Title: T Cell Transfection
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Materials

- Non-Tissue Culture Treated Plate, 24 Well, Flat Bottom with Low Evaporation Lid (Corning Life Sciences cat. no. 351147)
- Non-Tissue Culture Treated Plate, 96 Well, Flat Bottom with Low Evaporation Lid (Corning Life Sciences cat. no. 351172)
- Disposable Hemocytometer (Fisher Scientific cat. no. 22600100)
- Trypan Blue (Fisher Scientific cat. no. 15250061)
- 96 wells plate V-shape (Fisher Scientific cat. no. 720096)
- X-vivo 15 Culture Media (Lonza BEBP04-744Q)
- P3 nucleofection kit (Lonza V4SP-3096)
- Human serum (Fisher Scientific cat. no. MT35060CI)
- IL-15 (Miltenyi Biotec cat. no. 170-076-114)
- IL-7 (Miltenyi Biotec cat. no. 170-076-111)
- Steriflip Sterile Disposable Vacuum Filter Units (Fisher Scientific cat. no. SCGP00525)
- T cell TransAct beads (Miltenyi Biotec cat. no. 130-019-011)
- SpCas9 NLS recombinant protein
- sgRNA (source: IVT, Synthego)

Culture media for T cells. Supplement X-vivo 15 media with HSA to 5% (vol/vol) (or 20% (vol/vol) where indicated), filter sterilize. Add IL-15 with IL-7 to the final concentration 10 ng/ml each. Prepare fresh every time.

$CD4^+/CD8^+$ T cells

Thaw, then activate and culture $CD4^+/CD8^+$ T cells from frozen stock before nucleofection by following the steps below.

1 - Thaw cells and pipette into 50 ml tube, slowly add 10 ml of pre-warmed X-Vivo 15 media (without supplements). Count the cells with hemocytometer.

2 - Spin for 10 minutes at 300 x *g* at room temperature, remove supernatant, and resuspend cells at $1x10^6$ cells/ml in X-vivo 15 media, supplemented with 5% HSA and 10 ng/ml (each) of IL-7 and IL-15.

3 - Activate T cells with TransAct polymeric nanomatrix by adding the TransAct reagent directly to the cell culture at the specific dilution ratio specified by the manufacturer (1:100) and culture in a non-TC 24-well plate for 72 hours.

4 - Prior to nucleofection, pre-equilibrate a non-TC 96-well recovery plate(s) with 250 µl per well of filter sterilized complete X-vivo 15 media with 20% HSA and 10 ng/ml (each) of IL-7 and IL-15 in 37°C, 5% CO_2 incubator.

5 - For each nucleofection, use 75 pmol of the Cas9 protein and 3-fold molar excess of sgRNA in a final total volume of 5 µl. Incubate at room temperature for 20 minutes.

6 - Pool and count the cells with hemocytometer.

7 - Collect cells needed for nucleofection by centrifugation 10 min at 300 x *g*, room temperature. For each nucleofection, $3x10^5$ cells are resuspended in 20 µl of P3 buffer. We recommend making a single pool of cells resuspended in P3 buffer for all nucleofections. For example, for 10 nucleofections resuspend $3x10^6$ cells in 200 µl of P3 buffer.

8 - In a 96 well plate (V-shape), for each nucleofection combine 20 µl of the cell suspension and 5 µl of the RNP complex from step (5) .

9 - From the plate, for each nucleofection mix and transfer 20 µl of the mixture to one well of a 16 well Lonza 4-D strip. Nucleofect the samples using EO-115 pulse code on a Lonza 4-D nucleofector following the manufacturer's instructions.

10 - Incubate at room temperature for 7 minutes.

11 - Add 100 µl of media from the plate prepared in step (4) to the well.

12 - Transfer 120 µl of the content of well back to the plate, culture in CO_2 incubator for 72 hours before harvesting.