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Retrovirus Production and Mouse T Cell Transduction

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This protocol is a guide for ecotropic retrovirus production and mouse T cell isolation, activation, and retrovirus transduction. Here, we transfect Platinum-E cells (PlatE), a cell line based on HEK293T that can produce ecotropic (mouse cell infecting) retrovirus, with plasmid DNA encoding for a murine chimeric antigen receptor (CAR) gene in a pMMLV backbone linked to a green fluorescent protein (GFP) reporter gene by a 2A peptide sequence. To increase retrovirus production, we co-transfected the PlatE with pCL-Eco (gag/pol/env encoding helper plasmid, Addgene #12371). In parallel, we magnetically isolated T cells from mouse splenocytes, activated the T cells with anti-mouse CD3/CD28 beads and recombinant human interleukin-2 (rhIL-2) for 24h, and transduced the activated T cells with retrovirus co-incubated with protransduzin A or protamine sulfate.

Materials:

Retrovirus production

Platinum-E cells (Cell BioLabs)

DMEM medium with 10% Fetal Bovine Serum (FBS), Glutamax, Non-essential amino acids (NEAA), Sodium pyruvate, Penicilin-Streptomycin, HEPES (all from Gibco)

DPBS (Gibco)

Trypsin-EDTA (0.25%) (Gibco)

Chloroquine diphosphate (VWR)

PEI Prime Linear Polyethylenimine (Sigma)

Plasmid DNA

OPTI-MEM (Gibco)

RPMI medium with 10% Fetal Bovine Serum (FBS), Glutamax, Non-essential amino acids (NEAA), Sodium pyruvate, Penicilin-Streptomycin, HEPES (all from Gibco)

ViralBoost (AllStem)

Mouse T cell isolation, activation, and transduction

Mouse spleen

DPBS

5 ml plastic syringe plunger

ACK Lysis Buffer (Gibco)

Cell separation buffer (DPBS with 2% FBS and 1 mM EDTA)

Mouse T cell isolation kit (e.g. STEMCELL Technologies EasySep Mouse T Cell Isolation Kit)

RPMI medium with 10% Fetal Bovine Serum (FBS), Glutamax, Non-essential amino acids (NEAA), Sodium pyruvate, Penicilin-Streptomycin, HEPES (all from Gibco)

2-mercaptoethanol (ThermoFisher)

Recombinant Human IL-2 (Peprotech)

Anti-mouse CD3/CD28 Dynabeads (Gibco)

Protransduzin A (Immundiagnostic AG) or Protamine Sulfate (ThermoScientific)

Procedure:

Retrovirus production

PlatE cells are kept in culture by splitting and seeding 10^6 cells in a new 10 cm dish twice a week (Mondays and Thursdays). Cells are cultured in DMEM10 medium (DMEM with 10% FBS and additives listed above), harvested using trypsin, and counted with Trypan Blue. Ideally, PlatE are used for retrovirus production below passage 20 and at viability above 90%. The steps below describe a workflow where PlatE are seeded for transfection on Monday, transfected on Tuesday, medium changed on Wednesday, and retrovirus collected for mouse T cell transduction on Thursday.

Monday

1. Harvest PlatE cells with trypsin, count with Trypan Blue and seed 4 x 10^6 cells per 10 cm dish.
2. Seed a maintenance PlatE cell plate (10^6 cells per 10 cm dish). Keep track of passage number.

Tuesday

1. In the morning, add 25 μM chloroquine diphosphate to PlatE cells to be transfected by adding 10 μl of 25 mM chloroquine diphosphate stock (kept in single use aliquots at -20C) to each plate with 10 ml DMEM10.
2. In the afternoon, transfect PlatE cells with 10 μg of pMMLV plasmid (e.g. CAR-2A-GFP construct) and 4 μg of ecotropic retrovirus helper plasmid (e.g. pCL-Eco).
   - In one tube, add both DNA plasmids to 500 μl of OPTI-MEM (serum-free medium).
   - In another tube, add 42 μl PEI (3:1 ratio, PEI stock at 1 mg/ml in single use aliquots at -20C) to 500 μl of OPTI-MEM.
   - Add contents of PEI tube to DNA tube dropwise for a total volume of aprox. 1 ml.
   - Incubate at room temperature for 15 min.
   - Add the mixture (DNA-PEI complexes) to PlatE cells dropwise with a P1000 pipet.

