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High-Efficiency Generation of Antigen Specific Primary Mouse Cytotoxic T Cells for Functional Test in Autoimmune Diabetes Model.

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Dear Editors,

On behalf of my co-authors, I wish to submit our reversion article titled "High-Efficiency Generation of Antigen Specific Primary Mouse Cytotoxic T Cells for Functional Test in Autoimmune Diabetes Model." for consideration by the *Journal of Visualized Experiments*. I confirm that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

Developing a safe and effective antigen specific immunotherapy is a primary goal of much current type 1 diabetes research. In this manuscript, we described an optimized protocol to generate antigen specific T cells for treating autoimmune diabetes purpose. Following this protocol, we have successfully reprogramed the progression of type 1 diabetes in NOD mouse.

We believe that our approach are significant because it provides a method to generate antigen specific T cells starting from small number of naïve T cells with a large number of functional antigen specific T cells as a safe immune therapy.

We believe that this manuscript is appropriate for publication by the *Journal of Visualized Experiments.* The manuscript, tables and figures are prepared following the instruction of your journal instruction. We use JoVE EndNote style in preparing references.

I acknowledges that I am an inventor on a patent describing the therapeutic use of inhibitory antibodies targeting peptide/MHC complexes in autoimmunity. None of the other authors have any potential conflicts of interest to disclose.

Thank you for considering this manuscript!

Sincerely,

Li Zhang

TITLE:

High-Efficiency Generation of Antigen-Specific Primary Mouse Cytotoxic T Cells for Functional Testing in an Autoimmune Diabetes Model

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KEYWORDS:

Type 1 Diabetes, antigen specificity, monoclonal antibody, chimeric antigen receptor, transduction, insulin, epitope, CD8 T cell, NOD mouse

SUMMARY:

This article describes a protocol for the generation of antigen-specific CD8 T cells, and their expansion in vitro, with the aim of yielding high numbers of functional T cells for use in vitro and in vivo.

ABSTRACT:

Type 1 Diabetes (T1D) is characterized by islet-specific autoimmunity leading to beta cell destruction and absolute loss of insulin production. In the spontaneous non-obese diabetes (NOD) mouse model, insulin is the primary target, and genetic manipulation of these animals to remove a single key insulin epitope prevents disease. Thus, selective elimination of professional antigen presenting cells (APCs) bearing this pathogenic epitope is an approach to inhibit the unwanted insulin-specific autoimmune responses, and likely has greater translational potential.

Chimeric antigen receptors (CARs) can redirect T cells to selectively target disease-causing antigens. This technique is fundamental to recent attempts to use cellular engineering for adoptive cell therapy to treat multiple cancers. In this protocol, we describe an optimized T-cell

retrovirus (RV) transduction and in vitro expansion protocol that generates high numbers of functional antigen-specific CD8 CAR-T cells from low starting numbers of naive cells. Previously multiple CAR-T cell protocols have been described, but typically with relatively low transduction efficiency and cell viability following transduction. In contrast, our protocol provides up to 90% transduction efficiency, and the cells generated can survive more than two weeks in vivo and significantly delay disease onset following a single infusion. We provide a detailed description of the cell maintenance and transduction protocol, so that the critical steps can be easily followed. The whole procedure from primary cell isolation to CAR expression can be performed within 14 days. The general method may be applied to any mouse disease model in which the target is known. Similarly, the specific application (targeting a pathogenic peptide/MHC class II complex) is applicable to any other autoimmune disease model for which a key complex has been identified.

INTRODUCTION:

Given the likely reduced risk of unwanted off-target effects, antigen-specific immune therapies (ASI) are promising treatments for autoimmune diseases such as T1D. Accumulating evidence suggests that immune responses to (prepro)insulin may be particularly important in T1D[1]. In the past decade, studies from multiple groups, including our own, strongly suggest that presentation of an epitope containing insulin B chain amino acids 9 to 23 by specific MHC class II molecules (B:9-23/MHCII), plays an important role in the development of T1D in mice and humans[2-5]. To selectively target the B:9-23/MHCII complex, we generated a monoclonal antibody, named mAb287, that has no cross reactivity to the hormone insulin or complexes containing other peptides[6]. MAb287 blocks antigen presentation in vitro, and weekly administration of mAb287 to pre-diabetic NOD mice delayed the development of T1D in 35% of the treated mice[6]. To block antigen presentation in vivo, frequent injections are typically required in order to maintain a high circulating concentration. We hypothesized that we could overcome this difficulty by taking advantage of the high specificity of Ab287 to reprogram T cells, thereby providing an improved antigen-specific T cell therapy for T1D[7].

Cytotoxic T cells are reported to be able to kill their target if even a single copy of their cognate ligand is expressed[8-10]. Thus, B:9-23/MHCII specific CD8 T cells are expected to have higher efficiency in eliminating the unwanted antigen presentation than the parent antibody, which will likely need to bind to multiple complexes on the same APC to exert its effect. CAR T cells have been used for treating multiple human cancers[11-13], and may also be efficacious in autoimmunity[14]. However, CAR-T cells with specificity for pathogenic peptide-MHC complexes have not so far been used to modify the progression of T1D. By using the optimized CD8 T cell transduction technique described below, we recently demonstrated proof of principle that this indeed represents a viable approach[7].

In this protocol, we outline an efficient and streamlined transduction and expansion method. Our protocol is applicable to other studies requiring the generation of mouse CD8 CAR T cells with high efficiency.

PROTOCOL:

Mice were maintained under specific pathogen-free conditions at a Transgenic Mouse Facility, and all animal experiments were performed in accordance with protocols approved by the Baylor College of Medicine animal care and use committee.

NOTE: The experiment requires preparing the virus and the T cells in parallel. **Table 1** summarizes the protocol. The key reagents and buffers are listed in the **Table of Materials**. We focus on the generation and expansion of CAR-T cells targeting specific populations of APCs in this protocol.

