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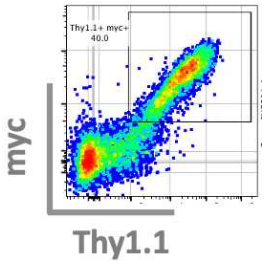
Retroviral transduction of primary murine CD8 T cells



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We use this protocol and it's working

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Abstract

This protocol will guide you through the process of how our group (Riddell Lab, Fred Hutchinson Cancer Center) generates murine CD8+ CAR T cells.

We normally begin this protocol on Monday, transduce the cells on Thursday, and immunophenotype them by the following Monday. The CAR T cells can then be used in standard cell-based assays (e.g. cytotoxicity, cell proliferation, or cytokine production) or for in vivo studies. See the following two protocols for details:
_ **51Cr Release Cytotoxicity Assay for murine CAR T cells**
_ **Standard cell-based assays for cytokine release and cellular proliferation of murine CAR T cells.**

The protocol will hopefully elaborate on the usual methods-section write-up that reads something like this:

Murine T cell viral transduction and adoptive transfer: CD8+ T cells were negatively selected (Stem Cell) from spleen and peripheral lymph nodes of 6–8 week-old CD45.1 mice, and stimulated with 1 mg/ml plate-bound anti-CD3 (145-2C11) and anti-CD28 (37.51) for 18-20 h at 37°C 5% CO2 in complete RPMI (RPMI-1640, 10% heat-inactivated FBS, 1 mM HEPES, 100 U/mL penicillin/streptomycin, 1 mM sodium pyruvate, and 50 µM b-mercaptoethanol) supplemented with 50 U/mL human IL-2 (IL-2+, Peprotech). Retrovirus was captured for 2 h at 2560rcf at 32°C onto wells pre-coated with RetroNectin (Takara) and CD8+ T cells were added at 1×106 cells/mL in IL-2+ complete RPMI and mouse T-activator Dynabeads (ThermoFisher) at 1:1 ratio. Plates were centrifuged at 800rcf for 30 min at 32°C and incubated overnight. T cells were then resuspended in 1.5x volume of IL-2+ media and incubated for an additional 24 h. T cells were resuspended in 3x volume of IL-15+ media and incubated for an additional 48 h. Activator beads were removed and transduction efficiency was determined by flow cytometry. CAR T cells were prepared for infusion by resuspending at the indicated number of CAR T cells per 100 µL serum-free RPMI-1640 and kept on ice prior to adoptive transfer. 6-8 week-old C57BL/6J female mice were pre-conditioned as indicated and 6 h later injected intravenously with 3×106 CAR T cells.

Attachments

XLSX

Retroviral transduct...

12KB

Guidelines

CD4 T cells can be isolated to generate CD4+ CAR T cells instead of CD8 equivalents, and the protocol can be used to engineer murine T cells by viral transduction to express proteins other than CARs as long as these proteins are encoded in a retroviral vector. Viral production cells other than plat-Es can be used as long as vectors encoding the gag, pol, env genes are included in the transfection workflow. Transfection reagents other than Xfect can be used according to their respective manufacturer's protocols. Lastly, large-batch retroviral supernatants can be generated in advance and snap-frozen in liquid nitrogen and placed at -80C long-term storage, instead of the fresh supernatants being generated as described below.

Large-batch production of murine CAR-encoding retroviruses carries the advantage of allowing for the careful titration of the dilution required for the transduction to achieve a desired transduction efficiency by the end of the culturing period. For example, if 50% CAR+ T cell cultures are desired, a pilot study with various dilutions of the freshly thawed supernatant can be performed to make that determination. The remainder of that retroviral sup lot can diluted at the same ratio every time a scalable CAR T cell transduction is performed.

Materials

See the attached spreadsheet titled "Reagent and equipment list" for details.

Protocol materials

EasySep Mouse CD8 T-Cell Isolation Kit **STEMCELL Technologies Inc.** **Catalog #**19853A

Xfect Transfection Reagent **Takara Bio Inc.** **Catalog #**631318

Troubleshooting

Before start

Prepare reagents listed below.

- 1L **cDMEM** [1000 mL DMEM (1x), 100mL FBS (heat inactivated), 25 mL HEPES (1M), 10mL L-Glutamine (200 mM), 10 mL Pen/Strep. Filter the mix through 500 mL Bottle top Filter (0.2 µm aPES membrane)].

