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

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Abstract

T cell dependent cytotoxicity assay for measuring bispecific T cell engager (BiTE®) activity.

Citation: Sandra L. Ross, Marika Sherman, Patricia L. McElroy, Julie A. Lofgren, Gordon Moody, Patrick A. Baeuerle, Angela Coxon, Tara Arvedson BiTE® T cell-dependent cellular cytotoxicity (TDCC) assay. **protocols.io**
dx.doi.org/10.17504/protocols.io.hweb7be
Published: 17 May 2017

Protocol

Overview

Step 1.

Two methods are described for measuring cytotoxicity – imaging (nuclear count) and CellTiter-Glo®. Set-up for the two assays are the same.

- 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 96-well plate assays, each well contains a final volume of 100 µl (10 µl 10X BiTE® dilutions), 40 µl T cells at desired E:T ratio and 50 µl target cells.
- In a typical 96-well assay, each well contains:
  - 10,000 target cells
  - 100,000 T cells/ well (10:1 E:T ratio)
  - BiTE® dilutions starting at 100 pM final
  - 2 columns of control wells (one with target cells, T cells and no BiTE® and the other with target cells only)
- Cytotoxicity is measured at 48h by imaging (nuclear count) and/or by luminescence (CellTiter-Glo)
- All steps are done in Biosafety laminar flow hood.

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
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
CellTiter-Glo®	Promega	G7572-100ml G7571-10ml
Hoechst 33342 nuclear dye	ThermoFisher	62249
Formaldeyde 16% (w/v) methanol-free	Pierce/ThermoFisher	28908

Thaw frozen T cells

Step 3.

- Keep cells on dry ice till ready for use
- In a 37°C water bath, warm medium (Assay Media) which contains 5% FBS.
- Clean the frozen vial with 70% alcohol before thawing. In a biosafety hood, twist the cap a quarter-turn to relieve pressure, and then retighten the cap.
- Thaw cells in a 37°C water bath for approximately 2 mins. Do not remove the vial until cells are almost completely thawed with a small ice-crystal still visible.
- Clean the outside of the vial with EtOH. In a biosafety hood, measure the cell suspension volume.
- Transfer all the cells to 50ml Falcon tube.
- Slowly rinse the vial with 1 ml of pre-warmed media original cell vial to ensure complete retrieval.
- Slowly add, dropwise, the 1ml of media + cells into 50ml Falcon tube.
- Add 40 ml of media slowly and with swirling,
- Remove 0.5 ml cells for counting on ViCell (Beckman Coulter; default cell setting and 50 images).
- Centrifuge at 1,000 rpm for 10 mins at room temp.
- Remove supernatant.
- Gently resuspend cell pellet in Assay Medium. Add enough medium to bring up to approximate working dilution (generally best to add about 10-20% less medium than calculated so cells can be further diluted).
- Transfer to TC flask and put into 37 deg incubator until ready for use.
- Observe thawing procedure at: http://www.allcells.com/support/BioTube-How-to-thaw-cells.wmv

Prepare BiTE® dilutions

Step 4.

- Determine number of wells needed and make sure to have extra volume for dead volume on electronic pipets or liquid handling automation.
- Use deep well plate for BiTE® dilution (for larger volumes) or 96-well polypropylene plate (for smaller volumes.
- For 96-well plate: 10 BiTE® concentrations + two control wells (no BiTE®)

- Highest working dilution concentration (10X) = 1 nM, 3-fold dilutions X 9 dilutions
- Highest final concentration = 100 pM
- Dilute each BiTE® to 1 nM in Assay Media, then titrate 1:3 across plate in cols. 1-10; Assay Medium only in cols. 11-12.
    - For example, add 300 µl 1nM BiTE® to col. 1 of plate and add 200 µl Assay Medium to cols. 2-12; remove 100 µl BiTE® from col. 1, add to col. 2 and mix; remove 100 µl BiTE® from col. 2, add to col. 3 and mix, and so on through col. 10.
- Volume each BiTE® concentration needed depends on number of cell lines and other parameters being tested and number of replicate wells or plates desired.

Prepare target cells

Step 5.