1. **Generation and validation of single chain Fab antibody (scFab)-CARs.**

NOTE: CARs typically contain 3 critical domains—an antigen targeting domain, a spacer/transmembrane domain, and a cytoplasmic signaling domain. The precise design of each CAR depends on the intended target, and so, apart from the key features of the construct relevant to the generation of the retrovirus, will not be described in detail in this protocol. The overall design of the CARs used for the studies described below is shown in **Figure 1**. In brief, the targeting domain comprises the entire light chain and variable and CH1 domain of the heavy chain from the parent monoclonal antibody linked by a semi-rigid linker. The spacer/transmembrane domain is from mouse CD28, and the signaling domain is a fusion containing elements from mouse CD28, CD137 (4-1BB), and CD247 (CD3ζ). These elements are assembled by standard molecular biology procedures such as splice overlap polymerase chain reaction (PCR), or the synthesis of an appropriate "gene block". Details of the generation of the mAB287 CAR are contained in Zhang et al.[7]. The cDNA sequences can be obtained from the authors upon request.

1.1. **Assembling the CAR construct**

1.1.1. Synthesize the targeting single chain Fab antibody (scFab) and combined spacer/signaling domains separately, and use a "3 point" ligation technique[15] to assemble the final construct (**Figure 1**).

NOTE: The key requirement for the CAR insert is that it should contain flanking restriction endonuclease sites allowing ligation into the retroviral expression vector pMSCV-IRES-GFP II (pMIG II), or a related derivative. We use a "3 point" ligation technique but other molecular cloning strategies [15] are also appropriate.

1.2. **Validation of CAR surface expression**

1.2.1. Transduce the hybridoma cells using pMIG II derived retroviral particles generated by a standard protocol (e.g., Holst et al.[16]).

1.2.2. Run flow cytometry analysis to detect the expression of GFP from the CAR vector[17].

1.2.3. Stain surface expression of the CAR of the transduced hybridomas using labeled antibodies against the mouse κ chain (e.g., clone RMK-45)[17].

NOTE: To confirm that the construct is functional, expression in a suitable T cell hybridoma capable of cytokine secretion is necessary[18].

1.3. **Validation of CAR specificity**

1.3.1. Stimulate the transduced hybridoma cells with appropriate plate-bound or cellular antigens. After overnight co-culture collect supernatants and secreted cytokines, and assayed by enzyme-linked immunosorbent assay (ELISA)[7].

NOTE: Ideally, each CAR should be independently validated before being used for transduction. At this step, the experiment may be paused and restarted later.

2. **Transfection of viral producer cells (day -4 to day 3)**

NOTE: Retrovirus is produced using Phoenix-ECO cells (see the **Table of Materials**)[19,20]. Use appropriate precautions for the generation of potentially infectious agents (preferably including a designated BSL-2 cabinet and separate incubator for culturing transfected/transduced cells).

2.1. **Thawing Phoenix cells (Day -4)**

2.1.1. Thaw 2 x 10^6 Phoenix-Eco cells. Scale up the number of Phoenix cells if multiple transductions are planned.

2.1.2. Plate them in a 10 cm tissue culture dish with 10 mL of medium (Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS)).

2.2. **Passage Phoenix cells (Day -3)**

2.2.1. Remove medium, and wash with 5 mL of Dulbecco's phosphate-buffered saline (DPBS).

2.2.2. Add 3 mL of 0.25% trypsin and incubate at 37 °C under 10% CO_2 atmosphere for 3 min.

2.2.3. Harvest the cells then pellet by centrifugation for 3 min at 200 x *g*. Re-plate cells with 10 mL fresh medium and incubate at 37 °C.

2.3. **Irradiation of Phoenix cells (Day -1 afternoon**)

2.3.1. To minimize further cell division, collect Phoenix cells as described in step 2.2, resuspend in 5 mL of medium, and gamma irradiate cells on ice (1000 rad).

NOTE: Caution should be used for radiation work to avoid personnel exposure.

2.3.2. Centrifuge the irradiated cells, resuspend in fresh medium, plate at 2 x 10^6 cells (in 10 mL of medium)/plate/CAR, and incubate.

2.4. **Transfection (Day 0 - morning)**

2.4.1. Aspirate the supernatant from the Phoenix cells, wash with 5 mL of phosphate-buffered saline (PBS), and carefully add 7 mL of reduced serum medium (e.g., Opti-MEM) dropwise to the sidewall of the plate to avoid disturbing the monolayer. Transfer cells back to incubator.

2.4.2. Take two 14 mL round bottom polypropylene tubes, and add 1.5 mL of reduced serum medium to each. To one tube, add 40 µL of transfection reagent (see the **Table of Materials**).

2.4.3. To the other tube, add 15 µg of Ab-CAR-plasmid (generated in step 1) and 5 µg of envelope and packaging plasmid (5 µg pCL-Eco). Incubate tubes at room temperature for 5 min.

2.4.4. Add the transfection reagent mixture from step 2.4.2 dropwise to the second tube without contacting the tube sides, and mix by pipetting the solution up and down gently 3 times. Incubate at room temperature for at least 20 min.

2.4.5. Add 3 mL of the mixture dropwise to the Phoenix cells, and place in a tissue culture incubator.

2.4.6. After 4–5 h add 1 mL of FCS. Culture cells overnight at 37 °C.

2.5. **Medium change (Day 1)**

2.5.1. Remove the supernatant containing the plasmid/transfection reagent complexes and dispose of them in accordance with institutional procedures for handling infectious material. Add 4 mL of fresh, pre-warmed culture medium to the cells.

2.6. **Harvest virus for Transduction (Day 2)**

2.6.1. Collect the virus-containing medium from the Phoenix cells with a sterile syringe, filter (0.45 µm) to remove residual cell debris, and collect in a new tube.

2.6.2. Add rhIL-2 stock to a final concentration of 200 IU/mL. Use the virus immediately for transduction (step 5.3). Add 4 mL of fresh medium to the Phoenix cells and place in the incubator.

2.7. **Repeat virus collection (Day 3)**

2.7.1. Repeat step 2.6, but discard Phoenix cells as infectious waste instead of adding fresh medium. This supernatant is used in step 5.4.

3. **Primary CD8 T cell isolation and activation (day -1 to day 0)**

NOTE: Previously, collect CD8 T cells from female NOD mice at 4–5 weeks, a time point before islet inflammation starts[21,22]. Handle all the mice following IACUC approved protocols. CD8 T cells are enriched from splenocytes using a commercial negative selection kit.

3.1. **Coating plates with CD3/CD28 antibodies (Day -1)**

3.1.1. Add 1 mL of a mixture of anti-mouse CD3 and CD28 antibodies (both at 1 µg/mL in PBS) to each well of a 24-well plate, and incubate at 4 °C overnight.

