EAE Induction by Active Immunization in SJL Mice

Recommended protocol for use with:

- Hooke Kit™ PLP\textsubscript{139-151}/CFA Emulsion (cat. no. EK-0120), or,
- Hooke Kit™ PLP\textsubscript{139-151}/CFA Emulsion PTX (cat. no. EK-2120)

**Note:** This protocol version is for use only with Hooke PTX lot numbers 1003, 1004, or 1005. (Future lots may require different PBS dilution.)

**Summary**

These kits are recommended for study of remitting-relapsing experimental autoimmune encephalomyelitis (EAE), including testing efficacy of potential therapeutics.

Each kit consists of:

- antigen (PLP\textsubscript{139-151}) in an emulsion with complete Freund’s adjuvant (in two pre-filled syringes)
- (EK-2120 only:) 1 vial of lyophilized pertussis toxin

EAE is induced in SJL mice by immunization with an emulsion of PLP\textsubscript{139-151} in complete Freund’s adjuvant (CFA). When using EK-2120, the initial immunization is followed by administration of pertussis toxin (PTX) in PBS on the day of immunization.

SJL mice develop remitting-relapsing paralysis, with typical EAE onset between 9 and 13 days after immunization (EK-2120, with PTX) and 11 to 15 days after immunization (EK-0120, without PTX), and peak of disease 1 to 2 days after disease onset for each mouse. The peak of disease lasts 1 to 3 days, and mice completely or partially recover within 7-10 days. Approximately 50 to 80% of mice will show an increase in EAE severity (relapse) after initial partial or complete recovery. This usually occurs 20 to 40 days after immunization.

For study of only the first wave of EAE, we recommend 10 to 12 mice/group.

For study of EAE relapses, we recommend 15 to 20 mice/group. This relatively large number of mice is recommended to ensure that enough mice will relapse for good statistical analysis.
Mice are typically observed for 40 to 45 days after immunization. This is sufficient for at least 50% of mice to relapse.

**Materials needed (per kit)**

<table>
<thead>
<tr>
<th>Qty</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hooke Kit™ PLP&lt;sub&gt;139-151&lt;/sub&gt;/CFA Emulsion (EK-0120) or Hooke Kit™ PLP&lt;sub&gt;139-151&lt;/sub&gt;/CFA Emulsion PTX (EK-2120) (See &quot;Kit selection&quot; below.)</td>
</tr>
<tr>
<td>1</td>
<td>50 mL sterile polypropylene tube (EK-2120 only)</td>
</tr>
<tr>
<td>10</td>
<td>SJL mice, females, at 9 to 10 weeks old (Jackson Laboratory strain SJL/J)</td>
</tr>
<tr>
<td></td>
<td>Phosphate buffered saline (PBS; EK-2120 only) (standard formulation, pH 7.4, calcium-free, magnesium-free)</td>
</tr>
</tbody>
</table>

**Method**

- Use female SJL mice at 9 to 10 weeks of age
- Acclimate mice for at least 2 weeks prior to immunization
- Minimize mouse stress (see “Mouse selection and handling” below)

On Day 0, each mouse will receive adjuvant emulsion s.c. *Only if using* Hooke Kit™ PLP<sub>139-151</sub>/CFA Emulsion PTX (EK-2120), adjuvant emulsion administration is followed by i.p. injection of PTX in PBS.

*Preparation of pertussis toxin solution (applies only if using EK-2120)*

The lyophilized PTX must be dissolved in PBS. Higher PTX concentration induces more severe EAE; the amount of PBS may be adjusted to achieve more or less severe EAE.

**Important:** *The same solution of PTX must be administered to all mice. If more than one kit is used in a study, pool the PTX solution before injection.*

To prepare the PTX solution:

1. Add 1.5 mL PBS for each 6 µg vial of PTX into a single 50 mL sterile polypropylene tube.

   **Note:** *The recommended dilution is for Hooke PTX lots 1003, 1004, or 1005 only; future lots may require different PBS dilution.*
2. Using 3 mL syringe and 18G needle, move 1.5 mL from the 50 mL tube into each PTX vial.

