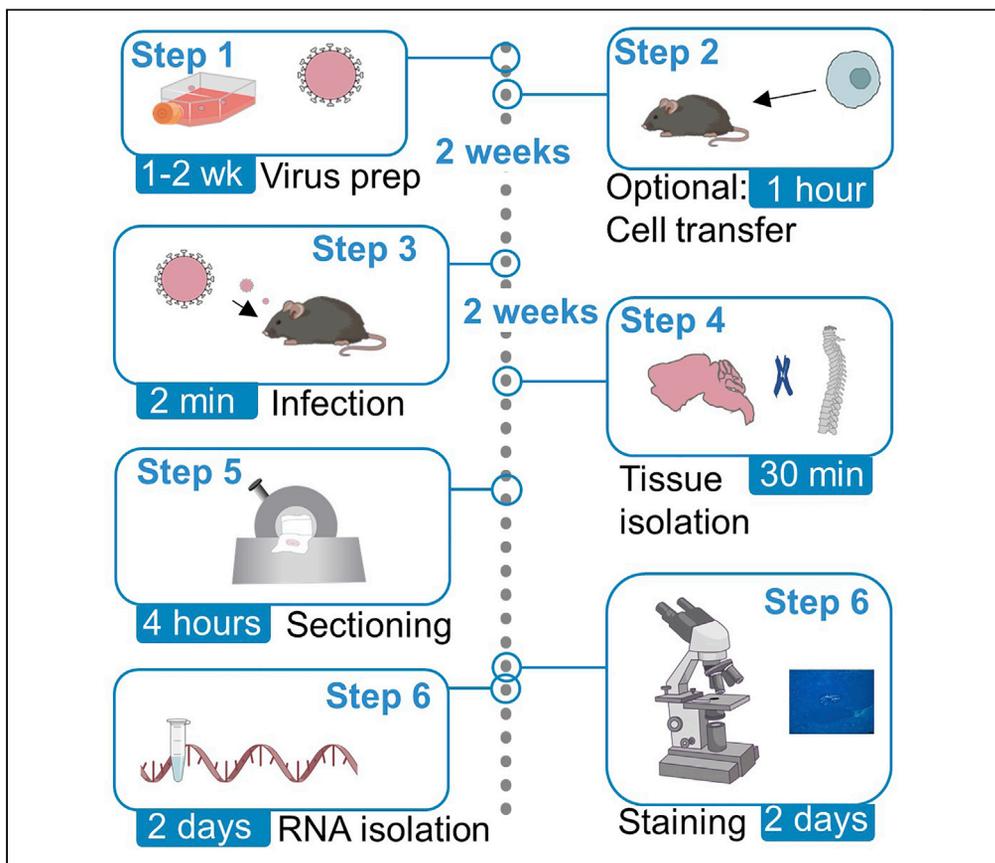


Protocol

Protocol for a mouse CNS demyelination model induced by a combination of HSV-1 and IL-2



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HIGHLIGHTS

We developed a model to induce demyelinating disease using HSV-1 expressing IL-2

Recombinant HSV-1 incorporates environmental (HSV-1) and immune factors (IL-2)

We describe the steps to induce and evaluate demyelinating disease using our protocol

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system. Although viruses have been suggested to be a contributing environmental factor, conventional experimental MS mouse models do not account for this aspect. Here, we describe a mouse model to induce and evaluate demyelinating disease with both a viral and an immune component via ocular infection with a recombinant herpes simplex virus expressing murine interleukin-2.

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Protocol

Protocol for a mouse CNS demyelination model induced by a combination of HSV-1 and IL-2

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SUMMARY

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system. Although viruses have been suggested to be a contributing environmental factor, conventional experimental MS mouse models do not account for this aspect. Here, we describe a mouse model to induce and evaluate demyelinating disease with both a viral and an immune component via ocular infection with a recombinant herpes simplex virus expressing murine interleukin-2. For complete details on the use and execution of this protocol, please refer to Hirose et al. (2020).

BEFORE YOU BEGIN

Experimental considerations

Note: This protocol for inducing demyelinating disease complements the EAE mouse model of MS and induces clinical abnormalities similar to those seen in human patients of MS.

Herpes simplex virus type 1 (HSV-1) is infectious to humans, therefore it is necessary to follow national and institutional guidelines for safe handling of HSV-1. Before obtaining virus stock, consult with the lab safety department and animal facility in your institution and complete necessary paperwork. Following safety guidelines, we handle HSV-1 in biosafety cabinets. After the experiments, bleach treatment is used to inactivate the virus and decontaminate the tools; waste is disposed of as biohazardous waste.

All animal procedures described here, including ocular HSV-1 infection and euthanasia of mice, require approval by institutional animal care and use committee (IACUC). Under the approved protocol, all infected animals are housed in a separate room in the animal facility with low air pressure. Dissection of the infected mice is performed in a safety cabinet and the carcasses of infected mice are discarded in a designated manner.

△ **CRITICAL:** You should practice cryosectioning until you are proficient at it because good quality sectioning and staining of tissues are essential for evaluating demyelination correctly.

Maintaining RS cells

⌚ **Timing:** 1–3 h



We use rabbit skin (RS) cells for amplifying and titering HSV-1 (Perng et al., 1994), since they grow very well and infect efficiently with HSV-1. RS cells are adherent cells that are grown on standard tissue-culture treated vessels without special treatment of the vessel surface, such as collagen coating. RS cells are cultured in 5% CO₂ incubator at 37°C and split every two to three days. Since culturing cells at low density results in poor cell growth, we recommend plating $>1 \times 10^4$ cells/cm². RS cells can be frozen in 80% fetal bovine serum (FBS) and 20% DMSO at –80°C. RS cells may not be readily available commercially, but can be requested from our laboratory.

Alternatives: The virus can also be amplified in other permissive cell lines for HSV-1, such as NIH3T3 and Vero cells, but requires optimization of culture conditions.