3.1.2. The next day, wash the plates with 1 mL of sterile PBS 3 times before adding the murine CD8 T cells (step 4.1).

NOTE: The number of wells to be coated will vary for each experiment, depending on the total number of activated CD8 T cells required.

3.2. **Collection of splenocytes (Day 0)**

3.2.1. Euthanize two NOD female mice aged 4–5 weeks using CO_2 inhalation followed by decapitation. Harvest the spleens and put them onto a cell strainer soaking in 10 mL PBS in a cell culture dish on the ice.

3.2.2. In a cell culture hood, cut each spleen into 3–5 pieces, press tissues with a sterile plunger of a 3 or 5 mL syringe to force spleen fragments apart and allow cells to pass through the wire mesh.

3.2.3. Gently remove red blood cells by resuspending splenocytes in 1:4 diluted red cell lysis buffer (1 mL of lysis buffer in 3 mL of PBS for one spleen), and incubating for 5 min at room temperature.

3.2.4. Then, dilute 10 µL of the cell suspension with trypan blue dye solution for counting cells with a hemocytometer, and pellet the rest of the cells by centrifugation at 350 x *g* for 7 min.

3.3. **Enrichment of CD8 T cells (Day 0)**

3.3.1. Enrich CD8 T cells by negative selection using a mouse CD8 T cell isolation kit, following the manufacturer's instructions.

NOTE: To ensure high purity always round up the cell numbers when calculating the volume of biotinylated-antibody to be added (e.g., use the volume of reagents suggested for 10^8 cells for a calculated 9.1 x 10^7 cells).

3.3.2. Suspend cell pellets in 400 µL of buffer and 100 µL of biotin-antibody cocktail per 1 x 10^8 cells, mix well and incubate for 5 min in the refrigerator (4 °C) to allow antibody binding.

3.3.3. Add 300 µL of labeling buffer and 200 µL of anti-biotin micro-beads per 1 x 10^8 cells, mix well and incubate for 10 min at 4 °C.

3.3.4. While waiting for the micro-bead binding, set up the separation column onto the separator. Wash column by rinsing with 3 mL of labeling buffer.

3.3.5. Pass 1000 µL of bead/cell mixture through a 40 µm cell strainer before loading onto the separation column to remove cell aggregates. Collect the column flow-through into a pre-chilled 15 mL tube.

3.3.6. Wash the column as instructed by the manufacturer, collecting all the effluent into the same tube. Determine the cell number (same as step 3.2.4) and collect by centrifugation at 350 x *g* for 5 min. Wash the cells by resuspending in 2 mL of complete T cell medium (RPMI-1640 containing FCS, 2-mercaptoethanol, rhIL-2 (200 U/mL), mIL-7 (0.5 ng/mL), ITS, HEPES and penicillin-streptomycin) and centrifuging at 350 x *g* for 5 min.

3.3.7. Resuspend the cells in pre-warmed (37 °C) complete T cell medium at a concentration of 0.25–0.5 x 10^6/mL.

4. **T cell activation (Day 0 to 2)**

4.1. Add 2 mL of the cell suspension (0.25–0.5 x 10^6/mL) to each coated well of the CD3/CD28 antibody coated 24-well plate from step 3.1.2. Use a swirling motion to dispense the cells evenly.

NOTE: Add the cells using a swirling motion to distribute them evenly and minimize edge effects. If the cells cluster along the edge of the wells, both the transduction rate and cell viability will be decreased.

4.2. As a control, plate the same number of CD8 T cells into a single non-coated well of the plate. Incubate the cells at 37 °C using a 10% CO_2 gassed incubator for 48 h.

NOTE: After 48 h, activation can be confirmed using a microscope; the activated cells will be larger than the cells that did not encounter anti-CD3/CD28 antibodies.

[place figure 2 here]

5. Transduction of activated CD8 T cells (days 1 to 3)

NOTE: This protocol uses a spin-transduction method. A centrifuge with a swing-out rotor and tissue culture plate adaptors that is capable of maintaining an internal temperature of 37 °C is required. To ensure maximum efficiency, on the day of transduction pre-warm the centrifuge to 37 °C before collecting the virus.

5.1. **Preparation of human fibronectin fragment coated plates (Day 1 to day 2)**

5.1.1. On day 1, add 0.5 mL of fibronectin (50 µg/mL in PBS) to the wells of a 24-well plate, and incubate overnight at 4 °C.

NOTE: Typically, two fibronectin-coated wells are required per plate of transfected Phoenix cells.

5.1.2. On day 2 remove the fibronectin solution, and replace with 1 mL of 2% bovine serum albumin (BSA) in PBS. Incubate at room temperature for 30 min to "block" non-specific binding sites.

5.1.3. Wash the treated wells with 1 mL of sterile PBS. After removing the wash solution, the plate is ready for use; or, can be sealed and stored at 4 °C for up to one week.

5.2. **Collection of activated CD8 T cells (Day 2)**

5.2.1. Harvest the activated CD8 T cells, count and calculate cell viability using trypan blue or a suitable automated instrument.

5.2.2. Collect cells by centrifugation and resuspend at 5 x 10^6 viable cells/mL for transduction. Maintain a small aliquot of cells in culture in the complete T cell medium in the CO_2 incubator to provide a control for subsequent fluorescence activated cell sorting of the transduced cells (step 6).

NOTE: After activation for 48 h, the total number of cells should have increased by approximately 1.5 fold, and have a viability greater than 95%.

5.3. **Transduction (Day 2)**

5.3.1. Add 100 µL of activated CD8 cell suspension per well (0.5 x 10^6 cells) to the fibronectin coated plate. Then add 1.5–2 mL of virus-containing medium (from step 2.6) to each well. Mix using a swirling motion to dispense the cells evenly (**Figure 2**).

5.3.2. Place the plate in a zip-lock plastic bag and seal (to provide secondary containment). Centrifuge at 2000 x *g* for 90 min at 37 °C.

5.3.3. Remove the plate from the centrifuge. In the biological safety, cabinet carefully remove the plastic bag and ensure that the outside of the plate is not contaminated with any medium.

5.3.4. Then transfer the plate to the dedicated 37 °C CO_2 incubator. After 4 h, remove 1 mL of the medium from each well and replace with 1 mL of pre-warmed complete T cell medium. Replace the plate in the CO_2 incubator.