Wednesday

1. If possible, check transfection of PlatE without disturbing the cells (e.g. GFP fluorescence).

2. In the morning, change medium of transfected cells from DMEM10 to 10 ml RPMI10 (RPMI with 10% FBS and additives as described above) with 20 μl of 500x ViralBoost (to enhance retrovirus production).

Thursday

1. Collect retrovirus-containing medium from transfected PlatE cells.
2. Centrifuge 300 g 10 min and decant retrovirus-containing medium into new tube (to remove any debris).
3. Use immediately for mouse T cell transduction.

Mouse T cell isolation, activation, and transduction

Mouse T cells are magnetically isolated from the spleen and/or lymph nodes of immunocompetent mice (e.g. C57BL/6 mice). They are then activated with anti-mouse CD3/CD28 beads and rhIL-2 in RPMI10 medium (DMEM with 10% FBS and additives listed above) with 2-mercaptoethanol. 24 hours later, mouse T cells are harvested, counted and transduced with retrovirus in the presence of either protransduzin A or protamine sulfate and centrifuged for 1.5 hours at 32C to increase transduction efficiency. Cells are fed with RPMI10 medium with 2-mercaptoethanol and rhIL-2 and split as appropriate until being used for experiments on Day 5 or Day 7 post-isolation and activation. The steps below describe a workflow where mouse T cells are isolated and activated on Wednesday, transduced with retrovirus on Thursday (to coincide with retrovirus production schedule), and expanded in vitro, with transduction efficiency being measured on the following Monday (Day 5).

Wednesday

1. Euthanize mouse and collect spleen (and/or lymph nodes).
2. Pre-wet 70 um filter mounted on 50 ml Falcon tube on ice with DPBS.
3. Add spleen on top of 70 μm filter and process it through the filter with a plastic syringe stopper.
4. Add DPBS on top to wash any remaining splenocytes through the 70 μm filter.
5. Centrifuge 300 g 10 min.
6. Discard supernatant and resuspend pellet in 2 ml ACK Lysis Buffer.
7. Incubate 5 min on ice.
8. Add 25 ml DPBS and centrifuge 300 g 10 min.
9. Discard supernatant, resuspend cells in 10 ml DPBS and count with Trypan Blue.
10. Centrifuge 300 g 10 min.
11. Discard supernatant, resuspend cells in appropriate volume of cell separation buffer, and purify mouse T cells using, for example, STEMCELL Technologies EasySep Mouse T Cell Isolation Kit.
12. Count mouse T cells with Trypan Blue and centrifuge 300 g 10 min.
13. Wash anti-mouse CD3/CD28 dynabeads twice with DPBS using a magnet. Beads are at a concentration of 40,000 beads/μl and are used at a 1 bead:1 cell ratio. For every 10^6 mouse T cells, 25 μl beads are needed (10^6 beads).
14. Resuspend cells at 10^6 cells/ml in RPMI10 with 2-mercaptoethanol (1000X), 100 IU/ml rhIL-2 (5000X),

and anti-mouse CD3/CD28 beads (1 bead:1 cell ratio, 40,000 beads/μl), disperse 1 ml of cell suspension per 24-well. For example, for 10^7 mouse T cells, resuspend cells in 10 ml RPMI10, 10 μl 2-mercaptoethanol, 2 μl rhIL-2, and 250 μl beads, and seed in 10 wells of a 24-well plate, 1 ml per well.

15. Put cells in tissue culture incubator.

Thursday

1. Pre-heat centrifuge to 32C with well plate rotor before transduction.
2. Harvest mouse T cells and count with Trypan Blue.
3. Distribute cell suspension such that there are 10^6 cells (or a multiple) per tube for each transduction.
4. Centrifuge cells at 300 g 10 min.
5. Mix retrovirus-containing medium with protransduzin A or protamine sulfate at 10 μg/ml (stocks at 10 mg/ml in single use aliquots at -20C), 2-mercaptoethanol (1000X), and rhIL-2 (5000X).
6. Resuspend activated mouse T cells with retrovirus. Each 10^6 mouse T cells are resuspended in 1 ml retrovirus and seeded in a well of a 24-well plate. Keep some cells as untransduced controls.
7. Centrifuge 24-well plate at 3000 rpm 1.5 hours at 32C.
8. Put plate in tissue culture incubator.

