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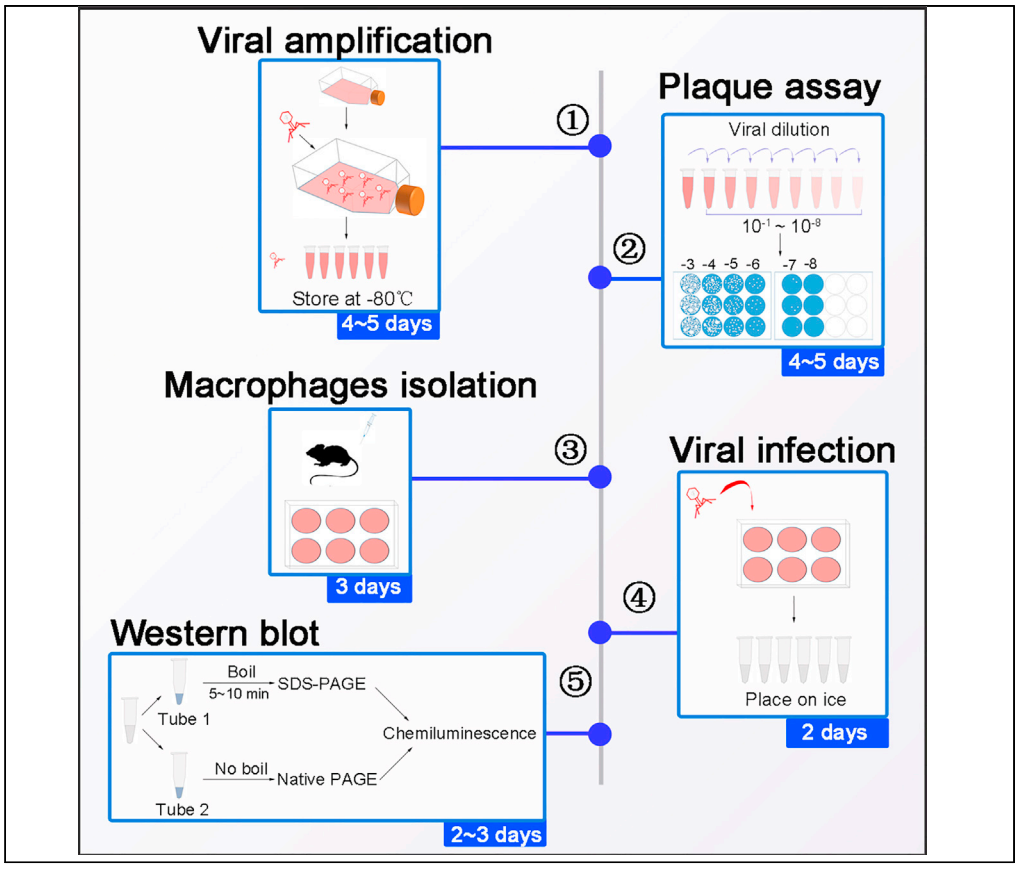
Protocol

Native-PAGE analysis of protein aggregation upon viral infection in mouse macrophages



Upon viral infection, several proteins in the innate signaling pathway form aggregates, which in turn promote the activation of innate antiviral immune response. In this protocol, we use herpes simplex virus type 1 (HSV-1) to infect mouse peritoneal macrophages, and show how to detect the aggregation of TBK1 upon viral infection. The protocol is adaptable for other proteins and other viruses.

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Highlights

Detailed protocol for HSV-1 virus amplification and titer detection

Detailed description of preparation and viral infection of mouse macrophages

Protocol for the detection of protein aggregation with Native-PAGE

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Native-PAGE analysis of protein aggregation upon viral infection in mouse macrophages

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SUMMARY

Upon viral infection, several proteins in the innate signaling pathway form aggregates, which in turn promote the activation of innate antiviral immune response. In this protocol, we use herpes simplex virus type 1 (HSV-1) to infect mouse peritoneal macrophages, and show how to detect the aggregation of TBK1 upon viral infection. The protocol is adaptable for other proteins and other viruses.
For complete details on the use and execution of this profile, please refer to Yan et al. (2021).

BEFORE YOU BEGIN

This protocol below describes the detection of TBK1 aggregation upon HSV-1 infection in mouse macrophages. However, this protocol is also adaptable for detecting aggregation of other proteins.