NOTE: Handle all media from the transduced cells as infectious waste.

5.4. **Second transduction (Day 3)**

5.4.1. In the dedicated biological safety cabinet, incline the plate containing the transduced cells by resting on the lid and carefully remove most of the medium (leaving 100–200 µL) making sure not to contact the cells at the bottom of the well.

5.4.2. Add the virus-containing medium collected in step 2.7, and repeat steps 5.3.2–5.3.4.

NOTE: In our experience, a third transduction rarely improves overall efficiency. In addition, the cell viability will likely drop significantly if a third transduction is used. If cells are plated at a higher concentration than 0.5 x 10^6/well, the T cells may reach confluence after the overnight incubation following the second transduction step. In this event, split cells after the 4 h incubation on day 3.

5.5. **Wash cells (Day 4)**

5.5.1. Remove 1 mL of medium from each well, resuspend the cells in the remaining medium and transfer to a 15 mL tube. Wash the wells with 1 mL of complete T cell medium and add to the tube containing the pooled cells from each transduction.

5.5.2. Centrifuge at 350 x g for 7 min, then wash twice by resuspending in 2 mL of complete T cell medium and pelleting. Finally resuspend in 2 mL of medium and determine the cell number.

NOTE: If 1 x 10^6 cells were originally transduced, the yield at this stage should be ~3 x 10^6.

5.6. Transfer

5.6.1. Transfer aliquots of 0.5–1 x 10^6 cells in 2 mL of complete T cell medium to the wells of a new 24-well plate and incubate at 37 °C. Approximately 48–72 h post-transduction the cells are ready for CAR expression analysis and cell sorting.

NOTE: The number of CAR-T cells usually doubles each 24 h at this stage. It is critical to never let them overgrow. Split the cells immediately if density is higher than 2 x 10^6/mL (or if the medium ever becomes bright yellow). In our experience the CAR-T cells proliferate more robustly in 24-well and 12-well plates than if transferred to a larger vessel.

6. **Purification of transduced cells by fluorescence-activated cell-sorting (FACS) (day 5 or day 6)**

6.1. Collect the cells.

6.1.1. Resuspend the cells by pipetting up and down multiple times (taking care not to cause frothing), transfer to 15 mL tubes and centrifuge at 350 x *g* for 5 min.

6.1.2. Resuspend in sorting buffer (2% BSA in sterile PBS containing gentamicin) at 1 x 10^6 cells/mL. Also harvest the control (un-transduced) CD8 T cells from step 5.2).

NOTE: From 1 x 10^6 cells at day 2, a yield of ~2 x 10^7 transduced cells is expected at this time point.

6.2. Wash the cells once with sorting buffer by centrifuging at 350 x *g* for 5 min, and resuspend at 1 x 10^7 cells/mL in sorting buffer. Remove a small aliquot for the Foxp3^{GFP} compensation control (to be used in step 6.4.1), and stain the remainder with labeled anti-mouse CD8 (clone 53-6.7; 0.2 µg of antibody/5 x 10^6 cells) by incubating for 20 min at 4 °C.

6.2.1. Similarly, stain an aliquot of the non-transduced CD8 T cells to provide a compensation control for the fluorophore labeling the anti-CD8 antibody.

NOTE: Avoid adding sodium azide to any buffer, as this is toxic to the cells.

6.3. Wash the labeled cells twice with sorting buffer, resuspend in cold sorting buffer at 1 x 10^7 cells/mL.

6.4. Sort the cells.

6.4.1. Sort CD8 GFP$^+$ positive cells into pre-chilled complete T cell medium (**Figure 3B**). Take a small aliquot for post-sorting analysis to determine the purity.

NOTE: To maximize the purity of the sorted cells tight gates should be used. Use a 100 µm nozzle to ensure high cell viability. Minimize the amount of time that the sorted cells are kept on ice. T cells that have been kept on ice for more than 3 h take much longer to recover than cells chilled for less than 2 h. Thus, if 3 transduced cell lines need to be sort, collect and label the second line while the first is being sorted and so forth rather than having the second and third lines spend an extended time at 0 °C. The expression of other T cell markers such as CD28 and CD3 can also be monitored (**Figure 3C**) but is not essential for sorting purposes.

6.4.2. (Alternative sorting strategy) Before staining the bulk population, analyze the CD8 expression of a small population of the transduced T cells. If the purity is >99% then the bulk population can be safely sorted solely on the basis of GFP expression.

7. Expansion of sorted CAR-T cells (Day 5 to 10)

7.1. **CAR-T cell expansion**

7.1.1. Wash the sorted CAR-T cells once, then resuspend in pre-warmed complete T cell medium at 2.5–5 x 10^5 cells/mL, and plate 2 mL aliquots in 24-well plates.

7.1.2. Count and split the cells every 1–2 days. Usually the cell number doubles every day until ~day 10, with viability remaining above 95%.

NOTE: Without re-stimulation, the CAR-T cells will stop proliferating around day 10 and eventually die. Thus, T cell functional assays and adoptive transfers should be scheduled accordingly.

7.2. **Alternative expansion strategy**

7.2.1. After sorting, culture the CAR-T cells in complete T cell medium containing rhIL-2 at 100 U/mL rather than 200 U/mL.

NOTE: The CAR-T cells proliferate at a slightly slower rate in this medium. However, they will often continue to proliferate until days 11 to 13 without re-stimulation. Thus, although this alternative expansion strategy does not generate a higher number of cells it provides a slightly longer time window for downstream assays to be performed.

8. Verification of the antigen specificity and functionality of the CAR T cells.

NOTE: The binding specificity of CAR T cells targeting peptide/MHC complexes can be verified by tetramer staining[7,23]. Similarly, their functionality can be confirmed by measuring cytokine secretion or cytotoxicity following stimulation by their cognate ligands. The NIH Tetramer Core Facility (TCF) at Emory University is a recommended source of "tetramers" and relevant staining protocols.

8.1. **Peptide-MHC Tetramer staining**.

8.1.1. Label aliquots of 2 x 10^5 transduced CAR-T cells in 100 µL of sorting buffer by incubating with ~0.6 µg of fluorescently labeled antigen-specific and control tetramers at 37 °C for 2 h.

