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Protein Pull-down Assay Using HiBiT-tag-dependent Luciferase Activity Measurement

Masashi Arakawa and Eiji Morita*

Department of Biochemistry and Molecular Biology, Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki-shi, Aomori, Japan
*For correspondence: moritae@hirosaki-u.ac.jp

Abstract

Co-immunoprecipitation or pull-down assays are frequently used to analyze protein–protein interactions. In these experiments, western blotting is commonly used to detect prey proteins. However, sensitivity and quantification problems remain in this detection system. Recently, the HiBiT-tag-dependent NanoLuc luciferase system was developed as a highly sensitive detection system for small amounts of proteins. In this report, we introduce the method of using HiBiT technology for the detection of prey protein in a pull-down assay. Using this protocol, we demonstrate the formation of a ternary complex consisting of Japanese encephalitis virus NS4B and two host factors, namely valosin-containing protein, and nuclear protein localization protein 4, which is a critical biological event during flavivirus replication in cells.

Keywords: Pull-down assay, HiBiT tag, Split nano luciferase, Flavivirus, Japanese encephalitis virus

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Background

Most proteins interact with their partner proteins to carry out their biological activity. To understand the role of proteins in cells, co-immunoprecipitation (co-IP) or pull-down assays are frequently used to characterize protein–protein interactions. Western blotting (WB) is commonly used to detect prey proteins in co-IP or pull-down assays. However, its sensitivity is low or dependent on the primary antibody, and it gives inaccurate quantification.

High Bit peptide of NanoLuc Binary Technology (NanoBiT) (HiBiT-tag, amino acid sequence: VSGWRLFKKIS) is a part of split NanoLuc luciferase that can reconstitute intact NanoLuc luciferase when another part of split NanoLuc luciferase, Large Bit peptide of NanoBiT (LgBiT), is present. Therefore, the addition of recombinant LgBiT protein and NanoLuc luciferase substrate and measurement of luminescence facilitates the detection of HiBiT-tag (Dixon et al., 2016). Since HiBiT-tag is a short peptide tag with a length of 11 amino acids, it has a minimum effect on the function of fused proteins. Furthermore, a HiBiT-tag-dependent NanoLuc luciferase detection system is useful for quantifying small quantities of protein, because the signal-to-noise ratio is significantly high in NanoLuc luciferase–dependent luminescence, which allows for small-scale experiments. The procedure is simple and therefore suitable for high-throughput assays. In this report, we introduce the method of using the HiBiT-tag-dependent NanoLuc luciferase system for the detection of prey protein in a pull-down assay. Generally, proteins form an oligomeric complex with various partners. In these cases, the depletion of key subunits significantly affects the formation of the entire complex. In this report, we demonstrate that depletion of a mediator protein affects ternary complex formation.

Japanese encephalitis virus (JEV), a single-stranded positive-sense RNA virus, is a human pathogenic flavivirus. In JEV-infected cells, endoplasmic reticulum membrane–derived large compartments (also called viral replication organelles) are observed. Viruses have been considered to efficiently replicate in these compartments, which may be a target for the development of antiviral reagents (Arakawa and Morita, 2019). Previously, our group reported that the host factor valosin-containing protein (VCP) is recruited to the viral replication organelle and helps in viral genome replication (Tabata et al., 2021). However, no direct interaction between VCP and viral proteins were detected. Our previous study revealed a mediator that bridges the interaction between them. We found that the interaction of nuclear protein localization protein 4 (NPL4), a VCP-associating co-factor, and NS4B, a nonstructural viral protein that localizes on the viral replication site, is important for the recruitment of VCP to the viral replication organelle. We have shown that depletion of NPL4 via siRNA knockdown significantly reduces the affinity between VCP and NS4B, through pull-down assay utilizing HiBiT-tag-dependent luciferase activity (HiBiT activity) measurement (Arakawa et al., 2022) (Figure 1A). HiBiT-tagged NS4B and One-Strep-FLAG (OSF)-tagged VCP were co-expressed in 293T cells and the VCP were affinity-purified using Strep-Tactin beads (Figure 1B). The amount of NS4B in the VCP-bound fraction was then determined in the presence of NPL4 and compared with that in the absence of NPL4 by measuring HiBiT activity (Figure 2C). This protocol is not only useful for studying virus–host interactions, but also has broader applications in the investigation of general protein–protein interactions.