3. Mix each PTX vial well but gently (do not vortex).

4. Let sit for 2 minutes, mix well again.

5. Using the same 3 mL syringe, move solution from each vial back into the 50 mL tube.

**EAE induction procedure**

Subcutaneous injection technique is important to successful EAE induction for both proper administration and consistent mouse stress. Because EAE susceptibility is influenced by mouse stress, the same person should dose all groups.

**Note:** Photos illustrate C57BL/6 mice; mouse handling is identical for SJL mice. These procedures are drafted for right-handed injection; reverse if injecting with the left hand.)

1. Grip mouse tail with finger and thumb of right hand (figure 1).

2. Restrain mouse against cage with three remaining fingers of right hand (figure 2).

3. With left hand, restrain mouse with two fingers behind head (figure 3).

4. Using right hand, inject mouse subcutaneously at 2 sites on the upper back of the mouse (one over each shoulder), with 50 µL of emulsion at each site (figure 4).

5. Using a similar technique, inject mouse subcutaneously at 2 sites on lower back (one injection over each hip/base of tail) with 50 µL of emulsion at each site (figure 5).

After each injection, keep the needle inserted
into the subcutaneous space for 10 to 15 seconds to avoid leakage of the emulsion. Alternatively, a light pull on the syringe plunger will prevent leakage.

Repeat (1-5) for all mice.

Perform steps 6-8 only if using Hooke Kit™ PLP139-151/CFA Emulsion PTX (EK-2120):

6. Draw 1 mL PTX solution into 1 mL syringe.

7. Mount a fresh 27G needle.

8. Inject each mouse i.p. with 0.1 mL/mouse.

Repeat (6-8) for all mice.

9. Check mice for signs of EAE daily starting on day 7 after the immunization. (See Appendix A - EAE scoring guide.)

10. As soon as the first signs of paralysis occur, provide mice with food pellets and wet food on the floor of the cage, and easily accessible water. HydroGel (ClearH2O, Portland ME) may be used as a source of water during the most severe paralysis.

All mice will develop obvious bumps of emulsion at the injection sites 2 to 4 days after injection.

In some mice, alopecia will develop at the site of injection 5 to 7 days after injection.

In most mice, the emulsion will remain at the site of injection for the duration of the experiment (typically 40-45 days). Approximately 10% to 40% of the mice will clear the emulsion by developing skin ulcers at injection sites. Most of the time, these ulcers do not require treatment – they usually heal in a few days and scars form. If concerned, consult attending veterinarian. Antibiotic ointment may be applied.

EAE development does not correlate with mice clearing or not clearing emulsion from the injection site.
Kit selection

Hooke Kit™ EK-2120 is supplied with pertussis toxin (PTX); kit EK-0120 is not. Both kits contain PLP\textsubscript{139-151} as the antigen.

Typically EAE will be induced in 90 to 100% of mice using either kit; the relapse rate will typically be 50 to 80% if PTX is not administered (EK-0120) and 40 to 60% if PTX is administered (EK-2120).

PLP\textsubscript{139-151} induced EAE in SJL mice is sensitive to small variations in mice and in laboratory environments. PTX administration increases the severity of the first wave of EAE and induces disease onset approximately 2 days earlier, but in some labs decreases the incidence and severity of relapses.

For most labs we recommended starting with kit EK-0120 (without PTX); if disease incidence and severity of the first wave of EAE is unsatisfactory, then we recommend using kit EK-2120 (with PTX).

Either method can be used for study of EAE development, including efficacy testing of potential therapeutics.

For information on choice of model and antigen, please see Hooke’s Learning Center (http://hookelabs.com/learning).

Mouse selection and handling

For the most uniform EAE development, use female SJL/J mice at 9 to 10 weeks of age. All mice should be the same age.

EAE susceptibility is influenced by mouse age, strain, and breeder, as well as mouse handling procedures and lab conditions.

Stress strongly decreases EAE susceptibility; minimizing mouse stress is very important for successful EAE induction.

For consistent EAE, minimize mouse stress as follows:

- Acclimate mice to your lab at least 2 weeks before starting.
- House mice in a quiet environment, without excessive noise or vibration.
- Avoid excessive handling of mice. Handle mice gently.
- Try to do all procedures in the mouse room. Avoid moving mice on carts.
- Follow recommended injection procedure. (See “EAE induction procedure” above).
Anesthesia is not recommended. However, if anesthesia must be used per veterinary requirements or to facilitate administration of emulsion, we recommend inhaled anesthesia to minimize stress.