8.1.2. Pellet the cells by centrifugation for 5 min at 350 x *g*, then wash twice by resuspending in 0.5 mL of sorting buffer and re-centrifuging. Finally, resuspend the cells in 300 µL of sorting buffer and analyze by flow cytometry (**Figure 4**).

NOTE: For these studies, we typically use BV421-labeled IA^{g7}-B:9-23(RE) (test) and IA^{g7}-HEL (control) tetramers. However, any fluorophore/tetramer combination that is appropriate for the CAR(s) under investigation can be used instead. In this case, the concentration and staining time should be optimized for each tetramer used. Both sorted and un-sorted CAR-T cells can be used for tetramer staining.

8.2. Specificity measurement by ligand stimulation.

8.2.1. Incubate 2 x 10^5 sorted CAR-T cells in 200 µL of cytokine-free T cell medium with appropriate plate-bound or cellular ligands.

8.2.2. After 6–24 h measure cytokine production by ELISA or intracellular staining using manufacturer's protocols.

NOTE: For our studies of IA^{g7}-B:9-23 redirected T cells, we culture the cells overnight with M12C3 murine B-cell lymphoma cells expressing IA^{g7}-B:R3 or "empty" IA^{g7} [24,25], then collect the supernatants and measure secreted mouse interferon gamma (IFN-γ) by ELISA[26] (**Figure 5**).

REPRESENTATIVE RESULTS:

Typically, the transduction efficiency using this protocol is ~60–90%. In the experiment shown in **Figure 3**, prior to sorting approximately, 70% of the CD8 T cells co-expressed GFP. They also co-expressed CD28 and CD3 (**Figure 3C**). Importantly, all of the "test" GFP^+ cells also co-stained with IA^{g7}-B:R3 tetramers, but not with the control tetramer (**Figure 4**). Similarly, the sorted test and control CAR-T cells each secreted high levels of IFN-γ only after co-culture with targets cells expressing their cognate ligands (**Figure 5**). This confirms that the transduced cells have a CD8 effector T cell phenotype directed towards the target of the parent antibodies.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the CAR retroviral construct. The CAR comprises a targeting domain derived from the Fab fragment of a suitable mouse monoclonal antibody, and a spacer/ membrane anchor/ signaling domain from mouse CD28, CD137 and CD247. The synthetic cDNA is inserted into the pMIG-II retroviral expression vector. Restriction endonuclease sites used for generating the mAb287-CAR are shown.

Figure 2: Effect of different plating methods on cell distribution. (Left) Cells pipetted using a swirling motion show an even distribution. (Right) Cells were pipetted directly into the center of the well. Images were captured after spinning at 350 x *g* for 5 min.

Figure 3: Flow cytometric analysis of transduced T cells. Cells were co-stained with PE-Cy7 conjugated anti-CD8, AF647 conjugated anti-CD3, and BV421 conjugated anti-CD28, as described in step 6.4. Profiles gated on single viable cells are shown. (**A**) Un-stained parental CD8 T cells. (**B**) PE-Cy7/GFP profile of transduced cells. The CAR expressing cells are identified by the GFP reporter. (**C**) Stained transduced cells were gated on PE-Cy7/GFP double positivity. The AF647/BV421 profile is shown.

Figure 4: Tetramer staining of un-sorted CAR-T cells. Cells were stained with BV421 conjugated tetramers as described in step 8.1. Profiles gated on single viable cells are shown. (**A**) Test IA^{g7}-insulin tetramer. (**B**) Control I-A^{g7}-HEL tetramer.

Figure 5: Antigen-specific cytokine secretion by CAR T cells. Sorted CD8 T cells expressing the test mAb287 or control mAb24.1 CAR were co-cultured with M12C3 cells expressing IA^{g7}-B:R3, "empty" IA^{g7}, or TFR-MBP-DTRL (the ligand for mAb24.1) as described in step 8.2. After 24 h,

secreted IFN-γ ELISA was quantified by ELISA. Specific stimulation of both T cell lines was observed. Data represent mean ± SD of 3 repeated experiments.

Table 1: Summary of the CAR-T generation protocol.

DISCUSSION:

This protocol describes an efficient method for producing antigen-specific CD8 CAR-T cells by retroviral transduction. The transduction efficiency of our protocol is typically high, and robust expression of the CAR is generally observed. The expanded CAR T cells retain the essential features of the parent-activated T cells, and antibody specificity, and are suitable for both in vitro and in vivo use. We have applied Ab-CAR CD8 T cells in reprograming Type 1 Diabetes in NOD mice[7].

Our protocol incorporates several critical modifications to previously described methods. First, we use an optimized T cell culturing medium that allows an extended activation time. The complete medium described contains optimal levels of several key supplements, and significantly improves both T cell viability and the extent of proliferation following activation. It should be noted that mouse IL-2 can be substituted for the human protein with equivalent results, although at present, human IL-2 is more affordable. Of note, a significantly higher transduction efficiency is obtained using T cells activated for 40–48 h than if a 24 h activation step is used.

Second, we use an improved transduction procedure that eliminates polybrene B (which is toxic to the T cells) and uses fibronectin instead. This further improves cell viability. It should be noted that to guarantee good transduction efficiency it is critical to maintain the T cells in an optimized medium at an appropriate cell density and to use fresh high-titer viral supernatants rather than previously frozen virus. Using our modified procedure, a third transduction step is unnecessary and indeed is undesirable as viability typically drops if a third spin infection step is included. It must also be emphasized that it is critical to never let the cells overgrow during the expansion phase. Once cells are overgrown, they tend to rapidly lose their phenotype and die.

In addition to the parameters described above, two other potential causes of low transduction efficiency/viability must be avoided. First, as antibiotics should not be present during the transfection steps it is important to make sure that the plasmids are prepared using an endotoxin-free kit, and dissolved in sterile water, and that good sterile technique is used at all times. Second, the presence of high levels of dead or dying T cells must be avoided. If the activated parental CD8 T cell suspension contains high levels of dead cells or cell debris this should be removed prior to transduction using commercial kits.

We have deliberately not included a CAR-T cell freezing step in this protocol, as in our experience a significant proportion of the transduced cells die during cryopreservation and thawing. Similarly, although the expanded CAR-T cells can be re-stimulated in vitro, they have an increased tendency to lose expression of the transgene. Accordingly, given the high degree of proliferation we observe using freshly sorted CAR-T cells, we highly recommend that only freshly generated CAR-T cells are used for functional assays and adoptive transfer.