Materials and Reagents

1. 1.5 mL tubes (WATSON, catalog number: 8064131815C)
2. 0.22 μm filter (AS ONE, catalog number: 033022SO-SFCA)
3. 6 cm cell culture dish (Thermo Scientific, catalog number: 150462)
4. Cell scraper (VIOLAMO, catalog number: 1-2249-01)
5. White 384-well immuno plates (Thermo Scientific, catalog number: 460372)
6. 293T cells (ATCC: CRL-3216)
7. Plasmid pCAG-NS4B-FLAG-HiBiT, which encodes HiBiT-tagged JEV NS4B protein (Arakawa et al., 2022)
8. Plasmid pCAG-OSF-VCP, which encodes One Strep–tagged VCP proteins or is empty (Arakawa et al., 2022)
9. siRNA-luciferase, which targets firefly luciferase (sense sequence: 3′-CGUACGCGGAAUACUUCGAtt-5′)
10. siRNA-NPL4, which targets NPL4 (sense sequence: 3′-CUGAAGUGGUCGAUGAAAUtt-5’)
11. Nano Glo HiBiT lytic detection system (Promega, catalog number: N3040)
12. Dulbecco’s modified Eagle’s medium (Nacalai Tesque Inc., catalog number: 08458-16)

13. Fetal bovine serum (FBS) (Thermo Scientific, catalog number: 10270-106)
14. Phosphate-buffered saline (PBS) without calcium and magnesium (Nacalai Tesque Inc., catalog number: 14249-24)
15. Benzylpenicillin potassium (Fujifilm Wako Pure Chemical Corporation, catalog number: 021-07732)
16. Streptomycin sulfate (Tokyo Chemical Industry, catalog number: S0585)
17. Strep-Tactin Sepharose 50% suspension (IBA Lifesciences GmbH, catalog number: 2-121-010)
18. Strep-tag elution (10× buffer E) (IBA Lifesciences GmbH, catalog number: 2-1000-025)
19. cOmplete, EDTA-free, protease inhibitor cocktail (Roche, catalog number: 11873580001)
20. Lipofectamine™ 3000 transfection reagent (Thermo Scientific, catalog number: L3000015)
21. Lipofectamine™ RNAiMAX transfection reagent (Thermo Scientific, catalog number: 13778150)
22. Opti-MEM (Thermo Scientific, catalog number: 31985062)
23. Tris (Tris[hydroxymethyl]aminomethane) (Nacalai Tesque Inc., catalog number:35406-91)
24. NaCl (Nacalai Tesque Inc., catalog number: 31320-05)
25. Triton X-100 (Nacalai Tesque Inc., catalog number: 35501-15)
26. 100× penicillin G + streptomycin stock solution (see Recipes)
27. Culture medium (see Recipes)
28. Pull-down washing buffer (see Recipes)
29. 100× concentrated cOmplete stock solution (see Recipes)
30. Lysis buffer (see Recipes)
31. HiBiT reagent (see Recipes)
32. 1× Strep-tag elution buffer (see Recipes)

Equipment

1. Humidified incubator (37 °C, 5% CO_2)
2. Microplate luminometer (Thermo Scientific, Varioskan LUX Multimode Microplate Reader)
3. Vortex mixer (Scientific Industries, model: Vortex-Genie 2)
4. Centrifuge machine for microtube (Thermo Scientific, model: Sorvall™ Legend™ Micro 21R)
5. Rotator (BIO CRAFT, model: BC-710I)

Procedure

1. Add 60 pmol siRNA-NPL4 (or siRNA-luciferase for negative control) to 250 µL of Opti-MEM. Mix well using a vortex mixer (Figure 1).
   Note: Steps 1–8 pertain specifically to the siRNA transfection protocol, while step 9 marks the beginning of the pull-down protocol. To perform siRNA transfection, please follow the "Reverse transfection protocol" of Lipofectamine™ RNAiMAX transfection reagent.