1. Dissociate RS cells. The amounts reported are for a 10 cm culture dish. Change amounts according to your culture vessel.
 - a. Aspirate RS cell culture medium.
 - b. Add 10 mL Dulbecco's Phosphate-Buffered Saline and aspirate it to wash out remaining culture medium.
 - c. Add 2 mL of trypsin.
 - d. Incubate at 37°C in a CO₂ incubator for 3 to 5 min.
 - e. Dissociate cells completely by pipetting gently.
 - f. Add 5 mL medium and transfer cell suspension to centrifuge tubes.
 - g. Centrifuge for 5 min at 300 × *g* at 22°C–25°C.
 - h. Remove supernatant and add warm culture medium.
 - i. Mix cells gently by pipetting.
 - j. Count cells using a hemocytometer or autonomous cell counter.
2. Seed cells at six-fold dilution to make the cells confluent in 2 days, or eight-fold dilution to make cells confluent in 3 days.
3. Place the culture vessels in a humidified 37°C incubator with 5% CO₂.

Note: The expected cell number from a 10 cm plate at 80% confluency is approximately $7\text{--}10 \times 10^6$ cells.

Preparation of HSV-1 stock

⌚ Timing: 1–2 weeks

We generally amplify virus stocks in-house. Stocks can be requested from our laboratory or other laboratories working on HSV-1. Typically, we obtain $>1 \times 10^8$ plaque forming units (PFU)/mL of HSV-1 from one T-75 flask.

4. Seed RS cells in several T-75 flasks at approximately 4×10^6 cells/flask and grow for 12–16 h.

Note: Count cells using a hemocytometer or autonomous cell counter after preparing the cell suspension (as in step 2c).

Note: The cells will be confluent for infection the following day (Figure 1A).

5. Replace growth media with 10 mL of fresh growth media 1 h before infection.
6. Thaw the virus stock on ice.
7. Inoculate virus at MOI 0.1 PFU/cell.
8. Incubate for 1 h in a humidified 37°C incubator with 5% CO₂, rocking flasks gently by hand every 15 min.
9. After 1 h, remove the media containing virus, and gently replace it with 10 mL of fresh culture media.

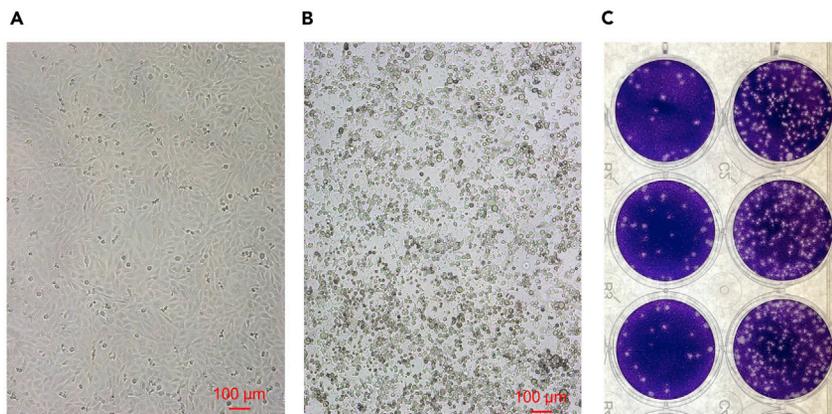


Figure 1. RS cell culture and HSV-1 infection

(A) Bright-field image of appropriate RS cell density on the day of infection. Images were taken using a 10× objective. Scale bar, 100 μm.

(B) Bright-field image showing rounding up of RS cells 2 days after infection with 0.1 PFU/cell HSV-1. Images were taken using a 10× objective. Scale bar, 100 μm.

(C) Plaque assay to determine HSV-1 stock titers. Image shows representative wells in a 12-well plate of RS cells infected with various concentrations of HSV-1 and stained with crystal violet.

10. Incubate in a humidified 37°C incubator with 5% CO₂ for 2–3 days until nearly all cells have rounded up and detached from culture vessel surface (Figure 1B).
11. Freeze flasks horizontally at –80°C until processing.

▣ **Pause point:** Store at –80°C before proceeding. We have stored flasks for a few days without problems, but we have not tried longer freezing times.

12. Collect cell extract containing HSV-1.
 - a. Freeze-thaw flasks twice to rupture cells at –80°C.
 - b. Transfer culture media from the flask to a 50 mL conical tube on ice.

△ **CRITICAL:** Keep viruses on ice at all times after this step.

- c. Centrifuge for 10 min at 4°C at 2,100 × g to pellet cell debris.
 - d. Transfer the supernatant to a new 50 mL conical tube.
 - e. Aliquot 0.25–0.5 mL supernatant into cryotubes and flash freeze on dry ice or liquid nitrogen.
 - f. Store aliquots in a –80°C freezer.
13. Determine the titer of the virus stock.
 - a. Seed 2×10^5 cells per well in a 12-well plate the day before infection.

△ **CRITICAL:** To accurately count the plaques made on the cell lawn, gaps between cells should be minimized. On the day of infection, the surface of the culture plate should be completely covered with cells, but not overcrowded as shown in Figure 1A.

- b. On the day of infection, replace cell culture medium with a reduced amount of warm fresh medium.
 - c. Thaw one of the virus vials on ice.
 - d. Prepare a dilution series using RS cell culture medium for dilution. Keep the tubes on ice.

Note: Typically, we prepare up to a 10^{-8} dilution. Plaques less than 50 can be counted easily in a 12-well plate.

△ **CRITICAL:** Infections can be done in a reduced volume of the medium to increase the chance of viral infection occurring. Make sure that all cells are covered with medium to prevent cell dehydration and death. Typically, we add 0.5 mL of fresh medium per well of a 12-well plate.

- e. Inoculate >10 µL of virus suspension per well in a 12-well plate.
- f. Incubate in a humidified 37°C incubator with 5% CO₂. Swirl the plate every 15 min by hand for 1 h.
- g. Remove the medium and add 1 mL/well of warm RS medium-1% methyl cellulose.