In summary, the significance of this protocol is that it describes a procedure that provides high transduction efficiency and generates large numbers of healthy antigen specific mouse CD8 T cells for use in vitro and in vivo. Our protocol thus provides a useful tool for researchers undertaking CAR-T cell studies in mouse models of disease.

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DISCLOSURES:

MAb287 and its derivatives are protected by a US patent issued in 2014.

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Figure 1

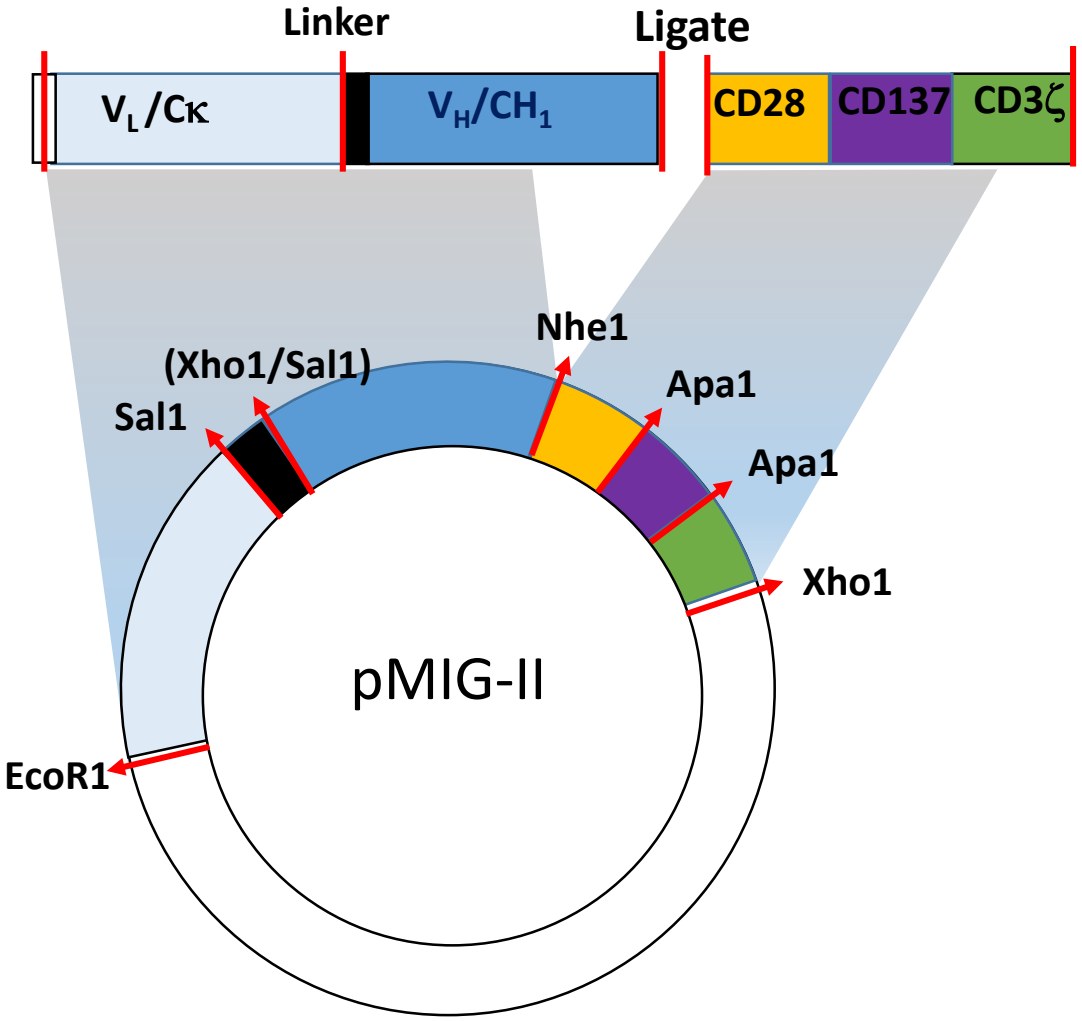
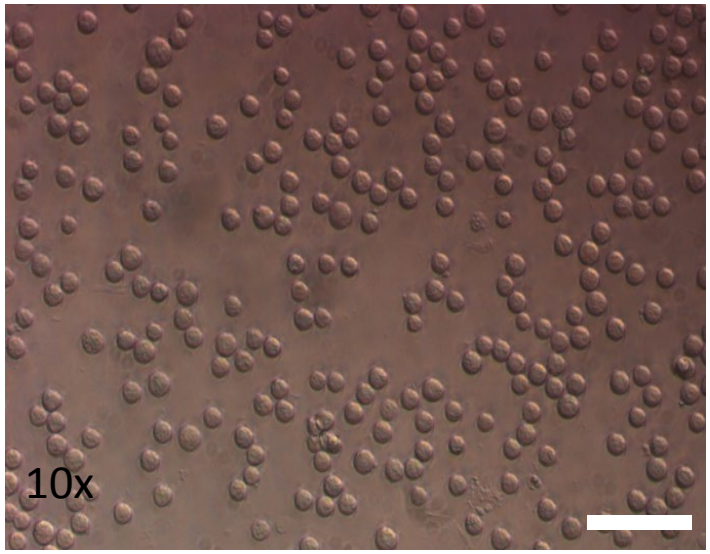