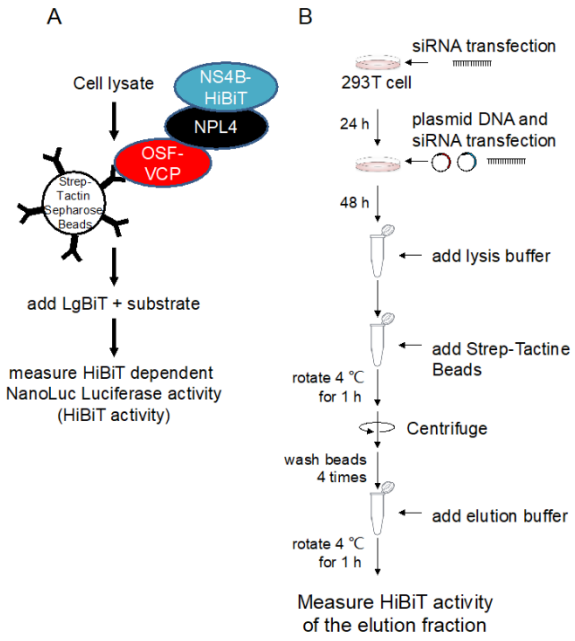

Figure 1. Overview of the protein pull-down HiBiT assay. A. Schematic illustration of the protein pull-down HiBiT assay. **B.** Flow diagram of the protein pull-down HiBiT assay.

2. Add 6 µL of Lipofectamine™ RNAiMAX transfection reagent to 250 µL of Opti-MEM. Mix well using a vortex mixer.
3. Add the mixture from step 1 to that from step 2 and mix well using a vortex mixer.
4. Incubate the mixture for 5 min at room temperature.
5. Seed 293T cells in a 6 cm dish at a density of 750,000 cells/well in 4.5 mL of culture medium.
6. Gently add approximately 500 µL of the siRNA-Lipofectamine mixture to the cell-containing dish.
7. Place the dish in a CO_2 incubator for 24 h.
8. Replenish culture medium at 24 h post transfection.
   Note: Slowly add medium to the side of the well to avoid cell detachment.
9. Add 1.5 µg pCAG-NS4B-FLAG-HiBiT, 1.5 µg pCAG-OSF-VCP, and 120 pmol siRNA-NPL4 (or siRNA-luciferase for negative control) to 250 µL of Opti-MEM. For siRNA transfection, repeat the initial transfection using the same siRNA. Mix well using a vortex mixer.
   (Optional) Set control experiments using an empty vector (pCAG-OSF) in place of pCAG-OSF-VCP.
10. Add 6 µL of Lipofectamine™ 3000 transfection reagent to 250 µL of Opti-MEM. Mix well using a vortex mixer.
11. Add the mixture from step 9 to that from step 10 and mix well using a vortex mixer.
12. Incubate the mixture for 10 min at room temperature.
13. Apply the mixture from step 12 to the cell-containing dish (~500 µL).
14. Place the dish in a CO_2 incubator for 48 h.
15. Aspirate the culture medium and add 1 mL of ice-cold PBS to each dish. Collect the cells using a cell scraper and transfer them to a 1.5 mL tube.
16. Centrifuge the 1.5 mL tubes at 500 × *g* for 10 min at 4 °C.
17. Aspirate PBS. Add 1 mL of ice-cold PBS to each tube and suspend by pipetting.
18. Centrifuge the 1.5 mL tubes at 500 × *g* for 10 min at 4 °C.
19. Aspirate PBS. Add 500 µL of ice-cold lysis buffer to each tube and mix by pipetting.
20. Centrifuge the 1.5 mL tubes at 20,000 × *g* for 10 min at 4 °C.

21. Transfer 5 µL of the supernatant to 1.5 mL tubes for input and add 45 µL of lysis buffer. Mix well using a vortex mixer.
22. Transfer 10 µL of diluted lysate in each well of a 384-well plate.
    Note: To ensure accurate measurements, it is recommended to use more than three wells per sample. If the obtained values are outside the reading range, a dilution series should be carried out.
23. Add 10 µL of HiBiT reagent to each well.
24. Incubate for 10 min at room temperature.
25. Measure the luminescence for 1,000 ms measurement time/well using a microplate reader.
26. Transfer 480 µL of the clear lysate to fresh 1.5 mL tubes.
27. Add 15 µL of Strep-Tactin Sepharose 50% suspension to the lysate and rotate at 4 °C for 1 h.
28. Centrifuge the 1.5 mL tubes at 20,000 × *g* for 1 min at 4 °C.
29. Aspirate the supernatant. Add 1 mL of cold pull-down washing buffer to each tube and mix via inversion.
30. Repeat steps 28 and 29 thrice.
31. Centrifuge the 1.5 mL tubes at 20,000 × *g* for 1 min at 4 °C.
32. Aspirate the supernatant. Add 450 µL of 1× Strep-tag elution and rotate at 4 °C for 1 h.
33. Centrifuge the 1.5 mL tubes at 20,000 × *g* for 1 min at 4 °C.
34. Transfer 400 µL of elution buffer to fresh 1.5 mL tubes.
35. Transfer 10 µL of elution in each well of a 384-well plate.
    Note: To ensure accurate measurements, it is recommended to use more than three wells per sample. If the obtained values are outside the reading range, a dilution series should be carried out.
36. Add 10 µL of HiBiT reagent from Nano Glo HiBiT lytic detection system to each well.
37. Incubate for 10 min at room temperature.
38. Measure the luminescence for 1,000 ms measurement time/well using a microplate reader (Figure 2).

