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Mitochondrial Antigen Presentation in RAW macrophages V.2

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We use this protocol and it's working

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Disclaimer

Protocol Particulars Video

The video below is a supplement with extra context and tips, as part of the Aligning Science Across Parkinson's (ASAP) Protocol Particulars video interview series, featuring conversations with protocol authors.

https://www.youtube.com/embed/92Vy76AakF0?si=Jmug8HphQanjC0pH

Abstract

This protocol details methods for 3-day Mitochondrial Antigen Presentation Assay in a murine macrophage cell line (RAW) that expresses a glycoprotein B (gB) of herpes simplex virus 1 (HSV1) targeted to the mitochondrial matrix (mito-gB). A gB-specific CD8+ T cell hybridoma recognizing the $gB_{498–505}$peptide loaded on MHC class I molecules is also used to monitor antigen presentation through a beta-galactosidase assay kit.

Materials

CELL LINES

- H-2K^{b}RAW macrophage cell line
- β-galactosidase-inducible HSV gB/K^b-restricted HSV-2.3.2E2 CD8+ T cell hybridoma (2E2)

REAGENTS

- DMEM media with 10% [v/v] fetal calf serum, penicillin (FCS) [100 units/ml], and streptomycin [100 µg/ml])
- RPMI-1640 medium supplemented with 5% (v/v) FCS, glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml).
- LPS stock (5mg/ml)
- PFA
- PBS
- Wash buffer: DMEM with 0.1M glycine + 10% iFBS
- Stock peptide
- 1x lysis buffer (stock = 5x, with triton, pH = 7.8) in dH2O
- 1M DTT
- β-galactosidase Assay (CPRG) kit containing lysis buffer, CPRG buffer and CPRG substrate

CONSUMABLES

- 96-well plates
- 50ml conical tubes

EQUIPMENT

- Incubator: 37°C with 5% CO2
- Centrifuge
- Cell counter
- Plate reader

Safety warnings

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

Day 1: cell plating

1d

1 Prepare a 24 well plate:
- Label appropriately (experimental condition – Heath shock (HS), controls, infection controls, etc.)
- Duplicate labelled wells for the peptide control

2 Scrape RAW cells and resuspend (pipette up and down 8x).

3 Count RAW cells and adjust concentration to **1 million cells/ml**.

4 Add 1 mL RAW cells to each well.

5 Check if you have good 2E2 hybridoma cells and passage them if necessary.

6 Incubate at 37 °C with 5% CO2 .

Day 2: Treatment, fixation and hybridoma incubation

1d

7 Treatments (Prior to beginning, check the cell's media red/orange = good, yellow = bad).

7.1 7h 45m

1. Heath shock treatment: incubate the plate in the water bath at 42 °C for 00:45:00
2. Place the plate at 37 °C for 04:00:00
3. Take the cells and re-plate in a 96 well plate. 1 well of a 24 well plate will be distributed in 6 wells = 3 wells of 250 µL and 3 wells of 50 µL for peptide
4. Incubate the cells at 37 °C for 03:00:00

7.2 6h

1. LPS treatment: 1.5 µL per ml (dilute 20 µL of LPS stock in 666 µL of media, then add 10 µL per 1 mL of media)
2. Place the plate at 37 °C for 03:00:00

3. Take the cells and re-plate in a 96 well plate. 1 well of a 24 well plate will be distributed in 6 wells = 3 wells of 250 µL and 3 wells of 50 µL for peptide
4. Incubate the cells at 37 °C for 03:00:00

7.3 6h

1. Bacteria treatment: bacterial infection is MOI 1. Usually 3 µL in 1 mL then add 100 µL per well
2. Place the plate at 37 °C for 03:00:00
3. Take the cells and re-plate in a 96 well plate. 1 well of a 24 well plate will be distributed in 6 wells = 3 wells of 250 µL and 3 wells of 50 µL for peptide
4. Incubate the cells at 37 °C for 03:00:00

8 Prepare [M] 1 % (v/v) PFA in PBS at Room temperature (pH 7.4).

9 Discard supernatant in the 96 well plate and add 50 µL 1% PFA .

9.1 Incubate at Room temperature for 00:10:00 . 10m

10 Prepare 2E2 cells while RAW cells are in 1% PFA:

10.1 Pour flask into 50 ml conical and centrifuge at 1500 rpm, 00:03:00 .

Note

To keep more 2E2 cells growing, add 50 mL RPMI media back into their flask and incubate at 37 °C , 5% CO2 .

10.2 Count 2E2 cells and adjust concentration to **0.4 million cells/ml**. Make enough for all the wells and split volume in 2 tubes (you will need 1 tube to add to cells in 250 µL of media and the other tube for the peptide control wells with 50 µL of media)

11 Add 200 µL wash buffer to each well in the 96 well plate and discard immediately.

! Make sure to wash the cells gently so as to not lose them. Add buffer against the wall of the well.

> Note
>
> Wash buffer = DMEM with 0.1M glycine + 10% iFBS.

11.1 Repeat 2x more: Add 200 µL wash buffer to each well in the 96 well plate and discard immediately. **(Wash 1/2)**

11.2 Repeat 1x more: Add 200 µL wash buffer to each well in the 96 well plate and discard immediately. **(Wash 2/2)**

12 Add 250 µL 2E2 cells per well in ½ of the samples (the non-peptide ones). Add an extra row of 2E2 cells alone as a negative control.

13 Dilute stock peptide (1 µg/ml) **5000 fold** in the 2E2 cells (final concentration: [M] 0.2 nanogram per milliliter (ng/mL)).

14 Add 250 µL peptide + 2E2 cell solution to the remaining wells. Add an extra row of 2E2 cells+peptide alone as a negative control.

15 Incubate at 37 °C , 5% CO2 for 16:00:00 (NO LONGER) . 16h

Day 3: Lysis and revealing 5m

16 Prepare 1x lysis buffer (stock = 5x, with triton, pH 7.8) in dH_2O.

16.1 Add 30 µL 1M stock of DTT in 10 mL lysis buffer .

17 Prepare CPRG solution (pH 7.8).

- 150 µL CPRG buffer
- 20.2 µL water
- 0.046 mg CPRG

18 Centrifuge 96 well plate at 2200 rpm, 00:01:00 .

19 Add 50 µL lysis buffer to each well.

20 When lysis is done, add 170 µL CPRG solution / well.

20.1 Incubate either at Room temperature or 37°C for 00:30:00 until the sample turns dark red (37°C speeds up reaction to about ~ 20 minutes for peptide samples). 30m

21 Transfer 150 µL colored solution to a new plate. Stop the reactions by adding 80 µL of stop solution to each well

Note

Take care not to transfer debris or make bubbles. Make sure all the wells are stop at the same time.

22 Read in a plate reader at a wavelength of 570–595 nm.

Note

* To stop reaction to leave overnight, incubate at 4 °C .