BiTE® bystander T cell-dependent cellular cytotoxicity (TDCC) assay

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Abstract

Protocol for detecting bystander cytotoxicity in mixed cultures of target-negative and target-positive cells treated with BiTE® antibody constructs. Two methods and one modification are described.

Citation: Sandra L. Ross, Marika Sherman, Patricia L. McElroy, Julie A. Lofgren, Gordon Moody, Patrick A. Baeuerle, Angela Coxon, Tara Arvedson BiTE® bystander T cell-dependent cellular cytotoxicity (TDCC) assay. **protocols.io**
dx.doi.org/10.17504/protocols.io.hwfb7bn
Published: 17 May 2017

Protocol

Overview

Step 1.

This assay is a modification of the standard TDCC assay (please refer to TDCC protocol). Target-positive and target-negative cells are mixed together in various ratios and the combined cells are used as the target cell population. At the end of the 48-hour incubation period, cytotoxicity is measured by one of two methods: (1) Cellular imaging - cells are stained with an antibody that detects the target (EGFR described here); cytotoxicity of target-positive and target-negative cells can thus be distinguished as populations below or above the target intensity threshold and each population is enumerated by counting nuclei; (2) Luciferase-labeled cells - target-negative cells expressing luciferase are generated using lentiviral transduction prior to mixing with target-positive cells; Steady-Glo® is then used to measure cytotoxicity of target-negative cells only.

In a modification of the bystander assay, BiTE®-activated T cells are generated by mixing with target-positive cells and BiTE® for 24 hours. T cells are then transferred to target-negative cells.

Assemble materials

Step 2.

Materials	Company	Cat.no.
Sterile 96-well clear V-bottom polypropylene plates	Greiner	651201
Flat-bottom white 96-well plates (CellTiter-Glo®)	Corning	3917
ViewPlate-96 black plate Packardview plate (imaging)	Perkin Elmer	6005182
Flat-bottom white 384-well plates (Steady-Glo®)	Corning	3570
Effector cells – unstimulated pan T cells	AllCells	PB009-1F
Target cells – SW620, NUGC4, MOLM13	Amgen cell bank	-
BiTE®s – EGFR, MEC14, CD33	Amgen	-
CellStripper™ Dissociation Reagent nonenzymatic 1X	Corning	25056CI

1x PBS	Gibco	14190
50 ml Falcon Tube	BD	35 2070
15 ml Falcon Tube	BD	35 2096
Growth medium: RPMI 1640 medium Supplements: 100U/ml penicillin/streptomycin 10% heat-inactivated fetal bovine serum (FBS)	Gibco Gibco Gibco	11875-093 15140-122 10082-147
Assay medium: RPMI 1640 medium Supplements: 1x nonessential amino acids (NEAA) 10mM HEPES 50µM 2-ß-mercaptoethanol 1mM sodium pyruvate 100U/ml penicillin/streptomycin 5% heat-inactivated fetal bovine serum (FBS)	Biochrom Gibco Gibco Sigma Gibco Gibco Gibco	FG1215 11140-050 15630-080 M6250 11360-070 15140-122 10082-147
Steady-Glo® Luciferase Assay System	Promega	E2510-10ml
Hoechst 33342 nuclear dye	ThermoFisher	62249
Formaldeyde 16% (w/v) methanol-free	Pierce/ThermoFisher	28908
Mouse anti-human EGFR antibody (Clone 199.12)	ThermoFisher	MA5-13319
Goat anti-mouse-AlexaFluor® 488	ThermoFisher	A-11029
Normal goat serum	Sigma	G9023

EGFR bystander imaging TDCC assay

Step 3.