**Expected results**

EAE will be consistently induced in 90-100% of the mice, with onset of paralysis between 9 and 13 days after immunization (EK-2120, with PTX) and 11 to 15 days after immunization (EK-0120, without PTX). The mean maximum score of the first paralytic episode will be 2.5 – 3.5.

Even mice which develop very severe first wave EAE (score 4) will almost always show some recovery within 2 or 3 days; therefore euthanasia due to severe EAE is not recommended. If mice are unable to reach water due to temporary paralysis, Ringer’s solution should be administered subcutaneously until recovery.

Mean maximum score of the second paralytic episode will be 1.5 to 3.5.

Daily dosing of mice with a potential therapeutic often delays and reduces relapses due to stress of dosing.

The following illustrates typical results:
<table>
<thead>
<tr>
<th>Exp.</th>
<th># mice</th>
<th>Mean day of onset ± SD</th>
<th>MMS of 1st wave ± SD</th>
<th>% EAE relapse</th>
<th>MMS of relapse* +/- SD</th>
<th>MMS of relapse period (Days 21-42)**</th>
<th>End score (Day 42)</th>
<th>Disease incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>13.3 +/- 5.6</td>
<td>3.2 +/- 0.5</td>
<td>87.0%</td>
<td>2.8 +/- 0.6</td>
<td>2.7 +/- 0.9</td>
<td>1.7 +/- 1.2</td>
<td>100 %</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>11.9 +/- 0.6</td>
<td>2.7 +/- 0.3</td>
<td>80.0%</td>
<td>2.9 +/- 0.6</td>
<td>2.3 +/- 1.3</td>
<td>1.4 +/- 1.3</td>
<td>100 %</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>12.3 +/- 1.7</td>
<td>3.3 +/- 0.9</td>
<td>80.0%</td>
<td>2.8 +/- 0.4</td>
<td>2.7 +/- 0.7</td>
<td>1.5 +/- 1.0</td>
<td>100 %</td>
</tr>
</tbody>
</table>

* Only for mice which relapsed
** For all mice in the group

Data are from three independent experiments using Hooke Kit™ PLP\textsubscript{139-151}/CFA Emulsion (EK-0120), with female SJL mice.

Similar results are obtained using PLP\textsubscript{139-151}/CFA Emulsion PTX (EK-2120).

**Troubleshooting**

Successful induction of EAE requires low-stress mouse handling and husbandry procedures, good injection technique (as recommended above), use of appropriate mice, and good quality, stable, antigen emulsion.

If you have difficulty inducing EAE, check the following:

- Mice should be acclimated for at least 2 weeks to your lab before starting.
- Use female SJL/J mice at 9 to 10 weeks of age.
- Follow recommended injection procedure (see “EAE induction procedure” above).
- Inject the emulsion at 4 sites, with 50 µL/site.
- Avoid excessive noise or vibration.
- Avoid excessive handling of mice. Handle mice gently.
- Try to do all procedures in the mouse room. Avoid moving mice on carts.
- Avoid use of anesthesia if possible. If anesthesia must be used, we recommend inhaled anesthesia.
Appendix A – Mouse EAE scoring guide

Typically, EAE is scored on scale 0 to 5. Most researchers also give mice “in-between” scores (i.e. 0.5, 1.5, 2.5, 3.5) when the clinical picture lies between two defined scores.

The scoring method differs slightly depending on the stage of disease (onset/peak vs. recovery), for each individual mouse.

Reliable EAE scoring requires skill which comes after considerable experience. To avoid unconscious bias in scoring, we strongly recommend that mice should be scored blind, by a person unaware of which mice have received which treatment.