△ **CRITICAL:** To reduce diffusion of the virus particles from the plaque, the media should be replaced with a viscous one by supplementing methyl cellulose. Otherwise, all the cells will be lysed. Make sure that the 2% methyl cellulose and 2× RS cell culture medium are well mixed. If they are not mixed well and the concentration of methylcellulose is lower than 1%, the medium might not be viscous enough to prevent virus diffusion and will result in whole cell lysis.

- h. Culture the plate for 2 to 3 days in a humidified 37°C incubator with 5% CO₂.

Note: Cytopathic effects can be observed under an inverted microscope. Plaques are best counted on day 2 to 3, when they have an adequate size and separated from each other. After day 4, the plaques grow larger and start to overlap.

- i. Remove the culture medium.
- j. Add 0.5 mL of crystal violet solution.
- k. Incubate at 22°C–25°C for at least 30 min.
- l. Discard the crystal violet solution in a designated waste container.
- m. Wash with running tap water several times, discarding waste per your institution’s protocol.

Note: Bi-distilled water is not necessary. The type of the water does not affect the quality of the staining.

- n. Count the plaques (Figure 1C) and calculate the titer as shown below.

$$[\text{titer (PFU mL}^{-1}\text{)}] = \frac{[\text{number of plaques in a well}]}{[\text{volume of inoculation (mL)}] \times [\text{dilution factor}]}$$

Alternatives: It is not necessary to remove the culture medium to stain cells with crystal violet solution, but staining will take approximately one day. Make sure the final methanol concentration is more than 30%, which is necessary to inactivate HSV-1.

Optional: If the titer of the virus is low, you can concentrate it using an ultracentrifuge. After precipitating the cell debris by centrifugation, transfer the supernatant to an ultracentrifuge tube, place it in a SW-28 rotor, and centrifuge at 87,000 × g for 1 h at 4°C. After centrifugation, decant the supernatant and resuspend the virus pellet gently by pipetting in the desired amount of cell culture media. See [Troubleshooting 1](#) for more tips.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
dLAT2903	Homayon Ghiasi, Cedars-Sinai Medical Center (Perng et al., 1994)	N/A
HSV-IL-2	Homayon Ghiasi, Cedars-Sinai Medical Center (Ghiasi et al., 2002)	N/A
Chemicals, peptides, and recombinant proteins		
1× Minimum essential medium (MEM)	Corning	Cat# 10-010-CV
Fetal bovine serum (FBS)	Omega Biosciences	Cat# FB-02

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Trypsin-EDTA (0.05%)	Thermo Fisher Scientific	Cat# 25300-054
Penicillin-streptomycin	Corning	Cat# 300-001-CI
2× MEM	Thermo Fisher Scientific	Cat# 11935-046
Methyl cellulose	MilliporeSigma	Cat# M0512
OCT compound	Sakura Finetek	Cat# 4583
Crystal violet	ACROS Organics	Cat# 405830250
Methanol	Macron Fine Chemicals	Cat# 3016-16
Acetone	Fisher chemicals	CatA929-1
Ethanol 200 proof	Decon Labs	Cat# 2701
Glacial acetic acid	JT Baker	Cat# 9508
Solvent Blue 38	MilliporeSigma	Cat# S3382
Lithium carbonate	MilliporeSigma	Cat# 255823
Cresyl echt violet	StatLab	Cat# AHCEV25
Xylene	CardinalHealth	Cat# C4330
Trizol reagent	ThermoFisher Scientific	Cat# 15596026
Chloroform	MilliporeSigma	Cat# 366919
Ethanol, molecular biology grade	MilliporeSigma	Cat# E7023
Nuclease-free water	QIAGEN	Cat# 129114
Critical commercial assays		
RNeasy Mini Kit	QIAGEN	Cat# 74104
DNase I kit	QIAGEN	Cat# 69989
Experimental models: cell lines		
Rabbit skin (RS) cells	Homayon Ghiasi, Cedars-Sinai Medical Center	N/A
Experimental models: organisms/strains		
Mouse: C57BL/6	Jackson Laboratory	Stock# 000664
Mouse: ILC2 ^{-/-}	Dr. ANJ Mackenzie (MRC Laboratory of Molecular Biology)	N/A
Other		
Ultracentrifuge tube	Beckman Coulter	Cat# 344058
SW 28 Ti swinging-bucket aluminum rotor	Beckman Coulter	Cat# 342207
Ultracentrifuge L8-70M	Beckman Coulter	N/A
Cryotube	Thermo Fisher Scientific	Cat# 368632
Mouse restrainer	Strategic Applications Inc	Cat# MR-01
Tissue-Tek Cryomold, 15 mm × 15 mm × 5 mm	Sakura Finetek	Cat# 4566
Tissue-Tek Cryomold, 10 mm × 10 mm × 5 mm	Sakura Finetek	Cat# 4565
Tissue-Tek Embedding rings	Sakura Finetek	Cat# 4151
Cryostat microtome	Major suppliers	N/A
Microtome blade	Sakura Finetek	Cat# 4689
Microscope slides	VWR	Cat# 48311-703
2 mL tube	QIAGEN	Cat# 990381
Metal beads	QIAGEN	Cat# 69989
Bead dispenser	QIAGEN	Cat# 69965
Tissuelyser LT	QIAGEN	Cat# 85600

MATERIALS AND EQUIPMENT

Alternatives: Reagents of equivalent grade could be used.

RS cell culture medium		
Reagent	Final concentration	Amount
1× MEM	1×	500 mL
Penicillin- streptomycin	1×	5 mL
FBS	5 %	25 mL
Total	n/a	500 mL

Alternatives: Lot testing of FBS is not necessary for RS cells.

Note: RS cell culture medium can be stored at 4°C for up to 1 month.

