
T Cell Activation, *In Vitro*

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Introduction

Mature T cells recognize and respond to the antigen/MHC complex through their antigen-specific receptors (TCR). The most immediate consequence of TCR activation is the initiation of signaling pathways including induction of specific protein tyrosine kinases (PTKs), breakdown of phosphatidylinositol 4,5-biphosphate (PIP2), activation of protein kinase C (PKC) and elevation of intracellular calcium ion concentration. These early events are transmitted to the nucleus and result in

1. clonal expansion of T cells
2. upregulation of activation markers on the cell surface
3. differentiation into effector cells
4. induction of cytotoxicity or cytokine secretion
5. induction of apoptosis

One of the most common ways to assess T cell activation is to measure T cell proliferation upon in vitro stimulation of T cells via antigen or agonistic antibodies to TCR.

This protocol is written as a starting point for examining in vitro proliferation of mouse splenic T-cells and human peripheral T cells stimulated via CD3. Critical parameters include cell density, antibody titer and activation kinetics.

Protocol A: Stimulation of mouse peripheral T cells

Materials

- 1X sterile PBS
- Anti-mouse CD3e, Clone 145-2C11 (Functional Grade, Cat. No. [16-0031](#), or Purified, Cat. No. [14-0031](#))
- Anti-mouse CD28, Clone 37.51 (Functional Grade, Cat. No. [16-0281](#))
- Complete RPMI-1640
- Sterile single-cell suspension of mouse spleen or lymph nodes
- 96-well flat-bottom microtiter plates with lids (Costar Cat. No. 3596)
- Concanavalin A, optional (ConA, Sigma Cat. No. C5275)

Instruments

- Pipettes and pipettors, Multichannel pipettor
- Centrifuge
- 37°C, CO₂ incubator

Experiment Duration

- 2-18 hours to coat antibody to flask or plate
- 20 minutes preparation of spleen single cell suspension
- 20 minutes to set up the assay
- 2-4 days incubation

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Experimental Procedure

Step I: Antibody Coating of the Assay Plate Microwells:

1. Prepare a 5-10 µg/mL solution of anti-CD3e (145-2C11) in sterile PBS. Calculate the number of wells required for each experimental condition and consider triplicate samples for each condition. For example, to coat one-half plate (48 wells) 2.6mL of antibody solution is required. *Note: We have performed titration studies and found these concentrations of 145-2C11 to induce a maximal response. However, a pilot experiment to determine efficacy of other concentrations of this antibody to induce cellular activation can be performed. For costimulation studies using antibodies to other antigens, a suboptimal activation with anti-CD3 may be required. To achieve suboptimal activation via anti-CD3, a 0.5-0.1 µg/mL 145-2C11 antibody solution can be used.*
2. Dispense 50 µL of the antibody solution to each well of the 96-well assay plate. For the control unstimulated wells, add 50 µL of sterile PBS.
3. Tightly cover the plate with Parafilm™ to avoid sample evaporation and incubate at 37°C for 2 hours or prepare the plate one day in advance and keep at 4°C overnight.
4. Just before adding cells, remove the 50 µL antibody solution with a multichannel pipettor.
5. Rinse each well with 200 µL of sterile PBS and discard PBS.
6. Repeat step 5 to remove all unbound antibody from each well.

Step II: Addition of Cells:

1. Harvest spleen and prepare a single cell suspension under sterile conditions. Follow the BestProtocols: [RBC Lysis of Mouse Splenocytes](#) protocol to remove red cells.
2. Count cells and resuspend in complete RPMI-1640 at 10⁶/mL. *Note: This density is optimal for TCR-mediated T cell activation in our experiments. A titration of cell densities (2-3x10⁶ cells/mL to 10⁵ cells/mL) is recommended for optimal activation in your studies.*
3. After washing the wells with PBS (step 6 above), add 200 µL of the cell suspension to each well and place in a humidified 37°C, 5% CO₂ incubator. *Note: For an additional stimulation control, incubate additional cells in 3 wells with Concanavalin A at 1-4 µg/mL of culture medium.*
4. Add soluble anti-CD28 to cells at 2 µg/mL.
5. Incubate for 2-4 days. *Note: Proliferation of cells between days 2 and 4 gives a good proliferation response in our hands; however, this incubation time should be optimized by the end-user.*
6. Cells can be harvested and processed for your assay of interest.