We recommend the following scoring guidelines for mice during onset and peak of EAE:

Mouse EAE scoring – onset and peak

<table>
<thead>
<tr>
<th>Score</th>
<th>Clinical observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>No obvious changes in motor function compared to non-immunized mice. When picked up by base of tail, the tail has tension and is erect. Hind legs are usually spread apart. When the mouse is walking, there is no gait or head tilting.</td>
</tr>
<tr>
<td>0.5</td>
<td>Tip of tail is limp. When picked up by base of tail, the tail has tension except for the tip. Muscle straining is felt in the tail, while the tail continues to move.</td>
</tr>
<tr>
<td>1.0</td>
<td>Limp tail. When picked up by base of tail, instead of being erect, the whole tail drapes over finger. Hind legs are usually spread apart. No signs of tail movement are observed.</td>
</tr>
<tr>
<td>1.5</td>
<td>Limp tail and hind leg inhibition. When picked up by base of tail, the whole tail drapes over finger. When the mouse is dropped on a wire rack, at least one hind leg falls through consistently. Walking is very slightly wobbly.</td>
</tr>
<tr>
<td>2.0</td>
<td>Limp tail and weakness of hind legs. When picked up by base of tail, the legs are not spread apart, but held closer together. When the mouse is observed walking, it has a clearly apparent wobbly walk. One foot may have toes dragging, but the other leg has no apparent inhibitions of movement. - OR - Mouse appears to be at score 0.0, but there are obvious signs of head tilting when the walk is observed. The balance is poor.</td>
</tr>
<tr>
<td>2.5</td>
<td>Limp tail and dragging of hind legs.</td>
</tr>
<tr>
<td>Score</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
</tbody>
</table>
| 3.0   | Limp tail with paralysis of one front and one hind leg. - OR - ALL of:  
- Severe head tilting,  
- Walking only along the edges of the cage,  
- Pushing against the cage wall,  
- Spinning when picked up by base of tail. |
| 3.5   | Limp tail and complete paralysis of hind legs. In addition to:  
Mouse is moving around the cage, but when placed on its side, is unable to right itself. Hind legs are together on one side of body. - OR -  
Mouse is moving around the cage, but the hind quarters are flat like a pancake, giving the appearance of a hump in the front quarters of the mouse. |
| 4.0   | Limp tail, complete hind leg and partial front leg paralysis.  
Mouse is minimally moving around the cage but appears alert and feeding.  
Often euthanasia is recommended after the mouse scores 4.0 for 2 days. However, with daily s.c. fluids most C57BL/6 mice may recover to 3.5 or 3.0, while SJL mice may fully recover even if they reach score 4.0 at the peak of disease. When the mouse is euthanized because of severe paralysis, a score of 5.0 is entered for that mouse for the rest of the experiment. |
| 4.5   | Complete hind and partial front leg paralysis, no movement around the cage. Mouse is not alert. |
Mouse has minimal movement in the front legs. The mouse barely responds to contact.

Euthanasia is recommended. When the mouse is euthanized because of severe paralysis, a score of 5.0 is entered for that mouse for the rest of the experiment.

Mouse is spontaneously rolling in the cage (euthanasia is recommended).

- OR -

Mouse is found dead due to paralysis.

- OR -

Mouse is euthanized due to severe paralysis.

In the recovery stage of EAE, most mice will have a tail that is no longer limp but is not normal either; it feels rigid and is “hooked”. The hind legs may start moving (pedaling), but the mouse cannot walk. Either change makes scoring difficult.

We recommend the following modifications to the above scoring criteria for these mice:

**Mouse EAE scoring – modified**

<table>
<thead>
<tr>
<th>Score</th>
<th>Clinical observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>When held by the base of tail, tail is somewhat “hooked” and rigid, but tail makes complete rotations around the body axis (“helicopter”). Mouse is healthy. No signs of wobbling.</td>
</tr>
<tr>
<td>0.5</td>
<td>Mouse appears normal but tail is “hooked” and rigid. Tail does not make complete rotations around the body axis (“helicopter”). Mouse is healthy. No signs of wobbling.</td>
</tr>
</tbody>
</table>
| 3.0   | Mouse is found on its side (as described for score 3.5 above), but there is excessive hind leg movement. Mouse cannot walk.  

- OR -  

Mouse has a wobbly walk (as described for score 2.5 above), and is unable to take more than two steps without falling on its side. The mouse is unable to right itself.  