- Follow TDCC assay protocol, substituting mixtures of $EGFR^+$ (e.g., NUGC4) and $EGFR^-$ (e.g., SW620) cells such that the total cell number is 10,000 cells/well
    - Dilute EGFR+ and EGFR- cells to 200,000/ml
    - Mix together in ratios in 15ml conical tubes:
        - 100% $EGFR^+$ = 6 ml $EGFR^+$ cells
        - 75% $EGFR^+$ + 25% $EGFR^-$ cells = 4.5 ml $EGFR^+$ + 1.5 ml $EGFR^-$ cells
        - 50% $EGFR^+$ + 50% $EGFR^-$ cells = 3 ml $EGFR^+$ + 3 ml $EGFR^-$ cells
        - 25% $EGFR^+$ + 75% $EGFR^-$ cells = 1.5 ml $EGFR^+$ + 4.5 ml $EGFR^-$ cells
        - 100% $EGFR^-$ cells = 6 ml $EGFR^-$ cells
    - Dispense 50 µl/well of each cell mixture as target cells in TDCC protocol (10,000 total target cells/well)
        - 100% $EGFR^+$ = 10,000 $EGFR^+$ cells/well
        - 75% $EGFR^+$ + 25% $EGFR^-$ cells = 7,500 $EGFR^+$ + 2,500 $EGFR^-$ cells/well
        - 50% $EGFR^+$ + 50% $EGFR^-$ cells = 5,000 $EGFR^+$ + 5,000 $EGFR^-$ cells/well
        - 25% $EGFR^+$ + 75% $EGFR^-$ cells = 2,500 $EGFR^+$ + 7,500 $EGFR^-$ cells/well
        - 100% $EGFR^-$ cells = 10,000 $EGFR^-$ cells/well
    - Assemble the TDCC assay as described in the TDCC protocol using black, clear-bottom PackardView plates, incubate 48 hours at 37°C.
    - Wash and fix cells: Wash cells two times with PBS, and fix with formaldehyde as described in the TDCC imaging assay protocol.
    - Prepare reagents while cells are fixing
        - Wash buffer: PBS + 5% normal goat serum
        - Primary antibody (anti-EGFR): 1 µg/ml in wash buffer
        - Secondary antibody (goat anti-mouse-AlexaFluor® 488) + Hoechst nuclear dye: 5 µg/ml secondary antibody + 5 µg/ml Hoechst in wash buffer
    - After cells are fixed, wash two times with PBS
    - Remove PBS and add 50 µl/well primary antibody solution; incubate 1 hour at room temp.
    - Remove primary antibody solution and wash 2X with 100 µl/well wash buffer

- Remove wash buffer and add 50 µl/well secondary antibody + Hoechst nuclear dye solution; incubate 1 hour at room temp. in the dark
- Remove secondary antibody/Hoechst solution and wash 1X with 100 µl/well wash buffer
- Remove wash buffer and wash 1X with 100 µl/well PBS
- Remove PBS wash and add back 100 µl/well PBS
- Seal plates and scan on ArrayScan™ using Target Activation bioapplication
    - Control wells containing $EGFR^+$ cells alone and $EGFR^-$ cells alone are used to set a threshold for AlexaFluor® 488 (EGFR) intensity such that >95% of $EGFR^-$ cells are classified as negative and >95% of $EGFR^+$ cells are classified as positive (see below).
    - The ArrayScan™ Target Activation bioapplication allows for classifying cells as "Type 1" and "Type 2" based on the average intensity in the EGFR channel (Channel 2). All nuclei are counted (Channel 1, Hoechst), and total nuclei in 16 10X fields are classified as Type 1 (below intensity threshold = $EGFR^-$) or Type 2 (above intensity threshold = $EGFR^+$).
    - Note: Any remaining T cells left in wells after washes are excluded from cell counts by the lower nuclear size threshold.

EGFR Bystander Steady- Glo® Luciferase TDCC Assay

Step 4.

- Use Corning 384-well plates for higher capacity and larger dose-response curves.
- Number of wells needed for each assay should be carefully calculated, depending on the number of cell lines, number of BiTE® antibody constructs, number of BiTE® dilutions, E:T ratios, controls and number of replicates.
- For 384-well plate assays, each well contains a final volume of 50 µl (5 µl 10X BiTE® dilutions), 25 µl T cells at desired E:T ratio and 20 µl target cells.
- In a typical 384-well assay, each well contains:
    - 2,500 target cells
    - 25,000 T cells/ well (10:1 E:T ratio)
    - BiTE® dilutions starting at 1 nM final
    - 2 columns of control wells (one with target cells, T cells and no BiTE® and the other with target cells only)
- For 384-well plates, liquid handling automation is used to dilute and dispense BiTE® (Bravo) and to dispense target cells and T cells (Multidrop)
- All steps are done in Biosafety laminar flow hood.
- Thaw T cells as described in TDCC protocol.
- Prepare target cells as described in TDCC protocol, except that cell density is 125,000 cells/ml (125,000 cells/ml X 0.02 ml = 2500 cells/well).
    - For the $EGFR^-$ AML suspension cell line, cells are counted, centrifuged at 300 x g for 10 min and resuspended in fresh Assay Medium, then recounted before making the dilution.
    - Mix together in $EGFR^+$ and $EGFR^-$ cell lines in ratios in 15ml conical tubes (10 ml total target cells + dead volume needed per 384-well plate):
        - 100% $EGFR^+$ = 10 ml $EGFR^+$ cells
        - 75% $EGFR^+$ + 25% $EGFR^-$ cells = 7.5 ml $EGFR^+$ + 2.5 ml $EGFR^-$ cells
        - 50% $EGFR^+$ + 50% $EGFR^-$ cells = 5 ml $EGFR^+$ + 5 ml $EGFR^-$ cells
        - 25% $EGFR^+$ + 75% $EGFR^-$ cells = 2.5 ml $EGFR^+$ + 7.5 ml $EGFR^-$ cells
    - 100% $EGFR^-$ cells = 10 ml $EGFR^-$ cells
- Prepare BiTE® serial dilution plates (EGFR test BiTE® and CD33 positive control BiTE® for AML