Friday

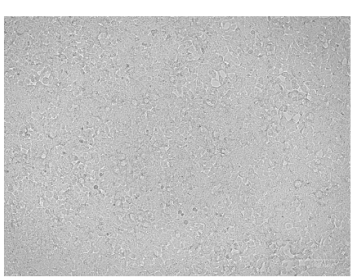
1. If possible, check mouse T cell transduction without disturbing the cells (e.g. GFP fluorescence).
2. Add 1 ml of fresh RPMI10 with 2-mercaptoethanol and rhIL-2 per well.
3. Monitor cell confluence and feed and split as needed over the next days.

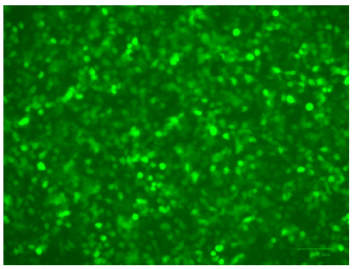
Monday

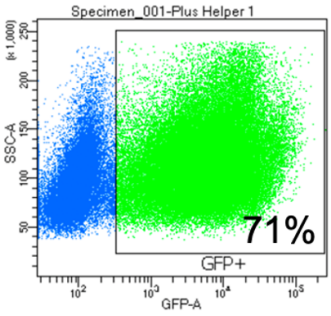
1. If possible, check mouse T cell transduction without disturbing the cells (e.g. GFP fluorescence).
2. Collect untransduced and transduced mouse T cells and assess transduction efficiency using flow cytometry.
3. Mouse T cells are usually used for experiments on Day 5 (Monday) or Day 7 (Wednesday) post-isolation.

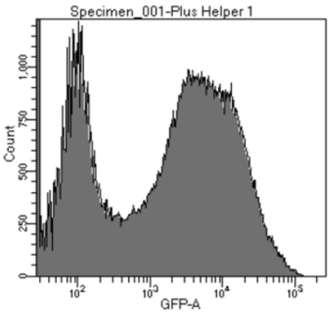
Expected results:

PlatE transfection (CAR-2A-GFP and helper plasmid, 48h after transfection)

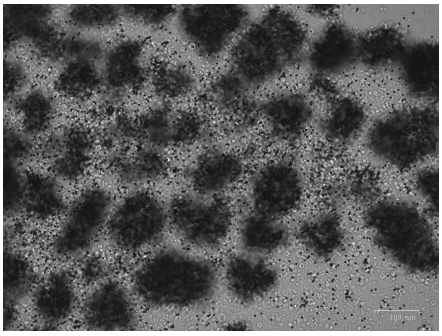




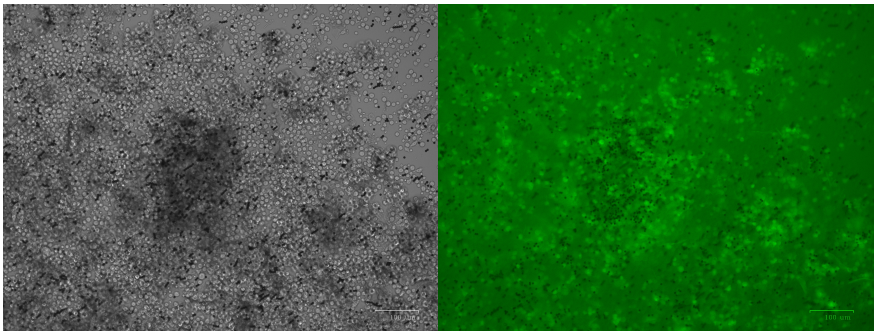




Mouse T cell activation (24h after activation)



Mouse T cell transduction (24h after transduction with CAR-2A-GFP retrovirus)



Mouse T cell transduction (4 days after transduction with CAR-2A-GFP retrovirus)