- Target cells are passaged 2-3X per week in Growth Medium and used for ≤15 passages before thawing a fresh vial.
- Determine number of wells needed and make sure to have extra volume for dead volume on electronic pipets or liquid handling automation.
- For suspension cells: Count cells and centrifuge needed number at 1,000 RPM or 300xg for 10 min; discard supernatant and wash once with Assay Media warmed to 37°C.
- For adherent cells: Use Cell Dissociation Solution (Sigma) to detach adherent cells, resuspend and count
- For 96-well plate assays, use 10,000 target cells/well
    - Dilute cells to 200,000 cells/ml (10,000 cells/well, 50 µl/well)

Dilute T cells

Step 6.

- Determine number of wells needed and make sure to have extra volume for dead volume on electronic pipets or liquid handling automation
- Determine desired E:T ratio, e.g., 10:1 = 10 T cells for each target cell
- Dilute T cells prepared in step 2 above to 2.5X (see example below)
    - For 10:1 E:T ratio with 10,000 target cells/well: 10X target cells = 100,000 T cells/well final; 2.5 X 100,000 = 2,500,000 cells/ml (2,500,000 cells/ml X 0.04 ml/well = 100,000 T cells/well)

Assay set-up

Step 7.

- For imaging assay, use black, clear-bottom PackardView plates; for CellTiter-Glo assay, use white Corning plates (see Materials).
- Add 10 µl of (10X) BiTE® dilutions to wells in cols. 1-10; add 10 µl Assay Medium to wells in col. 11; add 50 µl Assay Medium to wells in col. 12
- Add 50 µl target cells to wells in all columns (e.g., 200,000cells/ml X 0.05ml = 10,000 cells/well)
- Add 40 µl T cells to wells in cols. 1-11 (e.g., 2,500,000 cells/ml X 0.04ml = 100,000 cells/well)
- Mixing occurs upon addition of cells to wells
- Leave assay plates at room temperature in hood for 30 min. before placing in tissue culture incubator
- Incubate at 37°C for 48 hours

Cytotoxicity Assay

Step 8.

- **Imaging assay** (this method is often combined with an immunofluorescence assay to measure e.g., target, ICAM-1, FAS such that both cytotoxicity and expression patterns can be measured in the same assays – see separate protocols)
    - After incubation time, resuspend suspension cells in plate and remove with pipet (and save for subsequent assays) or vacuum manifold
    - Wash plates 2 times with PBS: Add 100 µl/well PBS to remove any debris or T cells with quick rinse. Remove PBS with pipet or vacuum manifold. Repeat with second wash.
    - In fume hood: Remove PBS and fix with 4% methanol-free formaldehyde in PBS, 15 min at room temperature (100 µl/well); remove formaldehyde and dispose in appropriate chemical waste; add back 100 µl/well PBS
    - Remove PBS and add 100 µl/well Hoechst nuclear dye (5 µg/ml in PBS); incubate for 30 min. at room temperature
    - Remove Hoechst dye and dispose in appropriate chemical wasted; add back add back 100 µl/well PBS
    - Read on ArrayScan™ using Target Activation bioapplication optimized for cell line in use. Count cells in 16 10X fields. Note: Any remaining T cells left in wells after washes are excluded from cell counts by the lower nuclear size threshold.
- **CellTiter-Glo® assay**
    - After incubation time, resuspend suspension cells in plate and remove with pipet (and save for subsequent assays) or vacuum manifold
    - Wash plates 2 times: Add 100µl/well Assay Medium to remove any debris or T cells with quick rinse. Remove media with pipet or vacuum manifold. Repeat with second wash.
    - Add 100µl/well of room temperature Assay Medium and add 100uL of room temperature Cell CellTiter-Glo® reagent
    - Incubate 10 minutes.
    - Read on luminometer.

Analysis of supernatants and T cells removed from cytotoxicity assay plates

Step 9.

- Crude supernatants (containing soluble factors and T cells) can be used directly in additional cytotoxicity assays, or used in Transwell® assays (see Transwell® assay protocol); alternatively soluble factors and T cells can be separated and analysed independently.

- Carefully remove supernatants (containing soluble factors and T cells) from wells into 96-well V-bottom polypropylene plates and centrifuge at 300 x g for 5 min
    - Test for soluble factors: Collect cell-free medium and test for cytokines by MSD or EIA according to Manufactures' instructions (medium can be stored at -80°C prior to testing). Please see additional protocols for cytokine detection.
    - Test for T cell activation: After removing cell-free medium (above), T cells are tested by flow cytometry for T cell activation markers (see T cell activation protocol)