2× RS cell culture medium		
Reagent	Final concentration	Amount
2× MEM	2×	500 mL
Penicillin-streptomycin	1×	10 mL
FBS	10%	50 mL
Total	n/a	500 mL

Note: 2× RS cell culture medium can be stored at 4°C for up to 1 month.

2% Methyl cellulose		
Reagent	Final concentration	Amount
Methyl cellulose	2%	20 g
ddH ₂ O	n/a	1,000 mL
Total	n/a	1,000 mL

Add a stir bar to the methyl cellulose and sterilize by autoclaving. It is not necessary to dissolve methyl cellulose before autoclaving, as it does not dissolve easily in water. After autoclaving, methyl cellulose usually forms a large solid mass, which can be dissolved by continuous stirring for 12–16 h at 4°C.

Note: 2% Methyl cellulose must be stored at 4°C, and is stable for up to 1 year.

RS culture medium – 1% methyl cellulose		
Reagent	Final concentration	Amount
2× RS cell culture medium	1×	250 mL
2% Methyl cellulose	1%	250 mL
Total	n/a	500 mL

Note: RS culture medium – 1% methyl cellulose can be stored at 4°C for up to 1 month.

Crystal violet solution		
Reagent	Final concentration	Amount
Crystal violet powder	1%	10 g
Methanol	50%	500 mL
ddH ₂ O	n/a	500 mL
Total	n/a	1,000 mL

Note: Crystal violet solution must be stored in tightly sealed bottles at 22°C–25°C for up to 6 months.

Luxol fast blue (LFB) solution		
Reagent	Final concentration	Amount
Ethanol 200 proof	95%	475 mL
Glacial acetic acid	1%	5 mL
Solvent Blue 38	0.1%	0.5 g
ddH ₂ O	n/a	20 mL
Total	n/a	500 mL

Mix vigorously. Solvent Blue 38 requires long time to dissolve.

Note: LFB solution must be stored in tightly sealed bottles at 22°C–25°C for up to 6 months.

Cresyl violet solution		
Reagent	Final concentration	Amount
Cresyl echt violet	0.1%	0.5 g
Glacial acetic acid	n/a	10 drops
ddH ₂ O	n/a	500 mL
Total	n/a	500 mL

Filter with vacuum-driven filter in order to remove undissolved powder.

Note: Cresyl violet solution must be stored in tightly sealed bottles at 22°C–25°C for up to 6 months.

STEP-BY-STEP METHOD DETAILS

Transfer of immune cells to mice

⌚ Timing: 1 h

To dissect the molecular and cellular mechanisms of demyelination induced by HSV1-IL-2, we infected HSV-IL-2 to various genetically engineered mice, or mice with adoptively transferred cells. We found that both type 2 innate lymphocytes (ILC2s) and T cells play a role in induction of demyelination, because mice lacking these cells did not develop demyelination following infection with HSV1-IL-2, whereas transfer of ILC2s isolated from wild-type mice into recipient ILC2-deficient mice (Hirose et al., 2020) or T cells isolated from wild-type mice into SCID mice (Zandian et al., 2011a) infected with HSV1-IL-2 restored the demyelination phenotype. In this section, we describe the method for intravenous injection of immune cells to test their function in this mouse model. After the transfer of the ILC2s or T cells, we waited two weeks for cells to settle down before infecting the mice with HSV-IL-2 virus. Female mice aged 7–9 weeks were used.

1. Isolate ILC2 cells as described previously (Hirose et al., 2020; Moro et al., 2015) and transfer into a 1.5 mL tube.
2. Centrifuge at $500 \times g$ for 10 min, 4°C.
3. Remove the supernatant with a p1000 pipetman.
4. Resuspend the cells with MEM to a concentration of 1×10^6 cells/mL.
5. Place a heat lamp above the mouse cage to help dilate the tail veins.
6. Gently place the animal in a restraining device and stabilize the tail between the thumb and forefinger.
7. Attach a 26G \times 1/2 inch needle to a 1 mL syringe and fill the syringe with 0.1 mL of cell suspension.

△ **CRITICAL:** Remove bubbles by tapping the syringe. Injected bubbles clog blood vessels and can cause the death of the mouse.

8. Wipe the tail with 70% isopropyl alcohol.
9. Start the injection at the middle or slightly distal part of the tail. With the tail under tension, insert the needle, approximately parallel to the vein, and with the bevel (angled surface of the needle) upward, and keep pushing the needle in until it is 3 mm into the vein.
10. Inject 0.1 mL of cell suspension slowly. The fluid should inject freely without resistance.
11. Wait for two weeks so that the cells settle down in the recipients.

Optional: Intraperitoneal (IP) injecting cells is an option if you know that the injected cells can move to the brain.

Ocular infection with HSV-IL-2

⌚ **Timing:** 2 min/mouse

In this section, we describe a method for ocular infection with HSV-1. The recombinant HSV-IL-2 is used to induce demyelination, and its parental virus dLAT2903 was used as a negative control. Both viruses are derived from the highly virulent HSV-1 strain McKrae, and therefore ocular scarification is not required (Ghiasi et al., 2002).

△ **CRITICAL:** This procedure must be done in a biosafety cabinet, and the infected mice must be housed in a separate space from uninfected mice as described in the animal protocol approved by IACUC.

12. Thaw virus stock on ice. Gently mix with pipetting.
13. Dilute the virus stock to 1×10^5 PFU/ μ L with RS medium.
14. Hold a mouse with the left hand and drop 2 μ L of virus stock into each eye with a 10 μ L tip attached to a P10 pipetman (Gilson) or similar device.
15. Return the mouse to the cage.

△ **CRITICAL:** Do not reuse the virus stock. The titer goes down significantly after refreezing.