- OR -  

Mouse has poor movement in the hind legs (as described for score 2.5 above), and has partial front leg paralysis evidenced by head held lower than normal and mouse’s inability to right itself when placed on its side. |
| All other scores | Subtract 0.5 from the score of all mice with either a rigid, “hooked” tail or pedaling of hind legs. |

**Relapses**

Relapses are expected only in SJL mice, and only when EAE is induced by active immunization (vs. adoptive transfer).
Hooke's recommended criterion for EAE relapse is an increase in a score by one (1) full point from the lowest score of the remission.

For example, if an animal has an initial peak of disease with a maximum score at 3 and its score then drops to 0.5, the animal should then be considered to have relapsed on the day its score first reaches 1.5 or higher.

A small error in scoring can result in an incorrect detection of relapse. To help verify relapses, we recommend special attention to tail tension. Upon relapse, mouse tails will almost always become limp, similar to the limpness observed at EAE onset (see description of score 1.0). Observation of the newly limp tail is a confirmation of relapse.

For example, a mouse which has recovered to score 0.5 may have further scores of 0.5 or 1.0 without meeting the criterion for a relapse, but an additional increase of just one-half point (score 1.5 instead of 1.0) indicates a relapse. Even if the scoring technician is very experienced, it can be difficult to say if this increase represents a real relapse or a small scoring error. Observation of a clearly limp tail will help confirm that the recorded increase was a true relapse.
Appendix B – EAE development in SJL mice

Because of its many similarities to multiple sclerosis (MS), EAE is used to study pathogenesis of autoimmunity, CNS inflammation, demyelination, cell trafficking and tolerance induction.

EAE is mediated by myelin-specific CD4+ T cells, but CD8+ cells and B cells may also play a role in some models of EAE.

The remitting-relapsing EAE model in SJL mice is the mouse model which most closely resembles human MS.

The model is induced by immunization with PLP\textsubscript{139-151} in CFA emulsion, which may be followed by administration of pertussis toxin (PTX) in PBS if desired to increase severity of the first wave of EAE. The emulsion provides antigen which initiates expansion and differentiation of PLP-specific autoimmune T cells.

PTX enhances EAE development by providing additional adjuvant and facilitating entrance of autoimmune T cells into the CNS. However, in some labs PTX also reduces the incidence and severity of relapses in SJL mice.

This model is usually used to test efficacy of compounds on development of relapses in EAE. It is less often used in a prophylactic treatment study because most mice recover after the first wave of disease, even without treatment.

EAE develops between 9 and 13 days after immunization (EK-2120, with PTX) and 11 to 15 days after immunization (EK-0120, without PTX). The first wave of EAE lasts several days and most mice fully or almost fully recover from it. After a disease-free period, which can last from one day to several months, most mice relapse.

Each individual mouse will have a different course of EAE after the first relapse. Most mice will only partially recover from the first relapse, but some may achieve full recovery. As mice are observed longer, more relapses will develop and less recovery will occur. During the first 5-7 weeks after immunization, 50-80% of mice will develop a relapse. After several months, almost all mice will have relapsed.

The first wave of paralysis occurs at the same time for all mice in the group, and is associated with a loss of body weight which is mostly regained on recovery from the first wave.

Different mice will experience succeeding waves of EAE at different times (not synchronized). In each wave there is body weight loss at onset (but not as dramatic as in the first wave), most of which is regained at recovery. After the first wave of disease, the average body weight of each group is relatively stable, with normal slow
increases with age. This is because individual mice experience relapses at different times, so only a few mice in each group are acutely sick at any given time. Therefore, statistically significant differences in relative body weight between groups are not usually found at the end of the study (typically 40-45 days from immunization).

Both EAE incidence and severity will be reduced if mice are exposed to stress any time after approximately 3 days before immunization. Compound administration is a major source of stress; the more frequent the administration and the less tolerated the vehicle, the greater the reduction of EAE development. Unlike the therapeutic EAE model in C57BL/6 mice, in this model the stress of treatment and administration of vehicle continues to affect disease even after clinical signs of EAE appear.

The most important readouts in this model are incidence of relapse and MMS of the relapse period, calculated for all mice. The MMS of only those mice which relapse rarely shows significant differences between groups because only a fraction of mice relapse and relapse severity tends to be variable even within a single group. The MMS of the relapse period is more sensitive because it is based on all mice in the group and reflects both relapse severity and residual EAE severity in those mice which do not relapse.

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