instruction (https://www.miltenyibiotec.com/US-en/products/cd117-microbead-kit-human.html) (optional).

17. Centrifuge at 400 x g for 5 min at 4°C. Aspirate supernatant.

18. Add 1 mL RPMI 1640 medium (containing 20% FBS, 20 ng/mL SCF, 20 ng/mL Flt-3, 10 ng/mL IL3 and 10 ng/mL IL6) per mouse to resuspend HSPCs cells into one well in 6 well plate. Count the cell number.

Note: Typically, 5–10 x 10^6 HSPCs, if there are no further enrichments, could be obtained from a mouse at 4 days after 5-FU injection. To obtain enough cells for viral infection and subsequent transplantation, pool cells isolated from 2–3 mice in one well.

19. Incubate the cells for 20–24 h in 37°C incubator supplied with 5% CO2.

Optional: A commercial HSPCs culture medium (StemSpan) is an option for growing HSPCs.

Retroviral infection of HSPCs

★ Timing: 1 day

20. Thaw one vial (200 μL aliquot) of frozen retrovirus and frozen polybrene in 37°C water bath.

21. Add 200 μL of viral suspension and polybrene (final concentration is 8 μg/mL) in one well of 6-well plate containing 2 mL cell suspension. Pipet up and down 3 times to briefly mix. Return the plate into incubator.

22. After a 30 min incubation, initiate spinoculation by centrifuging the plate at 500 x g for 90 min at 32°C.

23. Carefully return the plate into incubator and keep culturing for 2–4 h.

24. Repeat spinoculation once by adding 200 μL more concentrated retrovirus in same well as described above. Return the plate into incubator.

25. After 1–2 h incubation, collect cells in a 15 mL tube and count cell number.

   a. Aliquot approximately 10,000 cells in same medium and keep culture for 24 h. Next day, check transduction efficiency by FACS if the virus expresses fluorescence (e.g., GFP). For non-fluorescence expressing virus, estimate cell viability by antibiotics resistance gene screening (such as hygromycin or puromycin selection). Calculate cell viability as follow:

   \[
   \text{% cell viability} = \frac{\text{cell (#) after incubation with antibiotics for 24 h}}{\text{cell plated (#) (here 10,000)}} \times 100\%
   \]
b. Wash the cells that used for transplantation to remove virus and polybrene with RPMI medium supplied with 2% heat inactivated FBS.
c. Place the tube on ice until injection into murine recipients.

Note: For each retroviral infection of HSPCs, we recommend performing preliminary experiment to estimate the transduction efficiency or measure the functional effect through colony formation assay at 24 h after infection. Since pluripotency of HSPCs decreases with prolonged culture time, the cells should be transplanted into recipient mouse as early as possible (24–48 h post infection).

Bone marrow transplantation

 Timing: 1 day

26. Depending on the purpose of experiment, irradiate the recipient mice lethally (1100 Rads, 3 h apart) or sub-lethally (450 Rads once) from an X or gamma irradiator. Note that lethal irradiation leads to complete myeloablation which generally follows reconstitution of the whole hematopoietic system by hematopoietic stem cell transplantation.
27. Return irradiated mice into cages with fresh food and drinking water supplied with 0.2 mg/mL Baytril.
28. Resuspend cells with 10 mL cold RPMI 1640 medium containing 2% heat inactive FBS and filter the suspension through 40 μm Strainers. Centrifuge at 400 × g, 4°C for 5 min. Aspirate supernatant by vacuum.
29. Pipet up and down of cells with a proper volume of cold RPMI 1640 medium containing 2% heat inactive FBS.
30. Count and adjust cell number into a concentration of 0.2–1 × 10^6 cells/0.2 mL.

Note: Cell number that used for transplantation is dependent on the cell transduction efficiency and the purpose of experiment. Thus, we recommend performing a preliminary experiment to check transduction efficiency in similar condition (e.g., same batch of virus). The transduced cell number for each mouse could be calculated as: Transduced cell (#) = transplanted cell (#) × (%) transduction. For recipient mice with lethal irradiation, co-transplant with 0.2–0.5 × 10^5 healthy bone marrow cells as helper.
31. Inject 0.2 mL cell suspension into each irradiated recipient mouse via tail vein using a 1.0 mL syringe and 27G 1/2 inch needle.
32. Change cages with fresh food and drinking water (containing 0.2 mg/mL Baytril) one week post transplantation.

Note: Recipient mice are immunocompromised for the first two weeks post BMT, and therefore they should be housed under strict barrier conditions. The Baytril added in the drinking water can protect mice from bacterial infection.

Optional: After irradiation, the recipient mice could also be maintained on acidic (adjust to pH 3.0 by hydrochloric acid) drinking water for 2 weeks.

Expected outcomes
In this protocol, to produce high titer retrovirus with less cost, we use PEI as a mediator to deliver retroviral packaging plasmids (MSCV-IRES-GFP and pCL-Eco) into HEK293T cells, which produced comparable retroviral particles (up to 3 × 10^7/mL) after 20 fold concentration.