Isolation of organs for cryosectioning and luxol fast blue (LFB) staining

⌚ **Timing:** 4 days

The presence or absence of demyelination in the optic nerve (ON), spinal cord (SC), and brains of infected mice will be evaluated using luxol fast blue (LFB) staining of cryosections of these tissues as we described previously (Mott et al., 2013; Osorio et al., 2005; Zandian et al., 2009; Zandian et al., 2011a; Zandian et al., 2011b). LFB is used to visualize myelin in nerve tissue through blue staining. Myelin will be stained blue, and neurons will be stained violet. In this section, we describe the method of cryosectioning and LFB staining.

16. Isolate optic nerve (ON), spinal cord (SC) and brain from surviving mice on day 14 post infection. We have detected both focal and diffuse regions of demyelination in the HSV-IL-2 infected mice as early as day 10 post infection and as late as 60 days post infection (the final experimental time point) in all strains of mice tested (Zandian et al., 2009). See also [Methods videos S1](#) and [S2](#).
 - a. Euthanize infected mice with an IACUC approved method.

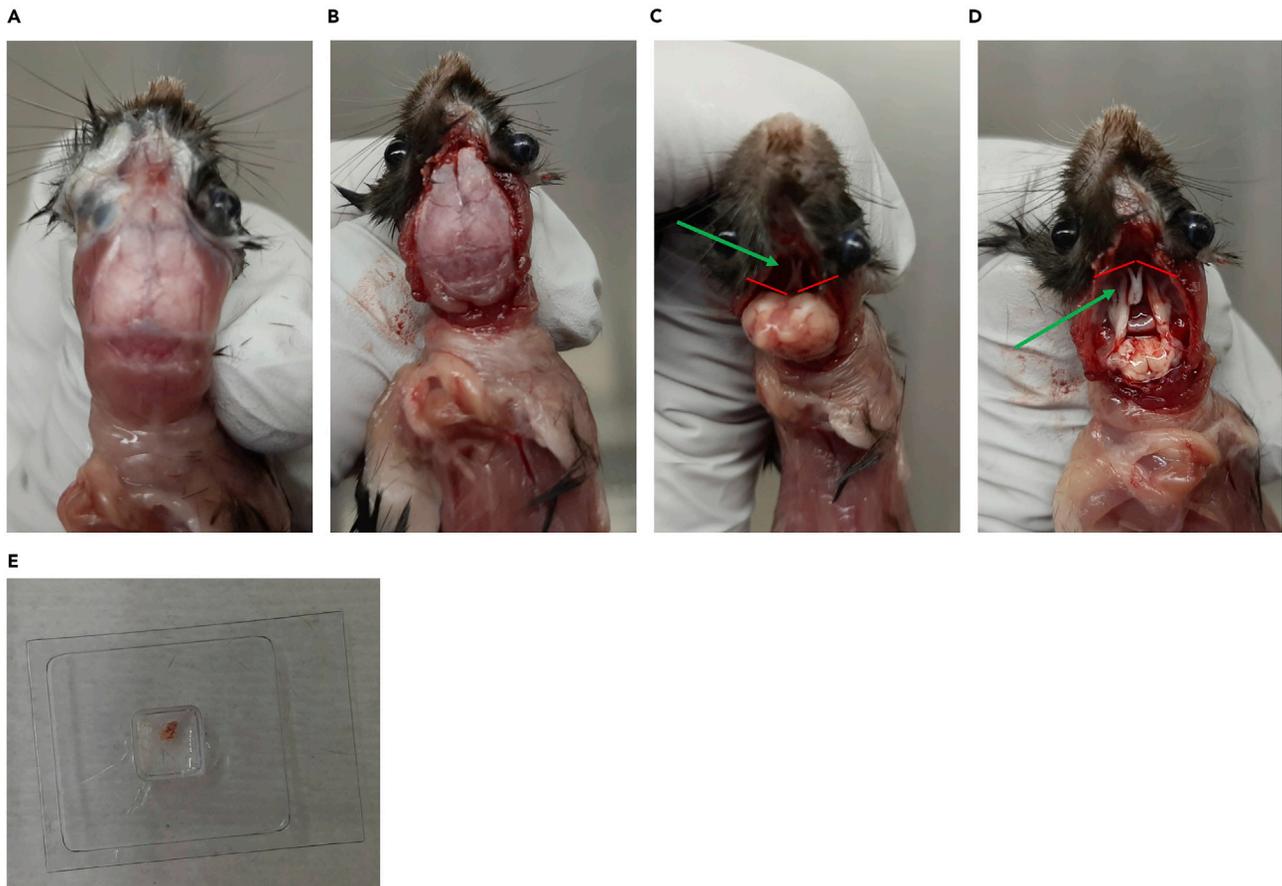


Figure 2. Isolation of brain and optic nerve for cryosectioning

- (A) Cut skin and peel back to expose the underlying tissue.
 (B) Use scissors to cut off the top of the skull and expose the brain.
 (C) Gently peel back the brain to reveal the X-shaped optic nerves (green arrow). Disconnect brain from optic nerves (red lines).
 (D) Optic nerves are exposed (green arrow) after brain removal. Cut along red lines to isolate them.
 (E) Place the optic nerve in mold and fill the mold with OCT compound.

⚠ **CRITICAL:** Euthanize one mouse at a time. We experienced that postmortem rigidity starts around 10 min after euthanasia and, once it happens, the spinal cord cannot be extruded from the spinal column with a syringe.

- b. Wet mouse fur by spraying with 70% ethanol.
- c. Using a pair of scissors, make a small cut along the back of the mouse.
- d. Grasp the skin along both sides of the cut with tweezers and pull in a cranial and caudal directions to tear the skin and expose the spine.
- e. Cut remaining skin with a pair of scissors along the dorsal midline to tip of the nose. Then peel skin to right and left in order to clear the dorsal aspect of the head (Figure 2A).
- f. Using scissors, cut the skull to expose the brain (Figure 2B).
- g. Insert a pair of angled tweezers between the olfactory bulb and cerebral cortex (Figure 2C) and gently lift the cerebrum without cutting the optic nerve. The optic nerves should be visible as X-shaped white fibers (Figure 2C, D, green arrow).
- h. Cut the rostral ends of the optic nerve in order to dissociate from brain.
- i. Remove the brain and wash it with PBS (Figure 2D).
- j. Cut the caudal ends of optic nerves, and pick it up with a pair of tweezers or a small needle.

