Human CD8 T cell transduction and rapid expansion protocol

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ABSTRACT

This protocol outlines the steps for generating human CD8 T cells expressing an engineered T cell receptor.

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Day 0 - Lentiviral supernatant production and transduction

1 **Prepare packaging cell line:**

1. Resuspend 3x10^6 293T cells with 10 mL LCL media and plate in a 10cm plate.
2. Incubate at 37C overnight.

Day 1 - Lentiviral supernatant production and transduction

2 **Transfect 293T:**

1. Thaw tubes of DNA. And prepare 5mL polypropylene tubes (1/plate).
2. Add 280.7 uL EC Buffer.
3. Add 2.5ug of M-Mix (2.6ul) and 1.5ug of DNA to EC Buffer.
4. Add 32uL of Enhancer and vortex for 1 second.
5. Incubate 5 min at Room Temp.
6. Add 60uL of Effectene. Vortex for 10 seconds.
7. Incubate 10 min at Room Temp.
8. Aspirate media from 293T plates. Add 7mL of CTL media to each plate.
9. Add 3 mL of CTL media to each tube of Effectene mixture. Pipette up and down to mix, then add slowly (dropwise) to each 293T plate.
10. Swirl gently to mix and return to 37C incubator overnight.

Day 2 - Lentiviral supernatant production and transduction

3 Aspirate media from the dish and replace with 10mL fresh CTL media. Incubate at 37C.

Day 3 - Lentiviral supernatant production and transduction

4 **Activate and transduce T cells:**

1. Collect the viral supernatant and filter thru a 0.45um filter (Thermo scientific Nalgene syringe filter cat #190-9945).
2. Add 10mL fresh CTL media to plates and return to 37C. Alternatively, use frozen viral supernatant.
3. Thaw CD8+ T cells and count. (Quick thaw at 37C and drop-wise (slow) dilution).
4. Resuspend $2x10^7$ T cells with $2x10^7$ anti-CD3 and anti-CD28 beads (1:1 T cell to bead ratio) (Dynabeads Cat#111.41D from Invitrogen).

Starting bead concentration = $1x10^8$ beads/ml.

5. Wash Dynabeads with 10mL CTL media.
6. Dilute the cells to a concentration of $5x10^6$ cells/mL, add IL-2 at 50U/mL and transfer into T25 flasks.
7. Incubate at 37C for ~4 hours
8. Spin down T cells at 369*xg* for 4-5 minutes at 4C and re-suspend in 10mL CTL media. Recount T cells post-stimulation.
9. Aliquot T cells into 15mL tubes (1 per transduction condition) and spin down at 369*xg* for 4-5 minutes at 4C.
10. Spin down cells at 369*xg* for 4-5 minutes at 4C.
11. Resuspend cells in viral supernatant and add 50 U/mL IL-2 and 5ug/mL Polybrene. Transfer 2mL per well to a 12-well plate.
12. Spin cells at 1265*xg* at 30C for 90 minutes.
13. Transfer plates to 37C, 5% CO2 incubator overnight.

Day 4 - Lentiviral supernatant production and transduction

5 **Transduce cells a second time:**

1. Collect and combine T cells. Spin down cells at 369*xg* for 4-5 minutes at 4C.
2. Resuspend cells in viral supernatant and add 50 U/mL IL-2 and 5ug/mL Polybrene. Transfer 2mL per well to a 12-well plate.
3. Spin cells at 1265*xg* at 30C for 90 minutes.
4. Transfer plates to 37C, 5% CO2 incubator overnight.

Day 5 - Lentiviral supernatant production and transduction

6 Add 2 mL per well fresh CTL media containing 100U/mL IL-2.

Day 6 - Lentiviral supernatant production and transduction

7 **Remove Dynabeads:**

1. Combine T cells into a 15mL tube and spin down at 369*xg* for 4-5 minutes at 4C.
2. Rinse the wells with 5mL CTL media and re-suspend the cells with this. Mix well to break up any clumps.
3. Use the DynaImagnet to remove Dynabeads. Let tube sit for ~2 minutes so the beads aggregate along the side of the tube.
4. Gently remove the media containing the cells, careful not to disrupt the beads. Wash the beads one more time with 5mL CTL media.
5. Collect T cells. Spin down cells at 369*xg* for 4-5 minutes at 4C.
6. Resuspend cells in 8 mL fresh CTL media containing 1:1000 dilution of IL-2. Transfer to one well of a 6-well plate.

Day 8 - Lentiviral supernatant production and transduction

8 Without disturbing cells on the bottom of the well, remove ½ of the CTL media. Add fresh CTL media containing 100 U/mL IL-2.

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Day 11 - Lentiviral supernatant production and transduction

9 Without disturbing cells on the bottom of the well, remove ½ of the CTL media. Add fresh CTL media containing 100 U/mL IL-2.

Day 13 - Lentiviral supernatant production and transduction

10 Without disturbing cells on the bottom of the well, remove ½ of the CTL media. Add fresh CTL media containing 100 U/mL IL-2.

Day 0 - Rapid Expansion Protocol (REP)

11 Prepare items for REP mix:
1. Thaw mixed donor PBMC, count and irradiate at 4000 rad.
2. Count and irradiate LCL cells at 8000 rad.

REP mix preparation depends on the number of CD8 T cells prepared.

	T25 (Per flask)	**6 well plate (Per Well)**	**96 well plate (Per Plate)**
CTL	2-5 x 10^5	1-2 x 10^5	(50-500) 0.5-5 cells/well
PBMC (irradiated)	2.5 x 10^7	1 x10^7	1 x10^7
LCL (irradiated)	5 x 10^6	2 x 10^6	2 x 10^6
OKT3 (1 mg/ml)	0.75 ul 1/33,333 dil	0.24 ul (1/33,333 dil)	(0.6 ul) 1/33,333 dil
IL-2 (5x10^4 U/ml)			20 ul
IL-15 (45 ug/ml)			***10 ul***
IL-7 (14 ug/ml)			***10 ul***
volume	25 ml	8 ml	20 ml

3. Combine all elements of REP mix and return to 37C incubator.

Day 1 - Rapid Expansion Protocol (REP)

12 Add 50 U/ml IL-2

Day 4-5 - Rapid Expansion Protocol (REP)

13 1. Gently pipette off 50-75% of the medium without disturbing the cells
2. Top up with CTL medium & a final concentration of 50 U/ml IL-2

Note: this step repeats every 2 days until day 10-14.

Day 6-7 - Rapid Expansion Protocol (REP)

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14 1. Gently pipette off 50-75% of the medium without disturbing the cells
2. Top up with CTL medium & a final concentration of 50 U/ml IL-2

Day 8-9 - Rapid Expansion Protocol (REP)

15 1. Gently pipette off 50-75% of the medium without disturbing the cells
2. Top up with CTL medium & a final concentration of 50 U/ml IL-2

Day 10-11 - Rapid Expansion Protocol (REP)

16 1. Gently pipette off 50-75% of the medium without disturbing the cells
2. Top up with CTL medium & a final concentration of 50 U/ml IL-2

Day 12-13 - Rapid Expansion Protocol (REP)

17 1. Gently pipette off 50-75% of the medium without disturbing the cells
2. Top up with CTL medium & a final concentration of 50 U/ml IL-2

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