Figure 2

Good cell distribution



Uneven/patchy cell distribution

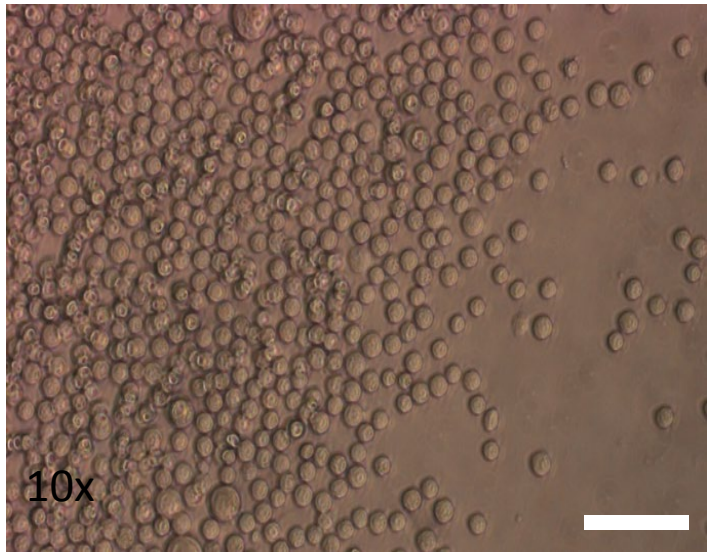


Figure 3

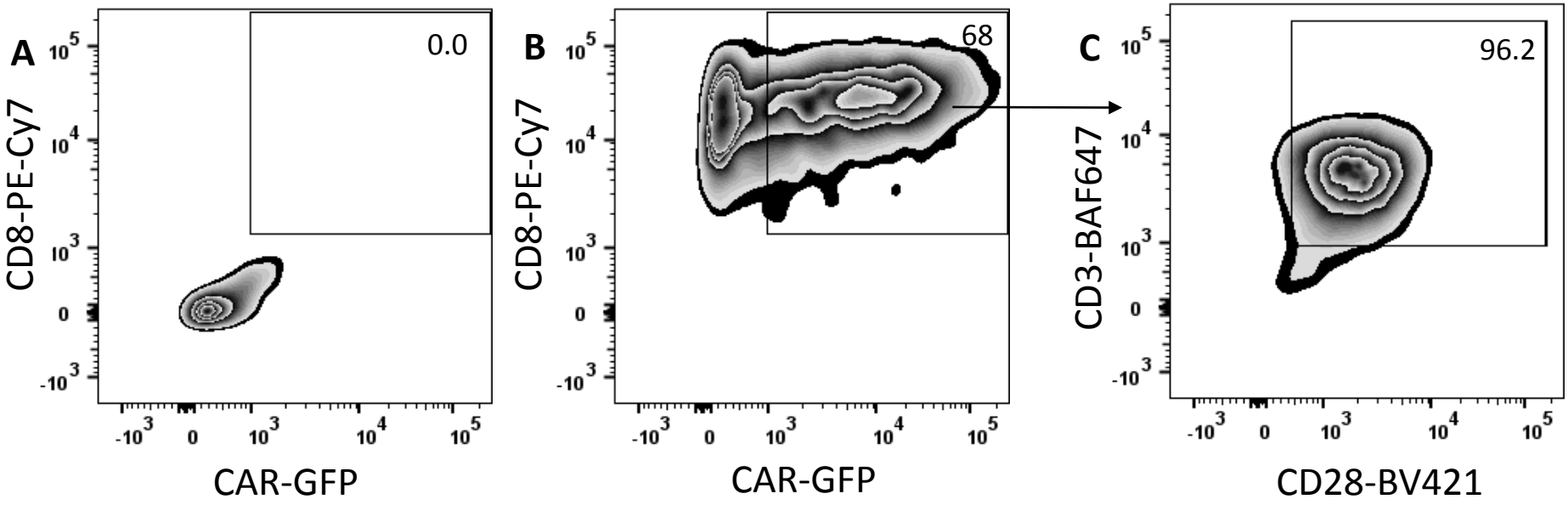


Figure 4

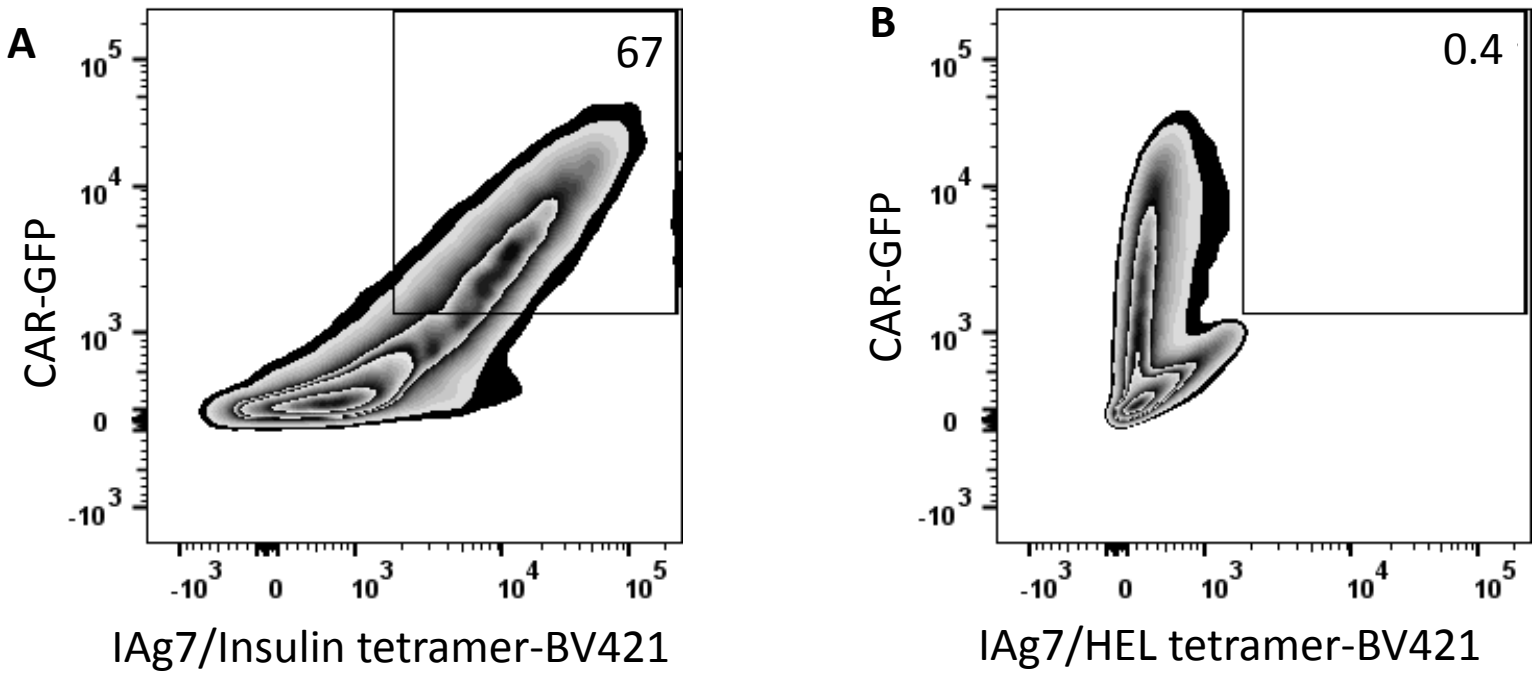
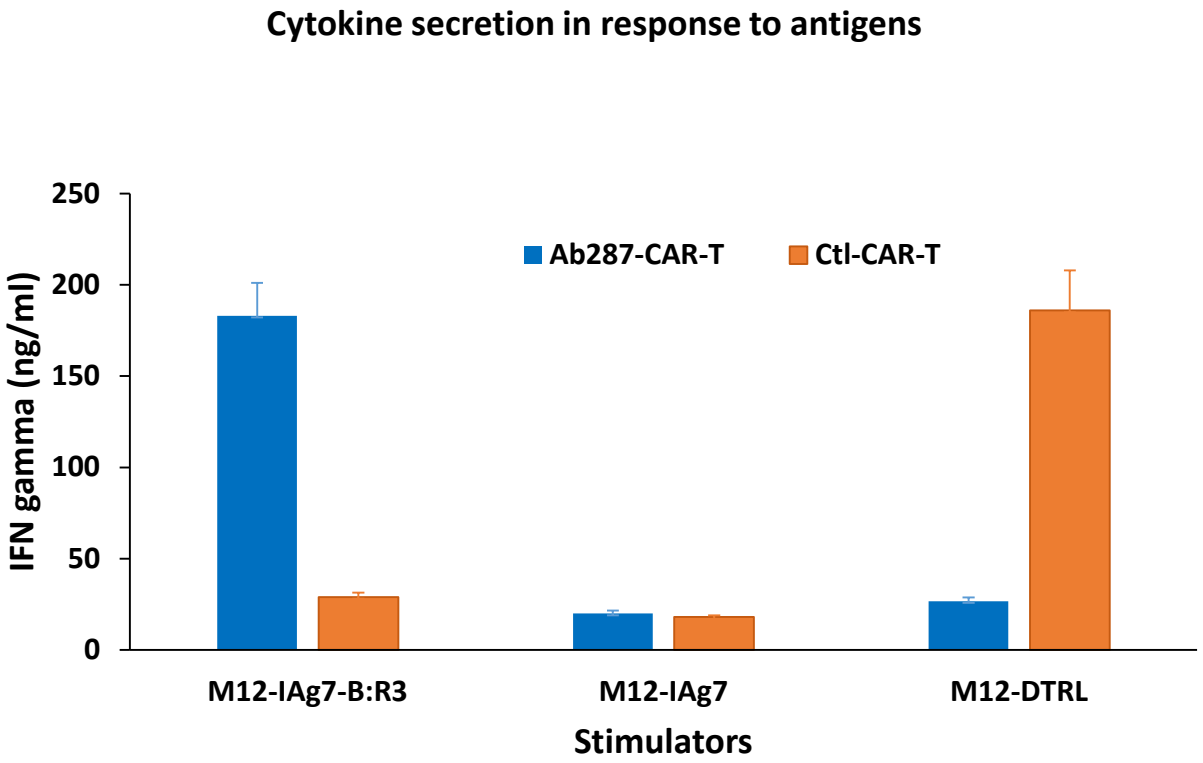


Figure 5



Name of Material/ Equipment	Company	Catalog Number
2-Mercaptoethanol (50mM)	Gibco	21985-023
5’ RACE PCR	Clontech	634859
anti-mouse CD28 antibodies	eBioscience	14-0281-86
anti-mouse CD3e antibody	eBioscience	145-2C11
Biotin Rat Anti-Mouse IFN-γ	BD Biosciences	554410
BSA	Sigma	A7030
Endo-free Maxi-Prep kit	Qiagen	12362
Gentamicin	Gibco	15750-060
Heat inactivated FCS	Hyclone	SH30087.03
HEPES (100X)	Gibco	15630-080
IAg7-CLIP tetramer-BV421	NIH tetramer Facility at Emory	per approval
IAg7-insulin P8E tetramer-BV421	NIH tetramer Facility at Emory	per approval
Insulin-Transferrin-Selenium-Ethanolamine (ITS 100x)	ThermoFisher	51500056
Lipofectamine 2000	Invitrogen	11668019
LS Columns	Miltenyi Biotec	130-042-401
MACS Separation Buffer	Miltenyi Biotec	130-091-221
Mouse CD8a+ T Cell Isolation Kit	Miletenyi Biote	130-104-075
Mouse CD8a+ T Cell Isolation Kit	Miltenyi Biotec	130-104-075
Opti-MEM medium	ThermoFisher	31985070
Penicillin-Streptomycin (5000U/ml)	ThermoFisher	15070063
Phoenix-ECO cells	ATCC	CRL-3214