- 1L **mTCM** [1000 mL RPMI1640 (w/ 20 mM HEPES) (1x), 100 mL FBS (heat inactivated), 10 mL Sodium Pyruvate (100 mM), 1 mL HEPES (1 M), 10 mL Pen/Strep, 100 uL 2-Mercaptoethanol (0.5 M). Filter the mix through 500 mL Bottle top Filter (0.2um aPES membrane)].

- 500 mL **EasySep Buffer** [500 mL D-PBS (1x), 10 mL FBS (heat inactivated), 1 mL EDTA (0.5 M). Filter the mix through 500mL Bottle top Filter (0.2 µm aPES membrane)].

- 500 mL **2% BSA blocking solution** [500 mL D-PBS (1x), 10 g dry weight Bovine Serum Albumin. Filter through 500 mL Bottle top Filter (0.2 µm aPES membrane)]. Combine and let dissolve for >15 minutes prior to filtering.

- 500 mL **Flow Buffer** [500 mL D-PBS (1x), 2.5 mL FBS (heat inactivated), 3.6 mL EDTA (0.5 M)]. No need to filter.

- Diluted **Retronectin** (12.5 µg/mL diluted in 1x D-PBS and aliquotes stored at -20C until use).

- 1000x **human IL-2** stock (50,000 IU/mL in 1x D-PBS; reconstituted per manufacturer). hIL-2 is cross-reactive and is more economical.

- 1000x **human IL-15** stock (10,000 IU/mL in 1x D-PBS; reconstituted per manufacturer). hIL-15 is cross-reactive and is more economical.

Day 1: Plating the plat-E cells

1 Approximately 20 hours prior to transfection of platinum-E (plat-E) cells, generate a working stock of [M] 0.001 % (v/v) poly-L-lysine (PLL) by diluting 0.1% PLL stock 1:100 in 1x D-PBS and coat a 6-well-plate well with 1.5 mL for 00:15:00 at room temperature . 15m

Note

A 6-well-plate well yields 2.5-3 mL retroviral supernatant (sup). The step can be scaled up as needed. (e.g. a 15 cm dish will yield approximately 30 mL retroviral sup. To coat a 15 cm dish, use 15 mL PLL).

1.1 Aspirate PLL and replace with 2 mL pre-warmed cDMEM.

Note

cDMEM recipe:
DMEM (gibco)
10% heat-inactivated FBS
100 U/mL penicillin/streptomycin
2mM L-Glutamine
25mM HEPES
Filter through 0.2um before use.

mTCM recipe:
RPMI 1640 (+HEPES)
10% heat-inactivated FBS
1 mM HEPES
100 U/mL penicillin/streptomycin
1 mM sodium pyruvate
50 µM b-mercaptoethanol
50 U/mL human IL-2 (Peprotech)
Filter through 0.2um before use.

2 Dissociate plat-E cells using Trypsin-EDTA (0.05%), resuspend the cells at 7.5×10^5 / mL in cDMEM, and transfer 1 mL to the PLL-coated well (final volume = 3 mL). Rock the cells to ensure even monolayer formation and incubate at 37 °C, 5% CO2, 95% humidity overnight.

Note

A detailed protocol for dissociation of adherent cells can be found **here**. Dissociated cells can be enumerated using a hemocytometer (**protocol link**) or an automated cell-counter (e.g. **Cellometer X2**).

For optimal viral production, plat-E cells should be 50-70% confluent at time of transfection.

Day 2: Transfection of plat-E cells for retroviral packaging

3 Transfect Plat-E cells for retroviral packaging using
Xfect Transfection Reagent **Takara Bio Inc. Catalog #**631318

3.1 Starting in the afternoon: In a sterile 1.7 mL microcentrifuge tube, dilute 7 µg of plasmid in Xfect buffer to a final volume of 100 µL. Vortex briefly and pulse centrifuge the tube.

Note

- e.g. If plasmid concentration is 1 mg/mL, use 7 µL DNA + 93 µL Xfect buffer for 7 µg plasmid in Xfect.
- The optimal amount used may vary by plasmid. If long-term plasmid consumption is a factor, a one-time serial titration of <20 µg plasmid can reveal a "sweet spot" for the transfection, yielding high retroviral titer with minimal plasmid amount.
- Make sure to add plasmid to the buffer ***before*** adding Xfect polymer.
- Do not use less than 2.5 µg of plasmid DNA, and at least 50% of the reaction volume must be Xfect reaction buffer.