HSV-1 is a biosafety level 2 pathogen and should be handled under appropriate conditions. The handling of HSV-1 was approved by the Institutional Biosafety Committee of Institute of Basic Medicine, Shandong Academy of Medical Sciences, Jinan, Shandong Province, China.

The mouse experiments are carried out following the general guidelines published by the Association for Assessment and Accreditation of Laboratory Animal Care. All of the mice are maintained under specific-pathogen free conditions with the approval of the Ethics Committee on Scientific Research of Shandong University Qilu Hospital, Jinan, Shandong Province, China. Permission-numbers: KYLL-2017(KS)-361.

HSV-1 amplification

Timing: 4–5 days

1. Seed 5 × 10^6 Vero cells (ATCC, CCL-81) in 30 mL DMEM cell culture medium (see materials and equipment section) in a 225cm^2 cell culture flask. Cells were cultured at 37°C under 5% CO_2 for 1–2 days.

   Note: In this protocol, we used a 225 cm^2 cell culture flask, however, any size of flask can be used as needed. Before plating the Vero cells for HSV-1 amplification, it is necessary to passage cells at least once. We usually passage two or three times.

2. At the time of cell density reach to ~ 100%, change the culture medium with 15 mL DMEM containing virus (see materials and equipment section) to provide an optimal MOI (MOI 0.1). Place the flask in humidified tissue-culture incubator at 37°C under 5% CO_2 for 2–3 days.

⚠ CRITICAL: Before changing the medium, confirm the cells were confluent monolayer. It is also important to avoid overgrowth of the cells.

3. Scrape cells from culture flask by a cell scraper (BIOLOGIX, 70–1250) without removing DMEM containing virus.
4. Transfer cells to a 15 mL centrifugation tube. Freeze the cells at −80°C.
5. After repeat freezing and thawing twice, spin at 500×*g* for 5–10 min at 4°C. Carefully move the supernatant into a new 15 mL centrifugation tube.
6. Aliquot and store the virus in a −80°C freezer and avoid repeated freeze-thaw cycles.

Note: This protocol is also suitable for the amplification of VSV.

HSV-1 titration by plaque assay

Timing: 4–5 days

7. Plate 3 × 10^5 Vero cells per well into 12-well plates. Incubate cells at 37°C under 5% CO_2 for 12–18 h.
8. Dilute the virus in serial 10-fold dilutions with DMEM culture medium (see materials and equipment section), ranging from 10^{-3} to 10^{-8}. For each dilution, dilute the HSV-1 stock into DMEM culture medium to a final volume of 1.2 mL.
9. Confirm the Vero cells to form confluent monolayer, change the culture medium with 1 mL virus dilution. Place the 12-well plates in 37°C under 5% CO_2 for 1–2 h.
10. Remove the culture medium and add DMEM medium containing 1% methylcellulose (see materials and equipment section). Incubate for 2–3 days in 37°C under 5% CO_2.
11. Carefully discard the cell culture medium, add 500 μL 4% formaldehyde per well and fix at 20°C–25°C for 15 min.
12. Wash the plate with PBS for 3 times. Carefully remove PBS from each well.
13. Stain the plate with 500 μL crystal violet solution per well for 15 min. Carefully remove the stain.
14. Wash the plate with PBS for 3 times and gently aspirate PBS.
15. Let the plate air-dry and count the number of plaque.

⚠ CRITICAL: Wells with 5–50 plaques are available for counting. To improve the accuracy of plaque assay, three replicates are preferred.

16. Calculate the titer as described (Shannon et al., 2021).

Viral titer (PFU/mL)=plaque number×dilution/absorption volume in mL

Preparation of starch broth

Timing: 2 h for step 17–24

17. Transfer 12 g of soluble starch to a thermostability glass bottle.
18. Transfer 1 g of sodium chloride to the bottle.
19. Transfer 2 g of tryptone to the bottle.
20. Transfer 0.6 g of beef extract to the bottle.
21. Add 200 mL of double distilled water.
22. Transfer the bottle to a water bath. Let the water bath slowly warm up from room temperature (20°C–25°C) to 90°C.

⚠ CRITICAL: The starch will coagulate into lumps if the water temperature is too hot in the beginning.

23. Keep stirring for 30 s every 3 min for a total of 30 min.

⚠ CRITICAL: After stirring for 30 min, the starch broth will be clear. If not, please prolong the heating time.