- k. Isolate the spinal cord as described by Richner and colleagues (Richner et al., 2017), except instead of using a pipette tip, use a 20G needle attached to a 10 mL syringe.
17. Place each tissue in a 10 mm × 10 mm × 5 mm mold (ON) (Figure 2E) or 15 mm × 15 mm × 5 mm mold (SC or brain), then embed the tissues in OCT compound. For brain, place an embedding ring after the brain is placed in the mold but before adding the OCT compound, in order to secure the tissue block on the microtome chuck adaptor.
18. Quickly freeze on dry ice. Store at -80°C .

▣▣ Pause point: Store frozen blocks at -80°C before proceeding. The blocks can be stored for years if the temperature is stable. At the chamber temperature of -18°C , tissues in the block will be damaged over time. Although others recommend using a sample block just once and discarding it after sectioning, we did not have any problems reusing the blocks that had been at -18°C for a few hours as long as they were stored at -80°C immediately after sectioning. We also did not have any problems using these sample blocks for isolating RNA.

19. Prepare cryosections.
 - a. Leave the frozen block inside the cryostat microtome chamber for 15 to 30 min to bring the temperature of the block up to that of the chamber.
 - b. Set the block on the microtome chuck adaptor with OCT compound and adjust the angle of the block.
 - c. Cut the frozen block with the cryotome in a transverse plane at 9 μm thickness and transfer the slices to glass slides.
- ▴ CRITICAL:** Obtaining good quality sections is the key to identifying genuine plaques of demyelination. Besides practicing, there are several points to keep in mind. See [Troubleshooting 2](#).
- d. Air dry sections on the slide at 22°C – 25°C for 12–16 h.
- e. Immerse the slide in acetone for 3 min, then air dry in a chemical hood.

▴ CRITICAL: Acetone is toxic and highly volatile. Work in a chemical hood. Follow the national and institutional guidelines for use and disposal.

- f. Store in slide boxes at -20°C .

▣▣ Pause point: Store slides at -20°C or -80°C before staining. In our experience, sections can be stored for at least a few months.

20. Stand slides in a slide holder and allow to thaw 22°C – 25°C for 10–20 min before transferring to LFB solution 22°C – 25°C .
21. Stain slides in LFB solution in a 56°C water bath 12–16 h.

Note: To avoid evaporation of LFB solution, we use parafilm to seal the container.

22. Remove surplus staining solution by transferring the slide to a 95% ethanol solution for 1–5 min.
23. Wash the slides in distilled water.
24. Wash out extra LFB staining from the slides in 0.05% lithium carbonate solution for 1–30 s.
25. Wash the slides in 70% ethanol for 1–30 s to remove excess LFB staining.
26. Keep the slides in distilled water. Check the slides under a microscope.

Note: Wash out residual 70% ethanol from the slides by moving the slide holder up and down in distilled water.

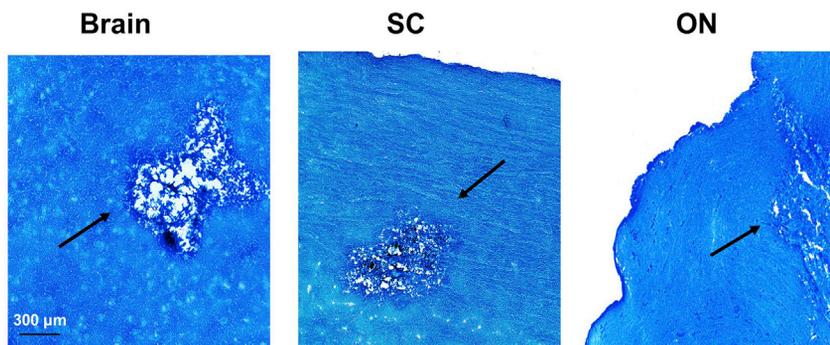


Figure 3. Demyelination in HSV-IL-2-infected mouse on day 14 post infection

Mice were infected ocularly with 2×10^5 pfu/eye of HSV-IL-2 virus. Typical demyelinating lesions detected by LFB staining are shown. The arrows indicate areas of demyelination (i.e., a plaque) in brain, SC, and ON of infected mice. A 10 \times objective lens was used. Scale bar, 300 μ m.

Optional: If the background staining is high, repeat the washing steps 7 and 8 (above).

Pause point: Slides can be stored up to 20 min or so until next step is ready.

27. Stain the slides in cresyl violet solution for 5–10 min.

28. Wash out excess cresyl violet solution from the slides using distilled water. See [Troubleshooting 3](#)

Pause point: Slides can be stored for up to 20 min or so until next step is ready.

29. Wash out extra cresyl violet staining from the slides in 95% ethanol for 5–20 min (check with a microscope).

30. Dehydrate slides in 100% alcohol for 5 min, twice.

31. Dehydrate slides in xylene for 5 min, twice. Air dry.

CRITICAL: Xylene is toxic and highly volatile. Work in a chemical hood. Follow the national and institutional guidelines for use and disposal.

32. Drop mounting medium on the slide and apply a cover glass.

Pause point: Slides can be stored at 22°C–25°C. In our experience, stained sections can be stored for at least one year when protected from light.

33. Observe slides by bright-field imaging with a 20 \times objective lens ([Figure 3](#)).

RNA purification from brain samples

Timing: 2 days

In this section, we describe the method to isolate RNA from frozen blocks of brain made for cryosectioning. The quality and quantity of the isolated RNA is sufficient for reverse transcription and subsequent qPCR. This protocol was modified from the RNeasy mini kit for samples containing OCT compound. Please review the manual for RNeasy kit before starting RNA preparation. This protocol may be applicable to other tissues stored as frozen blocks, although further optimization might be necessary.