3.2 Add 2.25 µL Xfect Polymer to each tube. Immediately vortex for at least 10 seconds at high speed. 10m

Incubate 00:10:00 at room temperature

Vortex again briefly and pulse centrifuge.

Note

Xfect Polymer freezes at 4 °C. Before use, allow the vial to warm up before you vortex and pulse centrifuge.

Xfect Polymer is also viscous; pipette patiently and plunge to the second stop to dispense the entire polymer volume into the tube.

3.3 Apply Xfect mixture dropwise to the respective well and gently rock the plate to mix.

Place transfection plate in the incubator (37 °C, 5% CO2, 95% humidity) overnight.

Note

The length of optimal incubation depends on the final viral yield and the health of the transfected cells at the end of the incubation period. We found overnight to be non-toxic to plat-E cells, but the incubation could be shortened to 6-12 hours by starting the transfection earlier in the day, if the viral yield is not ultimately affected.

3.4 Following the overnight incubation, exchange the transfection medium (on day 3) with fresh pre-warmed cDMEM and incubate the transfected plat-E culture for additional 24-30 hours. Harvest the supernatant (on day 4) and filter through 0.45 µm. Collected retroviral supernatant can be snap-frozen and placed at -80 °C long-term. Otherwise, the retroviral sup is ready for immediate use to transduce murine T cells.

Note

It is critical to use 0.45 µm filters and not 0.22 µm, as the latter affects the viral yield.

4 **Prepare the plate-bound antibodies [on day 2] for T cell stimulation for the following day [day 3]:**

Dilute anti-CD3 (1 mg/mL) and anti-CD28 (1 mg/mL) antibodies 1:1000 in PBS. Coat the required number of wells in a 6-well non-TC plate with 1 µg/mL anti-CD3 and 1 µg/mL anti-CD28 in PBS (4 mL/well). Incubate 4 °C Overnight .

Note

Coat 1 to 3 wells per mouse expected to be harvested on the following day. The isolation should yield approximately 1.2-1.5 x 10^7 murine CD8 T cells per mouse.

Day 3: Isolation of murine lymphocytes and their activation

5 Prepare the following for the trip to the vivarium:
- Ice bucket
- 6-well plate with 4 mL mTCM per well (1 well per mouse)
- Sterile, curved medium-point forceps
- Sterile, stainless steel surgical scissors

6 Euthanize mice per IACUC-approved protocol, and harvest the spleen and lymph nodes (inguinal LNs can be sufficient, but adding brachial, axillary, and superficial cervical LNs will increase the CD8 T cell yield).

Process harvested LN and spleen in a biological safety cabinet.

Note

A video tutorial on harvesting spleen and inguinal lymph nodes can be found here: https://www.youtube.com/watch?v=Mcmo8Z2u28s

