Supernatant Transfer Assay and Transwell® Assay

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Abstract

This protocol describes two methods for assessing whether soluble factors alone or a combination of soluble factors and T cells are responsible for bystander killing: (1) Supernatant transfer assays and (2) Transwell® assays.

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Protocol

Overview

Step 1.

This protocol describes methods to assess the contribution of soluble factors and BiTE®-activated T cells in bystander killing. Two methods are used:

(1) Supernatant transfer assays from BiTE®- + T cell-treated target positive cells to target-negative cells and (2) Transwell® assays in which a membrane separates BiTE®- + T cell-treated target-positive cultures from target-negative cultures, restricting movement of T cells but not soluble factors.

These assays are modifications of the standard TDCC protocol. Please refer to TDCC protocol for cell treatments and assay set-up.

Materials

Step 2.

See also Materials list in TDCC protocol.

HTS Transwell-96 System, PET membrane Corning #3392 or #3380 1 µm

HTS Transwell-96 Well Plate, PC membrane Corning #3387 or #3388 5 µm

HTS Transwell-96 receiver plate, tissue culture treated Corning #3382 clear plate

HTS Transwell-96 receiver plate, tissue culture treated Corning #3583 black plate, including bottom

Supernatant transfer assay

Step 3.

For 96-well assays, each well contains a final volume of 200 µl. Plan for setting up cells in separate ViewPlates to allow for:

- Supernatant transferred directly (medium + cells)
- Supernatant clarified by centrifugation (medium only)
- No transfer control

1. Prepare target-positive cells cells:

- Detach adherent target cells, count and dilute cells (e.g. NUGC-4) in a mixture with T cells at desired E:T ratio (10:1 typically used) in 160 µl, e.g. 10:1 = 10,000 target cells and 100,000 T cells per well
- Add 40 µl per well of BiTE® dilution or assay medium to plates
- Add 160 µl per well target cells plus T cell mixture to plates
- Allow plates to equilibrate for 30 min. at room temperature
- Incubate at 37°C, 5% CO2 for 48 hours

3. Prepare target-negative cell plates (e.g. SW620) on day of supernatant transfer:

- Detach adherent target-negative cells, count and dilute cells in a mixture with T cells at desired E:T ratio in 160 µl, e.g. 10:1 = 10,000 target cells and 100,000 T cells per well
- Add 160 µl target-negative cells plus T cell mixture to a fresh 96-well ViewPlate
- Allow plates to equilibrate for 30 min. at room temperature
- Incubate at 37°C, 5% CO2 for 5 hours to allow cells to adhere.

4. Set up supernatant transfer assay:

- Remove media from target-negative cells by inverting plate and blot edges on paper towel
- Plate 1: Transfer directly 0.160 ml crude supernatant from target-positive NUGC-4 plate to plate containing target-negative SW620 cells
- Plate 2: Transfer carefully 0.160 ml clarified supernatant from target-positive NUGC-4 plate to plate containing target-negative SW620 cells. (NUGC-4 plate first centrifuged at 400 x g for 5 min.)
- Plate 3: No transfer control-add fresh BiTE® dilutions and T cells
- Incubate at 37°C, 5% CO2 for 48 hours

5. Perform cytotoxicity assay (imaging or CellTiter-Glo® - see TDCC protocol).

Transwell® assay

Step 4.

For 96-well transwell assays, each well contains a final volume of 310 µl

- Bottom receiver plate: 200 µl target-negative (or target-positive for positive control) cells + 35 µl assay medium
- Top chamber insert: 35 µl 10X BiTE® dilutions + 40 µl target-positive cells/T cell mixture

(typically 10:1 E:T ratio)

1. Prepare target cells:

- Detach adherent target-positive cells, count and dilute to 10,000 cells/well, 200 µl/well. Also, dilute target cells in a mixture with T cells at desired E:T ratio in 40 µl, e.g. 10:1 = 10,000 target cells and 100,000 T cells per well

2. Set up assay:

- Add 35 µl assay medium to wells in bottom of receiver plate
- Add 200 µl target-negative (or target-positive for positive control) cells to wells in bottom of receiver plate (10,000 cells/well)
- Carefully place insert on top of the receiver plate
- Add 35 µl of BiTE® dilution to top chamber of insert
- Add 40 µl target-positive cells/T cell mixture to top chamber of insert
- Allow plates to equilibrate for 30 min. at room temperature
- Incubate at 37°C, 5% CO2 for 72 hours

3. Perform cytotoxicity assay on the cells in the bottom chambers/receiver plates (imaging or CellTiter-Glo® - see TDCC protocol).