

Detection of CLEC5A-JEV Interaction by ELISA

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[Abstract] JEV (*Japanese encephalitis virus*) belonging to *Flaviridae* interacts with CLEC5A (C-type lectin domain family 5, member A), a member of C-type lectin associated with DAP12 signaling protein and expressed on myeloid cell, as the same extent as *Dengue virus*. This protocol is used to perform and determine the interaction between purified JEV particles with human and murine CLEC5A.Fc fusion proteins by ELISA method. The different strain and batch of purified JEV as well as purity of CLEC5A.Fc fusion protein might affect the result of absorbance. Modifications are most likely needed if users intend to perform ELISA assay.

Materials and Reagents

1. The neurovirulent (RP-9) of *Japanese encephalitis virus* (JEV)
2. *Aedes albopictus* mosquito cell line C6/36
3. BHK21 cell line
4. 20% sucrose
5. Phosphate buffered saline (PBS)
6. FreeStyle 293-F cells (Life Technologies, Invitrogen™, catalog number: R790-07)
7. pcDNA3/CLEC5A_ECD.Fc plasmid (or plasmids carrying extracellular domain of other C-type lectin receptor)
8. 293 fectin Transfection Reagent (Life Technologies, Invitrogen™, catalog number: 12347-019)
9. Gibco FreeStyle 293 Expression Medium (Life Technologies, Invitrogen™, catalog number: 12338018)
10. Protein A sepharose beads (GE Healthcare, catalog number: 17-0780-01)
11. HRP-conjugated anti-human IgG (Fc) (Jackson ImmunoResearch Laboratories, 109-035-003)
12. 3,3',5,5'-tetramethylbenzidine (TMB) (BD Pharmingen, catalog number: 555214)
13. 1x RPMI-1640 (Life Technologies, Gibco®, catalog number: 31800-022)
14. Fetal bovine serum (FBS) (Life Technologies, Invitrogen™, catalog number: 11091-148)
15. BSA (Sigma-Aldrich, catalog number: A2153)

16. KH_2PO_4
17. Na_2HPO_4
18. Blocking buffer (see Recipes)
19. 1 L PBS buffer (see Recipes)

Equipment

1. TECAN ELISA Reader
2. Beckman Ultracentrifugation
3. Pasteur pipette
4. Centrifuge tubes (Corning Incorporated)
5. Microtiter plate (Corning, Costar®, catalog number: 9018)

Procedure

1. Infected C6/36 cells (1×10^7) with JEV (M. O. I. = 0.1) for 2 h followed by removing virus and replenishing fresh RPMI 1640 medium containing 5% fetal bovine serum.
2. After three days, supernatants were harvested and stocked; cells were further refilled with new fresh medium for another three days.
3. Collect viral supernatant (10 ml/tube) and centrifuging supernatant at 8,000 rpm for 30 min at 4 °C.
4. Add 10 ml of supernatant into centrifuge tube followed by 1 ml of adding 20% sucrose cushion at the bottom of centrifuge tube by using Pasteur pipette adding sucrose solution into bottom of the centrifuge tube.
5. Centrifuge supernatant at 28,000 rpm (SW28) for 3.5 h at 4 °C.
6. Remove supernatant and suspending the JEV particles in 0.5 ml PBS, and viral titer was determined by typical plaque assay using BHK21 cell line.
7. 30 µg of pcDNA3/CLEC5A_ECD.Fc plasmid or extracellular domain of other C-type lectin receptor (CLR) plasmid were transfected into suspended FreeStyle® 293-F cells (3×10^7 /30 ml) by using 30 µl of 293fectin® Transfection Reagent.
8. Supernatants were harvested at 48 h of post transfection and subjected to protein purification by protein A sepharose beads.
9. For Fc.fusion protein purification, supernatants were passed through a protein A column [pre-equilibrated with 0.05 M Tris-HCl (0.1 M, pH 7.0)], after binding, washed column with 20 ml of Tris-HCl (0.1 M, pH 7.0) and eluted with 4 ml of Tris-Glycine (0.1 M, pH 3.0) buffer.

10. 50 μ l of sucrose-cushion-purified JEV particles (5×10^6 pfu) in PBS were coated on microtiter plates for overnight at 4 °C, and washed with 250 μ l of PBS for 3 times before adding CLR fusion proteins and control protein.
11. Plate was filled with blocking buffer containing 1% BSA and 1% fetal bovine serum in PBS at room temperature for 1 h to reduce the detection background.
12. Both human and murine CLEC5A.Fc fusion proteins (0.05 mg/ml in PBS; 100 μ l/well) and hu-IgG1 control fusion proteins were added at the amount of 0.05 mg/ml; 100 μ l/well in room temperature for 2 h to interact with coated JEV particle.
13. Remove unbound JEV particle by gentle washing with 250 μ l of PBS twice.
14. HRP-conjugated anti-human IgG (Fc) (1:5,000) were used to detect fusion proteins for 1 h at room temperature followed by washing with 250 μ l of PBS twice and using 100 μ l of 3,3',5,5'-tetramethylbenzidine (TMB) as substrate and detecting the absorbance at 450 nm in ELISA reader.

Recipes

1. Blocking buffer
 - 1% BSA
 - 1% FBS
2. 1 L PBS buffer (pH to 7.2-7.4)
 - 900 ml H₂O
 - 8 g NaCl or 27.4 ml 5 M
 - 0.2 g KCl
 - 0.19 g KH₂PO₄ or 1.4 ml 1 M
 - 0.61 g Na₂HPO₄ or 4.3 ml 1 M

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