34. Cut frozen sections to a thickness of 9 μ m and brush them into a pre-chilled 2 mL tube.

Note: Bring a small box filled with dry ice into the chamber. Keep tubes on dry ice during and after collecting sections. Sections are thin and thaw quickly, which can result in RNA degradation.

Note: Sections do not need to remain flat and may form rolls. 20 to 40 rolled sections will fit into a 2 mL tube. The amount of tissue that can be used will be limited by the capacity of Trizol and the RNA purification column.

Alternatives: Other commercial reagents of the acid guanidinium thiocyanate-phenol-chloroform method could be used in a similar way as Trizol with optimization.

35. Add 0.5 mL of Trizol per tube containing 20 to 40 sections and vortex immediately to minimize section clumping.

⚠ CRITICAL: Trizol and chloroform are volatile and toxic. They must be used under chemical hood.

36. Leave tubes on ice for a few minutes, then vortex again.

Optional: All OCT compound should dissolve into Trizol. It may be necessary to add more Trizol to completely dissolve the sample. If the volume of Trizol is increased, also increase the volume of chloroform and 70% ethanol accordingly.

⏸ Pause point: In our experience, samples can be stored for at least one month at -80°C .

37. Thaw samples on ice.

Note: Keep samples on ice until homogenization is complete (step 38).

38. Add 1 bead (5 mm diameter) per tube using a bead dispenser.

Alternatives: We homogenize tissues using beads and a bead homogenizer, TissueLyser LT. Other commercial bead homogenizers or rotor-stator homogenizers can also be used. Optimization is needed for homogenizing samples completely.

39. Cool the bead homogenizer tube adaptor on dry ice for a few seconds.
40. Set sample tubes in the bead beater. Set oscillations to 50 Hz for 2 min.
41. After oscillation, check for the presence of large tissue fragments. If present, oscillate for another 2 min.
42. Leave tubes at 22°C – 25°C for 5 min (prepare 70% ethanol, spin columns, tubes).
43. Add 100 μL of chloroform to the tube and mix well.
44. Leave the tubes at 22°C – 25°C for 2 min. (Aqueous and non-aqueous phases will separate out.)

⏸ Pause point: Store at -80°C at this step or one step before.

45. Centrifuge the tubes at $12,000 \times g$ for 15 min at 4°C .
46. Transfer the upper phase (0.25 mL) to a new 1.5 mL tube.

Note: Do not take the middle debris layer made of proteins including RNase A, which easily refolds and becomes active in the absence of a denaturing reagent.

47. Add an equal volume (0.25 mL) of 70% ethanol and mix well.
48. Transfer up to 0.7 mL of the mixed solution into a RNeasy Mini spin column.

Alternatives: Other RNA purification columns can be used. For optimization, see the manual attached to the kit.

49. Centrifuge at $8,000 \times g$ for 15 s, and discard flow-through.

Note: If you increased the volume of Trizol at the homogenization step, repeat loading and centrifuging of sample until all the sample has been processed through the column.

50. Add 0.35 mL of Buffer RW1 to the column.
51. Centrifuge at $8,000 \times g$ for 15 s and discard flow-through.
52. Prepare DNase I master mix by adding 10 μ L of Qiagen DNase I with 70 μ L of Buffer RDD per sample.
53. Mix gently, as DNase I is sensitive to physical denaturation.
54. Add 80 μ L of the DNase I solution to the columns.
55. Leave the columns at 22°C–25°C for 30 min.
56. Add 0.35 mL of Buffer RW1 to the column.
57. Leave the columns at 22°C–25°C for 5 min.
58. Centrifuge at $8,000 \times g$ for 15 s, and discard flow-through.
59. Add 0.5 mL of Buffer RPE to the column.
60. Centrifuge at $8,000 \times g$ for 15 s, and discard flow-through.
61. Add 0.5 mL of Buffer RPE to the column.
62. Centrifuge at $8,000 \times g$ for 15 s, and discard flow-through.
63. Place the column in a new collection tube.
64. Centrifuge at maximum speed for 2 min to remove the residual liquid from the column.
65. Place the column in a new 1.5 mL collection tube.
66. Apply 30 μ L of nuclease-free water directly onto the membrane with a pipette.
67. Incubate at 22°C–25°C for 1 min.
68. Spin for 1 min at maximum speed to elute the RNA.
69. Measure the RNA concentration.

Note: Typically, we use 0.5 μ g of RNA for reverse transcription. cDNA derived from 10 ng RNA is sufficient for a qPCR assay. See [Troubleshooting 4](#).

▣▣ Pause point: Purified RNA can be stored in -80°C . In our experience, RNA can be stored for at least one month.

EXPECTED OUTCOMES

This protocol describes a method to induce demyelinating disease using a recombinant HSV-1 that expresses murine IL-2 (HSV-IL-2) as a model of human multiple sclerosis (MS), an autoimmune disease that involves chronic inflammation, demyelination, and neurodegeneration in CNS tissues.

We detected demyelinating plaques in the ON, SC and brain as “white” areas in LFB stained tissues ([Figure 2](#)). On day 14 post infection, 2 to 3 plaques per section were observed in brain and spinal cord from female mice infected with HSV-IL-2 ([Mott et al., 2013](#); [Osorio et al., 2005](#); [Zandian et al., 2009](#); [Zandian et al., 2011a](#); [Zandian et al., 2011b](#)). Demyelinating plaques were also detected after day 10 in the optic nerve of infected mice. No demyelination was detected in brain, spinal cord, or optic nerves of HSV-IL-2-infected mice on days 3 and 7 post infection. These lesions were not observed in mice infected with the parental virus, or similarly constructed recombinant viruses that express IL-4, IFN- γ , IL-12p35, or IL-12p40. Besides LFB staining, signs of demyelinating diseases can also be observed using other methods. Demyelination was confirmed by electron microscopy ([Osorio et al., 2005](#)), and optic neuropathy was shown by the detection of visual-evoked cortical potentials (VECPs) ([Zandian et al., 2009](#)).