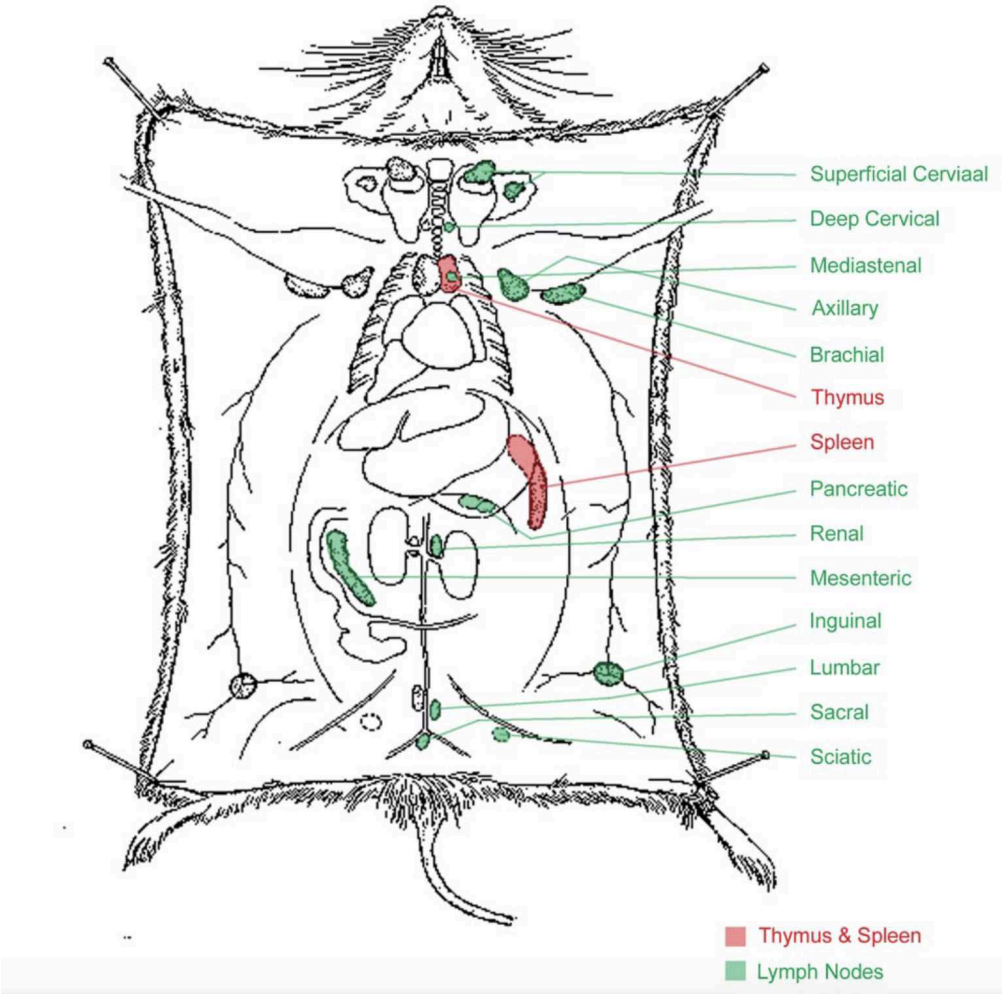

Diagram of Mouse Thymus, Spleen and Lymph Nodes (Creighton University; **link**)

7 Mechanically dissociate spleen and LNs by repeatedly pressing and grinding them between the frosted surfaces of two glass slides. Collect the dissociated tissue in a new 6-well-plate well with fresh mTCM.

Note

Proper mechanical dissociation of the lymph nodes and spleen is vital to an efficient cell isolation. If not properly ground, the reaction may produce a low concentration of cells. The lymph nodes should be soft; be gentle and try grinding a few at a time under medium pressure. Rinse the frosted slides off well with mTCM between groups. Splenocytes are very fragile; once the organ is crushed, rinse the slides and move on.

It's very easy to lose lymph nodes in the slurry of grinding, Use separate plates for 1) collecting lymph nodes and 2) grinding and rinsing the frosted slides.

8 Filter the cell suspension into a 15 mL conical tube through a 100 µm cell strainer. Rinse each well with with 1 mL mTCM and filter through the same strainer.

9 Isolate CD8 T cells using the

EasySep Mouse CD8 T-Cell Isolation Kit **STEMCELL Technologies Inc. Catalog #**19853A

:

9.1 Centrifuge the filtered cells (from step 8) 400 rcf, Room temperature, 00:05:00 . 15m

Remove supernatant with a pipet, being careful to avoid the cell pellet. Re-suspend in 1 ml StemCell buffer *per mouse-equivalent* in tube. Add 20 µL FcR blocker (equivalent to 50 µL Normal Rat Serum) and 50 µL CD8-isolation antibody cocktail each *per mouse-equivalent* and incubate 00:10:00 at room temperature .

Note

The slurry of all collected LN's and spleen from an individual mouse is equal to 1 mouse-equivalent.

E.g. 2 mouse-equivalents of cells in a single tube, add 40 µL FcR Blocker + 100 µL CD8 isolation antibody cocktail (~2 mL volume).

The StemCell CD8 isolation kit works well isolating up to 3 mouse-equivalents of CD8 T cells in a single 15mL conical at once. Avoid combining more than 3 mouse-equivalents per reaction.