24. Transfer the bottle to an autoclave, and sterilize at 121°C (0.1 MPa) for 30 min.
25. Cool the starch broth to 20°C–25°C before storing at 4°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TBK1/NAK Antibody	Cell Signaling Technology	Cat#3013; RRID: AB_2199749
Anti-Actin Antibody (2Q1055)	Santa Cruz Biotechnology	Cat#sc-58673; RRID: AB_2223345
Bacterial and virus strains		
HSV-1 (F strain)	A gift from H. Meng (Institute of Basic Medicine, Shandong Academy of Medical Sciences, China)	N/A
Chemicals, peptides, and recombinant proteins		
Beef Extract	AOBOX	Cat#01-009
Pen Strep Glutamine (100×)	Gibco	Cat#10378-016
Dulbecco's Modified Eagle Medium	Gibco	Cat#12100-038
FBS	Lonsera	Cat#S711-001S
TRYPTONE	Oxoid	Cat#2927114
Sodium deoxycholate	Sigma-Aldrich	Cat#302-95-4
Tween 20	Sigma-Aldrich	Cat#P1379
NaCl	SINOPHARM	Cat#7647-14-5
Soluble starch	SINOPHARM	Cat#9005-84-9
0.1% Crystal violet solution	Solarbio	Cat#G1064
Methylcellulose	Solarbio	Cat#9004-67-5
20×TBST buffer	Solarbio	Cat#T1081
6×Protein Loading Buffer	TRANS	Cat#DL101
Critical commercial assays		
Pierce™ BCA protein Assay Kit	Thermo Scientific	Cat#23225
Experimental models: Cell lines		
Vero	ATCC	CCL-81
Mouse peritoneal macrophage	This paper	N/A
Experimental models: Mice		
Mouse: C57BL/6J (8–12-week old male mice)	This paper	N/A
Software and algorithms		
FlowJo_V10	FlowJo, LLC	https://www.flowjo.com

MATERIALS AND EQUIPMENT

DMEM cell culture medium	
Reagent	Amount
DMEM	445 mL
FBS	50 mL
Pen Strep Glutamine (100×)	5 mL
Total	500 mL
Store at 4°C up to 1 month	

DMEM containing virus	
Reagent	Amount
DMEM	13.5 mL
FBS	1.5 mL
HSV-1 (1×10^7 PFU/mL)	3 mL
Total	18 mL
The medium need to be prepared just before use	

DMEM containing 1% methylcellulose	
Reagent	Amount
2% DMEM (Add DMEM powder to sterile distilled water)	225 mL
2% methylcellulose (Sterilize and store at 4°C for at least 3 days before use)	225 mL
FBS	50 mL
Total	500 mL
Store at 4°C up to 1 month	

Lysis buffer	
Reagent	Amount
NaCl	0.44 g
NP-40	500 μL
0.5 M Tris-HCl (PH7.5)	5 mL
0.5 M EDTA	5 mL
ddH_2O	35 mL
Total	50 mL
If necessary, please add protease inhibitor and phosphatase inhibitor before use	

6× Native-PAGE loading buffer	
Reagent	Amount
4×Tris-HCl/SDS (PH6.8)	7 mL
Glycerol	3 mL
Sodium deoxycholate	0.1 g
Bromophenol Blue	1.2 mg
Aliquot and store at −20°C up to 1 year	

7% Native Gel	
Reagent	Amount
30% Acr-Bis (29:1)	2.3 mL
H_2O	2.14 mL
1.5 M Tris-HCl (PH8.8) (NO SDS)	1.5 mL
10% APS	50 μL
TEMED	10 μL
Total	6 mL
The PAGE need to be prepared just before use	

Native-PAGE running buffer (cathode buffer)	
Reagent	Amount
Tris base	3.03 g
Glycine	14.4 g
Sodium deoxycholate	2 g
Total	1 L
The buffer need to be prepared just before use	

Native-PAGE running buffer (anode buffer)	
Reagent	Amount
Tris base	3.03 g
Glycine	14.4 g
Total	1 L
Store at 20°C–25°C up to 1 year	

10×SDS-PAGE running buffer	
Reagent	Amount
Tris base	30.3 g
Glycine	144 g
SDS	10 g
Total	1 L
Store at 20°C–25°C up to 1 year	

10×Western Transfer Buffer	
Reagent	Amount
Tris base	30.3 g
Glycine	144 g
Total	1 L
Store at 20°C–25°C up to 1 year	

1×Western Transfer Buffer	
Reagent	Amount
10×Western Transfer Buffer	100 mL
methanol	200 mL
Water	700 mL
Total	1 L
Store at 20°C–25°C up to 1 week	