We usually obtain 40–60 × 10^6 mononuclear bone marrow cells from each 8–12 weeks old mouse by following the protocol as mentioned above and else (Yang et al., 2019; Yang and Jiang, 2020). S-FU
is a nucleotide analogue that is incorporated into DNA during the S-phase of the cell cycle. Thus, it induces cell death of cycling cells and enriches HSPCs that have slow cell division in vivo. To enrich HSPCs, Intraperitoneal injection (IP) of 5-FU is mentioned in other publications (Zhu et al., 2016; Chen et al., 2019). However, we found that intravenous (iv) injection is more powerful and reliable for enrichment of HSPCs. For each mouse, around 5–10 $10^6$ mononuclear BM cells could be isolated at 4 days after iv injection of 5-FU. Those cells could be directly transduced with retrovirus or be further enriched by surface marker based enrichments such as c-Kit conjugated beads or flow mediated selection.

Herein, four days after administration of 5-FU, we further enriched c-Kit positive BM cells using a commercial CD117 magnetic beads and pooled cells isolated from 3 mice in one. After transduction with retrovirus expressing GFP, 0.4 $10^6$ total cells (CD45.2$^+$) together with 0.2 $10^6$ competitor BM cells (CD45.1$^+$) were transplanted into each lethal irradiated recipient. Percentages of GFP$^+$ cells in bone marrow and splenocytes were estimated by flow cytometry at 5 weeks post-transplantation. Results were analyzed by FlowJo software (Figure 4). More than half of CD45.2$^+$ BM cells, 25% B220$^+$; CD45.2$^+$ spleen cells were GFP$^+$, suggesting a significant number of HSPCs were transduced.

LIMITATIONS
This protocol was designed to produce retrovirus expressing fluorescent marker (e.g., GFP). The virus titer and transduction efficiency could be easily measured by flow cytometer. Therefore, researcher may optimize this protocol if using a non- fluorescent expressing retrovirus. Moreover, the use of CD117 magnetic beads to collect mouse c-Kit$^+$ cells (step 16), for some experiments, may not sufficient to enrich HSPCs. In this condition, a rigorous method (Yang et al., 2016) is needed.

TROUBLESHOOTING
Problem 1
Low production of retrovirus (step 2 of step-by-step method details)
Potential solution
Healthy and low passage (<20 passages) of 293T cells are essential for the production of high titer of retrovirus. In addition, check the integrity and isoforms of retroviral plasmids by agarose gel before transfection. High quality of plasmid (Figure 1) greatly improves the viral production.

Problem 2
Low yield of HSPCs (step 18 of step-by-step method details)

Potential solution
With 5-FU injection, around $5 \times 10^6$ HSPCs could be obtained from each adult mouse. Thus, cell recovery of less than $5 \times 10^6$ HSPCs per mouse is defined as low yield. To avoid this problem, we recommend: (1) Control the 5-FU dose at ratio of 150 mg/kg of body weight and sacrifice mouse at 4 days after administration; (2) Flushing out cells from all tibia, femur, and humerus; (3) lyse red blood cells on ice using ACK buffer, stop lysis immediately when it becomes clear by adding flushing buffer or equal volume of 2× PBS prior to centrifuging.

Problem 3
Bacteria contamination in HSPCs (step 19 of step-by-step method details)

Potential solution
Autoclave all the materials including scissors, forceps, and Kimwipes that used in BM isolation. Furthermore, we recommend that rinsing the mouse in 70% ethanol completely for at least 1 min after sacrifice. And then performing the cell isolation in a biosafety cabinet as mentioned above.

Problem 4
Low retroviral transduction efficiency in HSPCs (step 25 of step-by-step method details)

Potential solution
Check the viral titer before BM transplantation. Since low temperature reduces transduction efficiency, we recommend warming the frozen viral aliquot in 37°C water bath in a short time before adding them into cell culture. To minimize dilution of the culture media, in particular of the cytokines, the viral supernatant should not exceed 1/10 volume of the total medium. In addition, we highly recommend that spinoculation twice in 2–4 h apart and transplantation of transduced cell be performed in same day.

Problem 5
Recipient mice die after irradiation/BM transplantation (step 32 of step-by-step method details)

Potential solution
Recipient mice are quite fragile in the first two weeks after irradiation. Thus, they need to be housed under strict barrier conditions. In addition, we add antibiotic (Baytril) in the drinking water to prevent bacterial infection. To help mice reaching food, put some chow in cage and soften them by spraying a little drinking water containing Baytril. For lethal irradiation mice, co-transplant with healthy BM cells as competitor which will protect mouse from BM reconstitution failure.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zhenhua Yang (zhenhua@hust.edu.cn).

Materials availability
No unique reagent was generated in this study.
Data and code availability
No unique datasets or code was generated in this study.

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AUTHOR CONTRIBUTIONS
J.Q. performed the experiments and analyzed data. Z.Y. analyzed the data and wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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