9.2 Add 125 µL RapidSpheres *per mouse equivalent* and incubate 00:05:00 at room remperature . 5m

9.3 Add StemCell buffer up to a volume of approximately 8-9 mL, place uncapped 15 mL conical in "The Big Easy" magnet for 00:05:00 at room temperaure . 5m

9.4 Carefully pour the enriched T cell suspension into a new 15 mL conical. Centrifuge cells to pellet 400 rcf, Room temperature, 00:05:00 . Re-suspend in 10 mL mTCM. 5m

Enumerate T cells using a hemocytometer or automated cell counter.

10 Prepare cell culture media by diluting recombinant human IL-2 (IL-2) in mTCM to a concentration of 50 IU/mL (mTCM+IL-2)

Note

Ahead of time, prepare 500 µL aliquots of IL-2 at a concentration of 50,000 IU/mL. Store at -80 °C until use. Dilute IL-2 into mTCM at a ratio of 1 µL IL-2 : 1 mL mTCM.

11 Centrifuge cells to pellet 400 rcf, Room temperature, 00:05:00 . Re-suspend cells to a concentration to 1×10^6 cells/mL in mTCM + 50 IU/ml IL-2. 5m

12 Remove the PBS solution from CD3/28-coated plate (prepared on day 2 and stored at 4 °C) and add up to 5 mL T cell suspension to each coated well. Incubate at 37 °C overnight

13 **Prepare recombinant human fibronectin (RetroNectin)-coated plate in preparation for following day (day 4):**

Coat a non-TC-treated 24-well flat bottom plate with 400 µL 12.5 µg/ml RetroNectin in 1x D-PBS per well. Prepare 1 well per 1×10^6 T cells to be transduced, with the addition of 1 well for a non-transduced negative control.

Incubate 4 °C Overnight .

Note

Ahead of time, prepare 10 mL aliquots of RetroNectin at a concentration of 12.5 µg/ml diluted in 1x D-PBS. Store at -20C for long term use.
e.g. (2.5 mL RetroNectin)(1000 µg/ml)=(2.5 mL Retronectin + 197.5 mL 1x D-PBS)(12.5 µg/ml)

Day 4: Transduction of T cells

14 Transfer the RetroNectin solution (incubated from day 3) to a second non-TC-treated 24-well flat bottom plate.
Set aside and incubate 4 °C Overnight .

15 To the first RetroNectin-coated plate, add 500 µL 2% BSA in 1x D-PBS per well. Block the wells by incubating 00:30:00 at 37C . 30m

16 While the wells are being blocked, return to the transfected plat-E cultures to prepare the retroviral sup for T cell transduction. Following the 24-30 hour incubation (from day 3), collect retroviral sup from the Xfected Plat-E cells. Filter through 0.45 µm filter.

Note

It is critical to use 0.45 µm filters and not 0.22 µm, as the latter affects the viral yield.

The transfected cells continue to produce viral titers well beyond 24 hours. If needed, cDMEM media can be replenished and another round of sup collection can be performed at the 48 hr time-point. Collected retroviral sup can be snap frozen and placed at -80 °C long-term.

17 Rinse the blocked RetroNectin wells with 1 mL 1x D-PBS, aspirate, then add 1 mL fresh retrovirus sup to each blocked well.

Note

Viral titers may vary between batches and plasmids. As with the transfection reaction, for frozen products intended for long term use, a one time titration experiment (1:0, 1:2, and 1:4 ratio of virus diluted into complete media respectively) may reveal a "sweet spot" for the transduction.

18 Carefully wrap each covered 24-well plate in tin foil to minimize evaporation, balance, and centrifuge 2h
2560 rcf, 32°C, 02:00:00 , acceleration:3, deceleration:2

19 Before the retroviral capture is completed, collect the T cells activated on day 3. Suspend and enumerate cells by hemocytometer or automated cell counter. Centrifuge to pellet 400 rcf, Room temperature, 00:05:00 . After centrifugation, re-suspend at a concentration of 1×10^6/mL in fresh mTCM+IL-2. 5m

20 Rinse enough Gibco Dynabead mouse T-activator CD3/28 (Dynabeads) to achieve a final 1:1 ratio with T cells: aliquot Dynabeads to 9 mL fresh mTCM and place in the EastEight EasySep magnet. Incubate 00:05:00 at room temperature . Aspirate mTCM being careful to avoid Dynabeads. Combine the T cells with Dynabeads at 1:1 ratio and briefly set aside. 5m

Note

Before pipetting Dynabeads, vortex at high speed and work quickly to ensure homogeneity and accurate ratio with T cells.