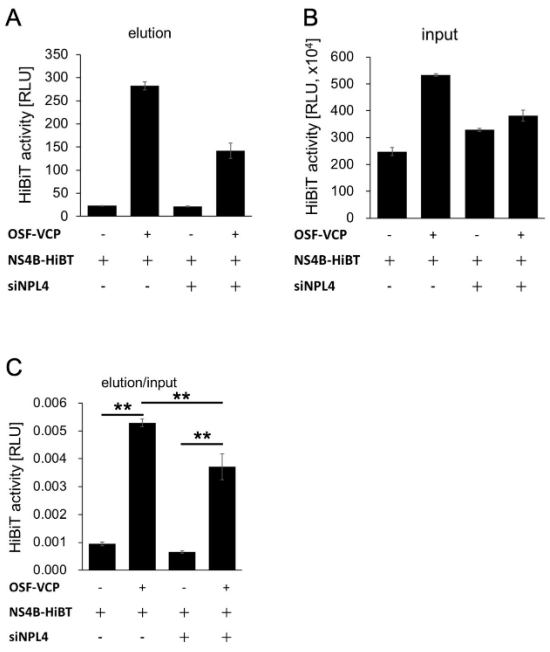

Figure 2. HiBiT activity of the Strep-Tactin-purified fraction and the input fraction. A. HiBiT activity of the Strep-Tactin-purified fraction. **B**. HiBiT activity of the input fraction. **C**. The compensated HiBiT value of the Strep-Tactin purified fraction. The HiBiT activity of the Strep-Tactin purified fraction was calculated by dividing its value by that of the input fraction. The means between two groups were compared using a Student's *t*-test. Differences were considered significant at **P < 0.01.

Notes

1. In the NanoLuc/HiBiT system, strong luminescence emission may be obtained and causes leaks to adjacent wells in a multi-well plate. Therefore, we recommend loading the samples in every other well.
2. The materials used in this study can be obtained from the corresponding author, Eiji Morita, upon reasonable request.

Recipes

1. 100× penicillin G + streptomycin stock solution

PBS 100 mL
Benzylpenicillin potassium 0.626 g
Streptomycin sulfate 1 g
Sterilize using a 0.22 μm filter and store in a refrigerator.

2. Culture medium

Dulbecco's modified Eagle's medium 500 mL
FBS, heat-inactivated via incubation at 56 °C for 45 min 50 mL
100× penicillin G + streptomycin stock solution 5 mL

3. Pull-down washing buffer

1 M Tris pH 7.5 20 mL
5 M NaCl 30 mL
Triton X-100 1.07 g
ddH_2O up to 1 L

4. 100×-concentrated cOmplete stock solution

cOmplete, EDTA-free, protease inhibitor cocktail 1 tablet
ddH_2O 500 μL
Store at -20 ℃

5. Lysis buffer

1 M Tris pH 7.5 20 mL
5 M NaCl 30 mL
Triton X-100 10.7 g
ddH_2O up to 1 L
Add 100× concentrated cOmplete stock solution just before use.

6. HiBiT reagent from Nano Glo HiBiT lytic detection system

Nano-Glo HiBiT lytic buffer 500 μl
Nano-Glo HiBiT lytic substrate 10 μl
LgBiT protein 5 μl

7. 1× Strep-tag elution buffer

Strep-tag elution (10× buffer E) 2 mL
ddH_2O 18 mL

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Competing interests

The authors have no competing interests directly relevant to the content of this article.

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