1×Wash Buffer	
Reagent	Amount
20×TBST buffer (Solarbio)	50 mL
Tween 20	1 mL
Total	1 L
Store at 20°C–25°C up to 1 month	

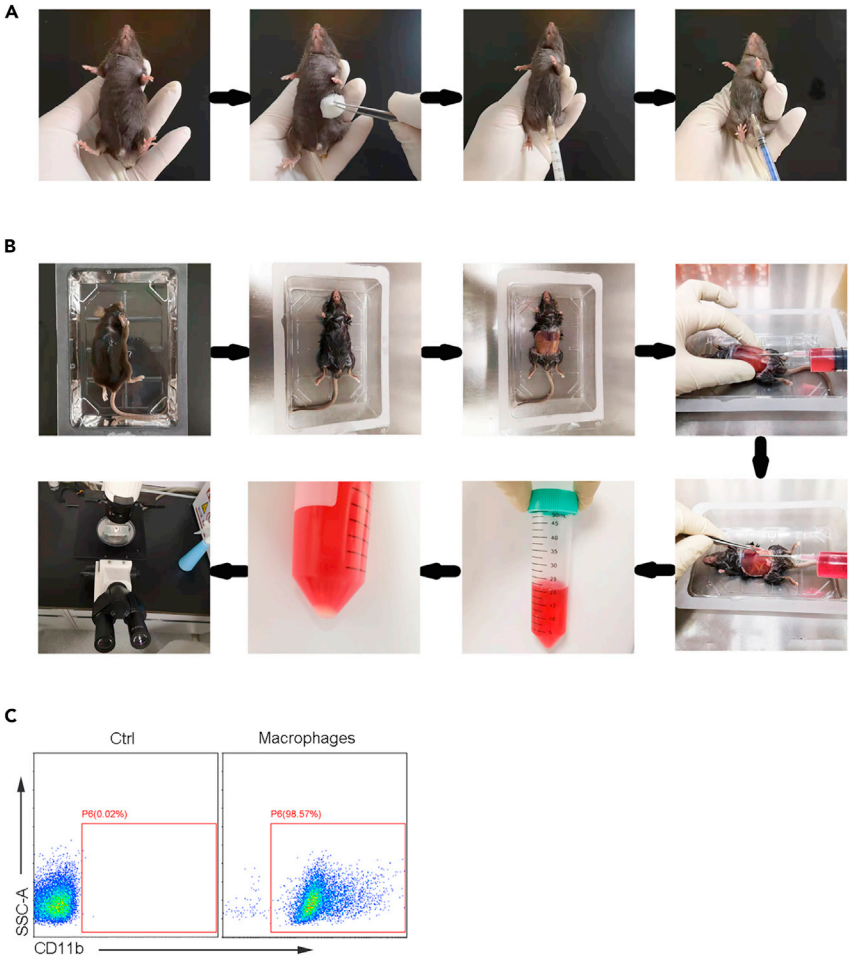

Figure 1. Collection of mouse peritoneal macrophages
(A) Intraperitoneally injection of starch broth.
(B) Collection of mouse peritoneal macrophages.
(C) Flow cytometry analysis of macrophages.

STEP-BY-STEP METHOD DETAILS

Collection of mouse peritoneal macrophages

Timing: 3 days

This section describes an efficient way to collect mouse peritoneal macrophages.

⚠ CRITICAL: Keep the collection steps under aseptic conditions.

1. Choose sex- and age-matched 8–12-week-old C57BL/6J mice. Sterilize abdomen skin with 75 % alcohol. Inject each mouse with 1 mL of starch broth intraperitoneally (Figure 1A and Methods video S1). Macrophages will greatly concentrate in mice's abdominal cavity, and reach to peak within 2–4 days after injection.

2. Euthanize the mice on day 3 by an appropriate method approved by the supervising institution. Soak the mice in 75 % alcohol for 5 min. Use sterile scissors and forceps to expose the peritoneum of the peritoneal cavity (Figure 1B).

3. Inject 10 mL DMEM into the peritoneal cavity with a 20 mL syringe.

⚠ CRITICAL: Be careful not to puncture any organs.

4. Gently massage the abdomen of the mice for 10–20 s.
5. Collect the peritoneal fluid containing peritoneal macrophages and transfer the cells to a sterile 50 mL centrifuge tube by a 20 mL syringe.
6. Repeat step 3–5.
7. Centrifuge the peritoneal exudate cells at 500×*g* for 5 min and discard the supernatant.