cell line)
    - 22 dilutions + 1 column with target cells, T cells and no BiTE® and one column with target cells only)
    - Starting 10X concentration is 10nM; serial dilutions in Assay Medium 2-fold across plate X 22 wells
        - Dispense 50 µl 10nM BiTE® into col. 1 and 25 µl Assay Medium into cols. 2-24
        - Transfer 25 µl from col. 1 into col. 2, and so on across plate through col. 22)
    - Prepare T cells as described in TDCC protocol, except that cell density is 1x10e6 cells/ml (1x10e6 cells/ml X 0.025 ml = 25,000 cells/well); 10 ml + dead volume needed per 384-well plate.
    - Dispense 20 µl target cells and 25 µl T cells into assay plates (Multidrop); add 5 µl BiTE dilutions into assay plates (Bravo); mix 3 times with slow aspirate (Bravo).
    - Leave assay plates at room temperature in hood for 30 min. before placing in tissue culture incubator.
    - Incubate at 37°C for 48 hours.
    - Steady-Glo® assay:
        - Thaw Steady-Glo® reagent to room temperature and reconstitute (10 ml needed per plate)
        - Add 25 µl/well of room temperature Steady-Glo® (Multidrop)
        - Incubate 30 min. at room temperature
        - Read on luminometer (EnVision with Ultrasensitive Lumi PMT)
        - Analysis: Only $EGFR^-$ cells are labelled, so the luminescence readout is a direct count of $EGFR^-$ cytotoxicity

TDCC assay using BiTE-activated T cells

Step 5.

- Overview: T cells are activated by incubating T cells (E:T = 10:1), EGFR BiTE® (100 pM) and $EGFR^+$ cells together in bulk for 24 hr. Control T cells are incubated with $EGFR^+$ cells but no EGFR BiTE®. T cells are then harvested, washed and resuspended for use in TDCC assays.
- Day 1: Plate target cells and prepare BiTE®-activated and control T cells
    - Plate EGFR+ (NUGC4) and EGFR- (SW620) cells in 96-well PackardView plates (for imaging) or Corning white plates (for CellTiter-Glo®) at 10,000 cells/well
        - Cells can be pre-treated +/- cytokines by replacing medium 8 hours after plating with Assay Medium containing IFNγ and/or TNFα at the desired concentrations (for upregulation of ICAM-1 and FAS, 10ng/ml IFNγ + 5ng/ml TNFα)
        - Incubate overnight
    - Prepare BiTE®-activated and control T cells
        - Thaw frozen T cells as described in TDCC assay protocol
        - Prepare EGFR BiTE®
            - Dilute stock to 2 nM (20X) in Assay Medium.
        - Prepare $EGFR^+$ NUGC4 target cells: Remove cells from flask with CellStripper™ and resuspend at 0.33 x 10e6 cells/ml (need at least 18 ml)
            - Transfer 3x10e6 cells (9 ml) to two 50 ml conical tubes (Tubes 1 and 2).
        - Prepare T cells
            - Dilute thawed T cells to 3x10e6/ml in Assay Medium.
        - Activate T cells
            - To the tubes containing 3x10e6 cells (9 ml) target cells prepared above, add 1 ml 20X (2 nM) BiTE® to Tube 1 (100 pM final) and 1 ml Assay Medium to

Tube 2 (0 pM final).
- Add 10 ml T cells at 3x10e6 cells/ml (30x10e6 total) to each tube and mix gently.
- Transfer the contents of each tube (20 ml total) to separate T75 flasks
    - Flask 1 (from Tube 1) = BiTE®-activated T cells = target cells + 100 pM BiTE® + 30x10e6 T cells
    - Flask 2 (from Tube 2) = 3x10e6 target cells without BiTE® + 30x10e6 T cells = control (resting) T cells
- Incubate at 37°C for 24 hours.

- Day 2: Harvest BiTE®-activated and control T cells and add to plated target cells
    - Harvest BiTE®-activated and control T cells
        - Collect T cells from each flask into two 50 ml conical tubes
        - Wash cells
            - Centrifuge at 300 x g for 10 min.
            - Remove supernatant and resuspend cell pellets in 20 ml fresh Assay Medium
            - Centrifuge at 300 x g for 10 min.
            - Remove supernatant and resuspend cell pellets in 10 ml fresh Assay Medium
            - Count cells and dilute to 2x10e6/ml
        - Remove medium from target cell plates (set-up on Day 1)
            - Add 50 µl Assay Medium or BiTE® at 2X in 50 µl Assay Medium to designated wells
            - Add 50 µl BiTE®-activated or control T cells (100,000 cells) to designated wells
        - Incubate at 37°C for 24 hours.
    - Day 3: Assay cytotoxicity by imaging or CellTiter-Glo® as described in TDCC protocol.
        - Cyototoxicity assays can be multiplexed (see separate protocols):
            - T cell activation
            - Soluble factors
            - Immunofluorescence to test for expression of cell-associated markers, such as ICAM-1 and FAS