With the RNA preparation method described here, 7–15 µg of total RNA were isolated from 20–40 sections. Since the portion of tissue varies among sections, the amount of RNA could be less, if the tissue within the sections is small. The A260/A280 ratios of the isolated RNA preparations were above 1.8 despite varying portions of OCT compound among the sections. Using the RNA isolated with this method, we performed RT-qPCR and observed good amplification of target genes including genes encoding cytokines and transcription factors.

Epidemiologic studies have suggested that certain viruses, as well as various genetic factors, are involved in the onset of MS. However, in traditional MS mouse models, such as experimental autoimmune encephalitis (EAE), demyelination is induced by injecting a myelin component together with adjuvant, without virus involvement. Our model incorporates both a viral (HSV-1) and a genetic (IL-2) component and allows us to study virus-mediated mechanisms of pathogenesis and host response during the development of demyelinating disease, which are lacking in traditional models. The insights obtained through this model may lay the basis of development of novel therapies for MS.

LIMITATIONS

Several viruses have been proposed to trigger autoimmune diseases such as MS. These viruses include several herpes viruses, such as HSV-1, HSV-2, HCMV, EBV, HHV-6, and HHV-7 (Daibata et al., 2000; Ferrante et al., 2000; Knox et al., 2000), as well as non-herpes viruses such as JC virus (JCV), Semiliki forest virus (SFV), human T lymphotropic virus (HTLV-1), Theiler's murine encephalomyelitis virus (TMEV), and mouse hepatitis virus (MHV) (Ferrante et al., 1998; Haider et al., 2000; Haring and Perlman, 2001; Jacobson et al., 1990; Miller et al., 2001; Morris et al., 1997; Parsons and Webb, 1989). However, no direct link between viral infection and MS has been established. There are two major limitations of this mouse model for MS. First, the lack of validation in humans. The second is the potential confounding effect of gut microorganisms in this disease model. Gut microorganisms is yet another factor recently associated with disease in the mouse EAE model (Miyauchi et al., 2020) and may influence the outcome in this disease model. Although the mice are usually kept in a specific pathogen-free environment, the gut microorganisms are not monitored and are known to vary among facilities. The variability in gut microorganisms may change the incidence of the disease among institutions.

TROUBLESHOOTING

Problem 1

Low titer of HSV-1 viruses.

Potential solution

The virus titer should be at least 1×10^5 pfu/µL for ocular infection. If the virus titer is lower, possible causes could be cell density or cell culture conditions. Consider using cells at a relatively low number of passages. We typically use cells below 20 passages. While cells should have enough media to completely cover the monolayer, too much media may result in dilute virus concentration. We have found that 10 mL of media works well for T-75 flasks. Reducing the medium immediately before freezing flasks did not improve the titer, probably because a significant portion of virus is already secreted into the media at this point. Another reason for low virus titer may be caused by ending virus production either too early or too late. For optimal results, cells should be frozen when most cells are rounded. We generally simply use ultracentrifugation to concentrate the virus.

Problem 2

Low quality sections.

Potential solution

Temperature during sectioning is important. If it is too low, sections will be shattered with countless number of slits. A chamber setting of -18°C when sectioning the ON, SC, and the brain works very well for us. Melt any ice from chamber including the thermal sensors to ensure they work properly.

Still, optimization of the temperature might be necessary, because of errors in thermal sensors or differences in instrumental environment. The condition of the blade is also important. Use one that is intact and handle it carefully in order not to damage it. Blades from some companies may require frequent changing. If you find that the tissue dissociates from the compound and rolls up, this is due to a gap forming between the tissue and compound. In this case, you can fill the gap with OCT compound using a plastering technique. Since brain is not too watery or fat-rich, there will be few difficulties in sectioning this tissue.

Problem 3

Poor staining.

Potential solution

Optimization of signal-to-noise ratio (steps 23, 24, 27) is necessary and potential solutions depend on your staining method. LFB destains quickly, so we go through steps 23 and 24 in a very short time. If you see precipitation on the specimen, it likely indicates that the LFB solution has precipitated and needs to be replaced. We typically store the solution in a tightly closed bottle and/or with parafilm sealing for up to six months at 22°C–25°C. Cresyl violet staining is more stable and produces non-specific staining, so it takes thorough washing to see good signal over the background. If the specimen is stained too long, it will be dark, and the excess staining will not come off even with multiple washes. Low signal or faint staining could be the result of repeated reusing of the Cresyl violet stain, or using a stain that is too old.

Problem 4

Low RNA yield.

Potential solution

Preventing RNase contamination is essential. Make sure that all the reagents and tools are RNase free. Also, bacterial contamination is a potential source of RNase. Consider replacing reagents with RNase-free, fresh ones if possible. Aliquot reagents for one time use only. If the tool is not disposable, wipe with decontaminating reagent such as RNase away. Alternatively, tools can be soaked in H₂O₂, chloroform, or 0.1 M NaOH-1 mM EDTA depending on their resistance to these chemicals. Use caution when handling reagents during the experiment. Change your gloves frequently and make sure that your fingers do not contact the inside of tubes and containers. Another major reason for low RNA yield is that tissues were either not homogenized enough in Trizol and/or warmed up before homogenization was complete. Make sure that there are no large pieces left in the homogenate and keep it on ice.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Homayon Ghiasi (ghiasih@CSHS.org).

Materials availability

RS cells, HSV-IL-2, and the parental virus stocks are available upon request by contacting the Lead Contact.

Data and code availability

This protocol does not include newly generated datasets or codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2020.100287>.

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AUTHOR CONTRIBUTIONS

S.H., K.T., and J.Y. contributed to the optimization of protocol and writing of the manuscript. M.K. and H.G. edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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