Note: To remove red blood cells (RBC), re-suspend cell pellets with 3 mL RBC lysis buffer per mouse. After 5 min, add 3 mL PBS, and mix gently. Centrifuge at 500×*g* for 5 min, and discard the supernatants.

8. Wash cells with PBS and centrifuge at 500×*g* for 5 min, discard PBS, and re-suspend the peritoneal macrophages in 10 mL DMEM cell culture medium (see materials and equipment section).
9. Count the cells and adjust the concentration to 1 × 10^6 cells/mL in DMEM cell culture medium.

Cell seeding and viral infection

Timing: 2 days

This section describes the detailed steps for mouse macrophages infection.

10. Seed 2 × 10^6 macrophages per well to 6-well plates containing 2 mL DMEM culture medium each well. Incubate cells at 37°C with 5% CO_2.
11. Four hours later, the macrophages are well adhering to the wall. Discard cell culture medium and wash gently with PBS to remove non-adherent cells. Add fresh DMEM culture medium and incubate cells at 37°C with 5% CO_2 for 12–18 h.

Note: The macrophages are available for further experiments within 48 h.

Note: The purification of macrophages can be analyzed by flow cytometry (Figure 1C).

12. Aspirate culture medium and add 2 mL fresh DMEM culture medium containing virus (The final concentration of HSV-1 is 10 MOI).

Note: MOI means multiplicity of infection. Thus, 10 MOI means there are 10 viral genomes for each cell.

13. At the desired time post infection, discard the medium from each well and wash with PBS buffer for 3 times.

Samples preparation

Timing: 1–2 h

This section describes the protein samples preparation for Native-PAGE and SDS-PAGE.

14. Add 100 μL cold Lysis buffer (see materials and equipment section) per well and place the plate on ice.
15. After 10 min, scrape and transfer the cells to 1.5 mL pre-cold centrifuge tubes. Mix the lysates gently and incubate on ice for 10–15 min.

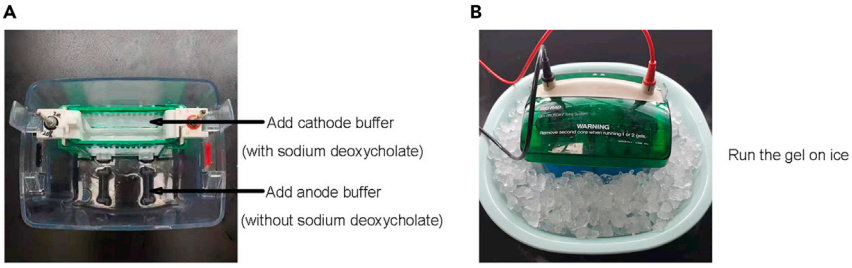

Figure 2. Electrophoresis tank set up to perform Native-PAGE
(A) Add different running buffers to different part of electrophoresis tank.
(B) Run the Native Gels on ice.

16. Spin at 12000×*g* for 10 min at 4°C.
17. Transfer the supernatants to new 1.5 mL pre-cold centrifuge tubes and determine the protein concentration by using a BCA Protein Assay Kit.
18. Make sure the total protein of 100 μL cell lysate in each tube is same.
19. Divide the lysate into two tubes and each tube contains 50 μL cell lysate.
20. Add 10 μL 6×SDS loading buffer to tube 1, and boil for 5–10 min.
21. Add 10 μL 6×Native-PAGE loading buffer (see materials and equipment section) to tube 2. Place tube 2 on ice.

⚠ CRITICAL: Tube 2 do not boil.

Native-PAGE analysis of protein aggregation

Timing: 2–3 days

This section describes the Native-PAGE analysis of protein aggregation.

22. Pre-run 7% Native Gels (see materials and equipment section) with Native-PAGE running buffer (see materials and equipment section) at 45 mA for 30 min on ice (Figures 2A and 2B).

Note: The cathode buffer and anode buffer are different.

23. Load the tube 2 samples into gels and run the gels on ice at 25 mA for 3 h.

⚠ CRITICAL: The gel running time should be adjusted according to the protein size.

