Immunofluorescence: EGFR, ICAM-1 and FAS detection

Sandra L. Ross, Marika Sherman, Patricia L. McElroy, Julie A. Lofgren, Gordon Moody, Patrick A. Baeuerle, Angela Coxon, Tara Arvedson

Abstract

This protocol describes methods for detecting cell-surface markers by indirect immunofluorescence after treatment with BiTE®. Cells are analyzed by cellular imaging.

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Protocol

Overview

Step 1.

Detection of EGFR, ICAM-1 and FAS by immunofluorescence is multiplexed with the TDCC imaging assay. After incubation with T cells + EGFR BiTE® or BiTE®-activated T cells or cytokines (IFNγ and TNFα), cells are fixed, and molecules are detected by indirect immunofluorescence. Nuclei are counted in the same assay to get cell counts (a measure of cytotoxicity).

Assemble materials

Step 2.

Materials	Company	Cat.no.
ViewPlate-96 black plate Packardview plate (imaging)	Perkin Elmer	6005182
Effector cells – unstimulated pan T cells	AllCells	PB009-1F
Target cells – SW620, NUGC4	Amgen cell bank	-
BiTE® – EGFR, MEC14	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	Gibco	11875-093
Supplements:		
100U/ml penicillin/streptomycin	Gibco	15140-122
10% heat-inactivated fetal bovine serum (FBS)	Gibco	10082-147
Assay medium: RPMI 1640 medium	Biochrom	FG1215
Supplements:		
1x nonessential amino acids (NEAA)	Gibco	11140-050
10mM HEPES	Gibco	15630-080
50µM 2-ß-mercaptoethanol	Sigma	M6250
1mM sodium pyruvate	Gibco	11360-070
100U/ml penicillin/streptomycin	Gibco	15140-122
5% heat-inactivated fetal bovine serum (FBS)	Gibco	10082-147
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
Mouse anti-human ICAM-1 (CD54) antibody (Clone MEM-111)	Abcam	ab2213
Mouse anti-human FAS (CD95) antibody (Clone DX2)	ThermoFisher	MA1-20163
Goat anti-mouse-AlexaFluor® 488	ThermoFisher	A-11029
Normal goat serum	Sigma	G9023

TDCC assay set-up

Step 3.

- Please refer to TDCC or Bystander TDCC protocols for assay set-up
    - Note: Because target cells can be rapidly killed by BiTE®-activated T cells, shorter incubation times (≤ 24 hours) is recommended

Immunofluorescence

Step 4.

- At the end of the assay, wash and fix cells: Wash cells two times with PBS, and fix with formaldehyde as described in the TDCC imaging assay protocol. Dispose formaldehyde in appropriate chemical waste.
- Prepare reagents while cells are fixing
    - Wash buffer: PBS + 5% normal goat serum
    - Primary antibody in wash buffer
        - Anti-EGFR used at 1 µg/ml
        - Anti-ICAM-1 used at 5 µg/ml
        - Anti-FAS used at 5 µg/ml
    - 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™ (16 x 10X fields) using Target Activation or Spot Detectpr bioapplications.
    - Control wells containing cells +/- treatment are used to set a threshold for AlexaFluor® 488 intensity and % positive cells is determined for each well.
    - Nuclei are counted using with size threshold to exclude any adhering T cells.