Buffer Recipes

Complete RPMI-1640:

- 900 mL RPMI-1640 (Hyclone Cat. No. SH30027.02)
- 100 mL FBS (Hyclone Cat. No. SH30151.03) Heat inactivated (10% final)
- 1 mL 2-mercaptoethanol (Gibco BRL Cat. No. 21985-023)
- 10 mL L-Glutamine (Hyclone Cat. No. SH30034.01)
- Antibiotic cocktail (optional)

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Protocol B: Stimulation of human peripheral blood mononuclear cells

Materials

- 1X sterile PBS
- Anti-human CD3:
 - Clone OKT3 (Functional Grade Cat. No. [16-0037](#)) or Clone HIT3a (Functional Grade Cat. No. [16-0039](#))
- Anti-human CD28:
 - Clone CD28.2 (Functional Grade Cat. No. [16-0289](#))
- Complete RPMI-1640
- Sterile PBMC
- 96-well flat-bottom microtiter plates with lids (Costar Cat. No. 3596)

Instruments

- Pipettes and pipettors, Multichannel pipettor
- Centrifuge
- 37°C, CO₂ incubator

Experiment Duration

- 2-18 hours to coat antibody to flask or plate
- 30 minutes preparation of PBMC
- 20 minutes to set up the assay
- 2-4 days incubation

Experimental Procedure

Step I: Antibody Coating of the Assay Plate Microwells:

1. Prepare a 5-10 µg/mL solution of anti-CD3e (OKT3 or HIT3a) in sterile PBS. Calculate the number of wells required for each experimental condition and consider triplicate samples for each condition. For example, to coat one-half plate (48 wells) 2.6 mL of antibody solution is required.
Note: We recommend that you run a pilot experiment to determine efficacy of other concentrations of this antibody to induce cellular activation. For costimulation studies using antibodies to other antigens, a suboptimal activation with anti-CD3 may be required. To achieve suboptimal activation via anti-CD3, a 0.5-0.1 µg/mL OKT3 or HIT3a antibody solution can be used.
2. Dispense 50 µL of the antibody solution to each well of the 96-well assay plate. For the control unstimulated wells, add 50 µL of sterile PBS.
3. Tightly cover the plate with Parafilm™ to avoid sample evaporation and incubate at 37°C for 2 hours or prepare the plate one day in advance and keep at 4°C overnight.
4. Just before adding cells, remove the 50 µL antibody solution with a multichannel pipettor.
5. Rinse each well with 200 µL of sterile PBS and discard PBS.
6. Repeat step 5 to remove all unbound antibody from each well.

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Step II: Addition of Cells:

1. Prepare PBMC and resuspend the cells at $1-2 \times 10^6$ /mL of complete RPMI.
Note: This density of PBMCs is optimal for TCR-mediated T cell activation in our experiments. A titration of cell densities ($2-3 \times 10^6$ cells/mL to 10^5 cells/mL) is recommended for optimal activation in your studies.
2. After washing the wells with PBS (step 6 above), add 100 μ L of the cell suspension to each well. For each condition, use triplicate wells.
3. Add soluble anti-CD28 to cells at 2 μ g/mL.
4. Place in a humidified 37°C, 5% CO₂ incubator.
5. Incubate for 2-4 days. *Note: Proliferation of cells between days 2 and 4 gives a good proliferation response in our hands; however, this incubation time should be optimized by the end-user.*
6. Cells can be harvested and processed for your assay of interest.

Buffer Recipes

Complete RPMI-1640:

- 900 mL RPMI-1640 (Hyclone Cat. No. SH30027.02)
- 100 mL FBS (Hyclone Cat. No. SH30151.03) Heat inactivated (10% final)
- 1 mL 2-mercaptoethanol (Gibco BRL Cat. No. 21985-023)
- 10 mL L-Glutamine (Hyclone Cat. No. SH30034.01)
- Antibiotic cocktail (optional)