24. Transfer the proteins to a 0.45 μm PVDF membrane in 1× Western Transfer Buffer (see materials and equipment section) at 300 mA for 90 min or at 30 V overnight (approximate 700 min) on ice.
25. After blotting, disassemble the setup and place the membrane in 3% BSA for 60 min at 20°C–25°C or for 12 h at 4°C with gently agitating.
26. Incubate the membrane with anti-TBK1 antibody (1:1000) at 4°C for 12 h.
27. Wash the membrane 3 times with 1×wash buffer (see materials and equipment section) at 80 rpm for 5 min at 20°C–25°C.
28. Discard the wash buffer and incubate the membrane with a secondary antibody (1:5000) at 40 rpm for 1 h at 20°C–25°C.
29. Repeat step 27.
30. Quantify the fluorescence of the secondary antibody by using a SAGECREATION Mini-Chemi610 or GE Amersham Imager 600 RGB (Figure 3).

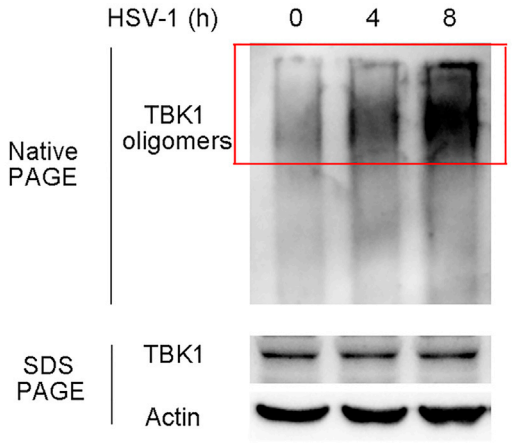

Figure 3. TBK1 form oligomers upon HSV-1 infection
Representative image of TBK1 upon HSV-1 infection in Native-PAGE and SDS-PAGE.

Note: The antibodies in step 26 and step 28 can be reused, please store at −20°C. Once you find the fluorescence of the protein in step 30 is too weak, please change the antibodies.

⚠ CRITICAL: If the fluorescence of the protein is too weak or too strong, we suggest adjusting the amount of proteins loading. We also suggest to run two or more gels once to test the same samples. The samples used in this experiment must be fresh.

Confirm the total protein level by SDS-PAGE

Timing: 2 days

This section describes the SDS-PAGE analysis of protein level.

31. Load the tube 1 samples (step 20) into a 10% SDS-PAGE. Load a pre-stained protein marker to estimate the size of the proteins.
32. Run the gel at 80 V in 1×SDS-PAGE running buffer (see materials and equipment section) for 1 h, and then at 120 V for 1 h.
33. Repeat step 24–30. Change the antibodies used in step 26 and step 28 to the indicated antibodies.

EXPECTED OUTCOMES

The titer of HSV-1 reach to at least 1×10^7 PFU/mL. The purification of mice macrophages reach to at least 97%. Upon HSV-1 infection, TBK1 form oligomers. The oligomerization of TBK1 is undetectable in control macrophages. The fluorescence of the proteins is proper.

LIMITATIONS

This protocol for determine protein aggregation has strict requirements for protein samples. Cell lysates must be on ice during the preparation of protein samples for Native-PAGE. The protein samples must be fresh and avoid storage. During the running of Native-PAGE, the protein size cannot refer to the pre-stained protein marker. It is hard to determine the gel running time.

TROUBLESHOOTING

Problem 1

The HSV-1 plaque is too small to count (before you begin, step 15).

Potential solution

Incubate Vero cells for a longer time after DMEM medium containing 1% methylcellulose addition.

Problem 2

Mice die during the starch infection (step 1).

Potential solution

Make sure the starch broth used in this experiment is sterile. Disinfect mice abdomen skin with 75 % alcohol before injecting starch broth. Most importantly, be carefully when inject starch broth to avoid puncturing mice organs.

Problem 3

Low yield of macrophages (step 9).

Potential solution

The starch broth used in the experiment must be prepared within a month. Make sure you inject starch broth intraperitoneally, but not inject into the chest or under skin.

Problem 4

Low protein concentration (step 17).

Potential solution

Add protease inhibitor and phosphatase inhibitor to the lysis buffer before used. Ensure the whole process of samples preparation is on ice. Increase the number of macrophages if necessary.

Problem 5

High background on the western blot image (step 30).

Potential solution

Lower the concentration of primary or secondary antibody used in the experiment. Ample and stringent washes of membrane is necessary. We also suggest prolonging the blocking time or increasing the BSA concentration.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Chengjiang Gao (cgao@sdu.edu.cn).

Materials availability

All reagents generated in this study are available from the lead contact upon request.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.101080.

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AUTHOR CONTRIBUTIONS

C.G. conceived and designed research; Z.Y. and H.L. performed experiments; Z.Y. and H.L. analyzed the data and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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