Phosphate-buffered saline (PBS)	Gibco	10010-023
pMIG II	Addgene	52107
pMSCV-IRES-GFP II	Addgene	52107
Purified Rat Anti-Mouse IFN-γ	BD Biosciences	551216
Red cell lysis buffer	Sigma	R7767
RetroNectin	Takara	T100A
rhIL-2 (stock concentration 10^5 IU/ul)	Peprotech	200-02
rmIL-7 (stock concentration 50ng/ul)	R&D	407-ML-005
RPMI-1640	Gibco	11875-093
Sterile Cell Strainers	Fisher Scientific	22-363-548
Tryple	Gibco	12605-028

Comments/Description
50 uM
final concentration at 1µg/ml
final concentration at 1µg/ml
Working concentration at 0.5 µg/ml
Final 50 µg/ml.
Final 10% FCS
1X
Working concentration at 6 µg/ml
Working concentration at 6 µg/ml
Final concentraion is 1x
50 U/ml

Working concentration at 3 µg/ml
Working concentration at 50 µg/ml in PBS
Final concentration at 200 IU/ml
Final concentration at 0.5ng/ml

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The changes can be summarized as

1. Remove the unnecessary change in the title. We keep the original title.
2. Added references per request.
3. Added all the details per request.

We do notice the highlighted part are changed from 2.5 page to about 3 pages after the Editor's changed it to current format. If it is more than allowed, we can un-highlight the Step 2.

Thank!

Li Zhang

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