Gibco Dynabead mouse T- Activator CD3/28 stock concentration is $4x10^7$/mL.

e.g. To activate $2x10^7$ CD8 T Cells, combine 500 µL of well-vortex'ed Dynabeads ($4x10^7$ /mL) with 9mL mTCM.

21 Collect viral capture plate from centrifuge. 30m

Work quickly - *one well at a time* - such that wells do not dry out.

Aspirate virus supernatant and rinse the well with 1 mL 1x D-PBS. Aspirate PBS using the pipette, then transfer 1 mL homogenous cell suspension onto the virus-captured well.

Once T cells are added, re-wrap the virus-coated plate in foil to minimize evaporation. Centrifuge 800 rcf, 32°C, 00:30:00 .

When complete, unwrap foil, place the cells in the incubator 37 °C Overnight .

Day 5: Culturing T cells in IL-2 mTCM

22 Collect T cells from incubator. Mix the T cells well with a P-1000 pipette and transfer into sterile micro-centrifuge tubes. Centrifuge samples 400 rcf, 00:05:00 . Aspirate supernatant while being careful to avoid the cell pellet. Re-suspend each cell pellet in 1.5x the initial volume, 1.5 mL mTCM+IL-2. 5m

23 Retrieve the second RetroNectin plate that was placed into 4 °C (on day 4). Aspirate the old Retronectin and transfer T cells into respective wells. Incubate 37 °C Overnight .

Day 6: Culturing T cells in IL-15 mTCM

24 Prepare cell culture media by diluting recombinant murine IL-15 (IL-15) in mTCM to a concentration of 10 IU/mL (mTCM+IL-15)

Note

Ahead of time, prepare 500 uL stock aliquots of IL-15 at a concentration of 10,000 IU/mL. Store at -80 °C until use. Dilute IL-15 into mTCM at a ratio of 1 µL IL-15 : 1000 µL mTCM.

25 Mix T cells well with a P-1000 piepette and transfer to a 15 mL tube. Rinse plate-well with 1 mL mTCM and transfer to respective tube. Enumerate T cells by hemocytometer or automated cell counter. Centrifuge 400 rcf, 00:05:00 . Re-suspend to adjust concentration to 1×10^6 cells/mL mTCM+IL-15. 5m

Incubate 37 °C 48 hours

Note

e.g. Choosing appropriate TC vessel:
< $1x10^6$ cells --> 24-well tissue culture treated plate.
$1\text{-}5x10^6$ cells --> 6-well tissue culture treated plate.
> $5x10^6$ cells --> upright T25 flask.

Day 8: Immunophenotyping to assess the transduction efficiency 5m

26 Transfer each cell culture to 15 mL conical tubes. Remove Dynabeads by placing the tubes onto EasyEight EasySep Magnet for 2 minutes. Transfer the "de-beaded" culture by pipetting into new 15 mL conical tube. Enumerate by hemocytometer or automated cell counter.

27 Immunophenotype the samples with the appropriate markers (e.g. CD8, transduction marker, congenic marker, etc..) by flow cytometry.

If desired, further enrich transduced cells by sorting or using magnetic isolation kits targeting a transduction marker.

28 Prepare cells for *in vivo* experiment and/or setup *in vitro* assays. See the following two protocols for details: 5m

_ **51Cr Release Cytotoxicity Assay for murine CAR T cells**

_ **Standard cell-based assays for cytokine release and cellular proliferation of murine CAR T cells.**

If not needed immediately, CAR T cells can be cultured for an additional day before use. To culture for an additional day, centrifuge cells 400 rcf, 00:05:00 , then re-suspend them at a concentration of 1×10^6/mL mTCM+IL-15. Place back in incubator 37 °C Overnight .

Protocol references

Takara Bio Xfect reagent protocol:
https://www.takarabio.com/documents/User%20Manual/Xfect%20Transfection%20Reagent%20Protocol/Xfect%20Transfection%20Reagent%20Protocol-At-A-Glance_103012.pdf

StemCell Murine CD8 T-cell negative selection isolation protocol:
https://cdn.stemcell.com/media/files/pis/10000003748-PIS_03.pdf

Retronectin reagent preparation protocol:
https://takara.co.kr/file/manual/pdf/T100A_B_e.v1705.pdf