

A simple method for short-term maintenance of neonatal mice without foster mothers

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ABSTRACT

Mice are typically weaned from their mother between 21 and 28 days of age, or at 10 grams of body weight. However, some biochemical experiments need to be done before the weaning days, and the mother might cannibalize or ignore those manipulated pups. Here, we provide a detailed protocol for maintenance of neonatal mice without the presence of their mothers for biomedical research. The basic instinct of neonate mice to hide under covers is harnessed for their survival in a mother-free environment. When covers are soaked with milk and the only targets for hiding, the neonates would acquire their nutrients at least in an involuntary fashion. The protocol is simple and can be used for neonatal rodent studies for short periods of times, and assures the accuracy of the biomedical experiments if survival rate of neonates is critical.

Keywords: neonate, rodent, baby mice, maintenance, foster mother

BACKGROUND

Over the last century, the role of animals in research is essential to the development of new and more effective methods for diagnosing and treating diseases that affect both humans and animals. It is important to stress that majority of animals necessary for biomedical research in the United States are rodents—rats and mice [1-5].

Neonatal mice have great potential for mechanistic and genomic research in human postnatal development and related diseases [6]. However, females of some strains are poor mothers and can cannibalize and/or ignore their young, especially when the neonates are handled by humans. In such cases, litters may need foster care to survive. The foster mothers have to be a healthy and well-fed litter of her own that is within one or two days of age of the fostered pups. Additional step to be taken is to make sure the foster mother recognizes the new pups. For example, researchers need observe the foster mother and the pups carefully for a day or two if litter survival is crucial for the experiments [7]. Those approaches require a careful planning and make experiments more complicated and expensive.

Some neonatal experiments, such as viral infection studies, do not need a very long term maintenance of the neonates. At the same time, the survival rates are a key readout. We present a protocol for maintaining

neonatal mice without their biological or foster mothers for studying biomedical changes. This method harnesses the instinct of neonates to hide under covers when they are alone. With a stable temperature and plenty of food (milk) soaked in the covers, neonates would acquire certain milk at least in an involuntary fashion. With the method, the postnatal day one (P1) baby mice can survive up to four days without foster mothers. We recognize the simplicity of the protocol and expect a wide usage in various biomedical applications with rodent neonates.

MATERIALS

Reagents

Milk powders (Enfamil ProSobee Soy Sensitive Baby Formula, Dairy-Free Lactose Free Plant Protein Milk Powder), 70% Ethanol, Clidox, Cotton balls, Cardboard box or plastic box, cardboard for making grids, Surface protectors

Equipment

Biosafety Cabinet Class II, Microisolator cages (HEPA filtered) for mice and rat, Heating pad (Sunbeam Xpress Heat Heating Pad)

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PROCEDURE

CAUTION: All animal experiments must be performed in accordance with all relevant institutional and governmental ethics guidelines and regulations. This protocol was approved by the Institutional Animal Care and Use Committee of the University of Nebraska-Lincoln.

For the safety of the handler and the animal, proper methods for handling and restraining laboratory animals should be followed. Proper protection and handling techniques should be chosen based on the biosafety level of the experiments. These practices should be found in Biosafety in Microbiological and Biomedical Laboratories 5th edition, available on the CDC website (<http://www.cdc.gov/od/OHS/biosfty/bmb15/bmb15toc.htm>). For the experiment shown here, a lab coat and gloves will be worn when handling the neonatal mice, in accordance with procedures for handling BSL-2 agents.

Transferring of neonatal mice

1. Sanitize a biosafety cabinet (BSC) with Clidox for working surface and 70% ethanol for side walls.
2. Remove a cage from the rack and spray with 70% Ethanol to sanitize cage outside before moving into the BSC.
3. Place the cage into sanitized BSC and spray Clidox onto the outside surface of the cage and wipe with Clidox towel.
4. Take an autoclaved microisolator cage (has HEPA filter on top of the cage) into the BSC. If necessary, place a preheated heating pad into the cage to keep neonatal mice warm.
5. Open both cages and put the lids leaning against cage with inner side (clean side) out.
6. Transfer neonatal mice using long forceps one by one from holding cage to the transfer cage, and then close both cages. A preheated heating pad may be a necessity in cold weather.
7. Put the transferring cages into a secondary container with locking lid, close the secondary container, spray 70% ethanol on the outside of the secondary container.
8. Properly transport the secondary containers into designated handling rooms, and take out a cage from the secondary container.

Setting up apparatus

CAUTION: All materials need to be autoclaved or sterilized by other methods before uses.

9. Prepare a BSC Class II for handling by clearing the BSC of any unnecessary objects and sterilizing the surface with Clidox and 70% EtOH as described above.
10. Take a large autoclaved microisolator cage for rat with HEPA filter on the top into BSC. Place a heating pad into the cage and a thermometer. Close the cage and adjust the temperature with the heating pad temperature settings. Find one that can keep the inner cage temperature at 29°C–31°C.

TIPS: Make sure to turn off auto shut-off functions in the heating pad and to keep the same temperature all the time.

11. Place a water-resistant surface protector pad at the bottom of a cardboard or plastic box, and divide the box properly with cardboard. The size of the box and grid are based on the numbers of neonatal mice needed and their sizes for the experiments (**Fig. 1**). Usually 3 × 3 × 3 cm³ would be sufficient for two postnatal day 1 (P1) baby mice. The sizes can be adjustable based on the sizes of the pups. It is recommended to have a 1 cm gap between neonates and cotton balls. The height of the grid should be higher enough to prevent the escape of the mice from the grid. Place the boxes into the cage in BSC.
12. Milk powders (Enfamil ProSobee Soy Sensitive Baby Formula, Dairy-Free Lactose Free Plant Protein Milk Powder) are dissolved in autoclaved water at approximately 10% concentrations. Using a large petri dish, place cotton balls insider (Equate Beauty Jumbo Cotton Balls), and soak the cotton balls with milk. Based on the sizes of cotton balls, 5–10 ml milk per cotton ball is sufficient. A cotton ball can be split into several smaller pieces. Place 2–4 pieces of cotton balls soaked with soymilk into the corners of the grids described in previous step.

TIPS: (1) The cotton ball should be wet but soft and allow pups to hide below; (2) mice like corners. Place cotton balls on all corners would help their survivals; and (3) Different sources of milk may have an impact on neonate's immune system [8,9]. The specific milk (Enfamil ProSobee Soy Sensitive Baby Formula) may enhance baby's immune response, however, due to the short duration time (up to 7 d), the effects may be marginal.

13. Place neonatal mice into the grids. It is recommended to put no more than two neonatal mice in one grid (Fig. 1).

CRITICAL STEP: Fewer mice would prevent neonates huddling to each other to form a ball. Once the neonates are huddled together, they seem to be less interested in searching for food and covers. Without huddling, neonates have an intrinsic desire to hide under certain covers. The cotton balls are their only and prime targets for cover-ups. Because cotton balls are soaked with soymilk, the neonates would get their milk in an involuntary fashion.

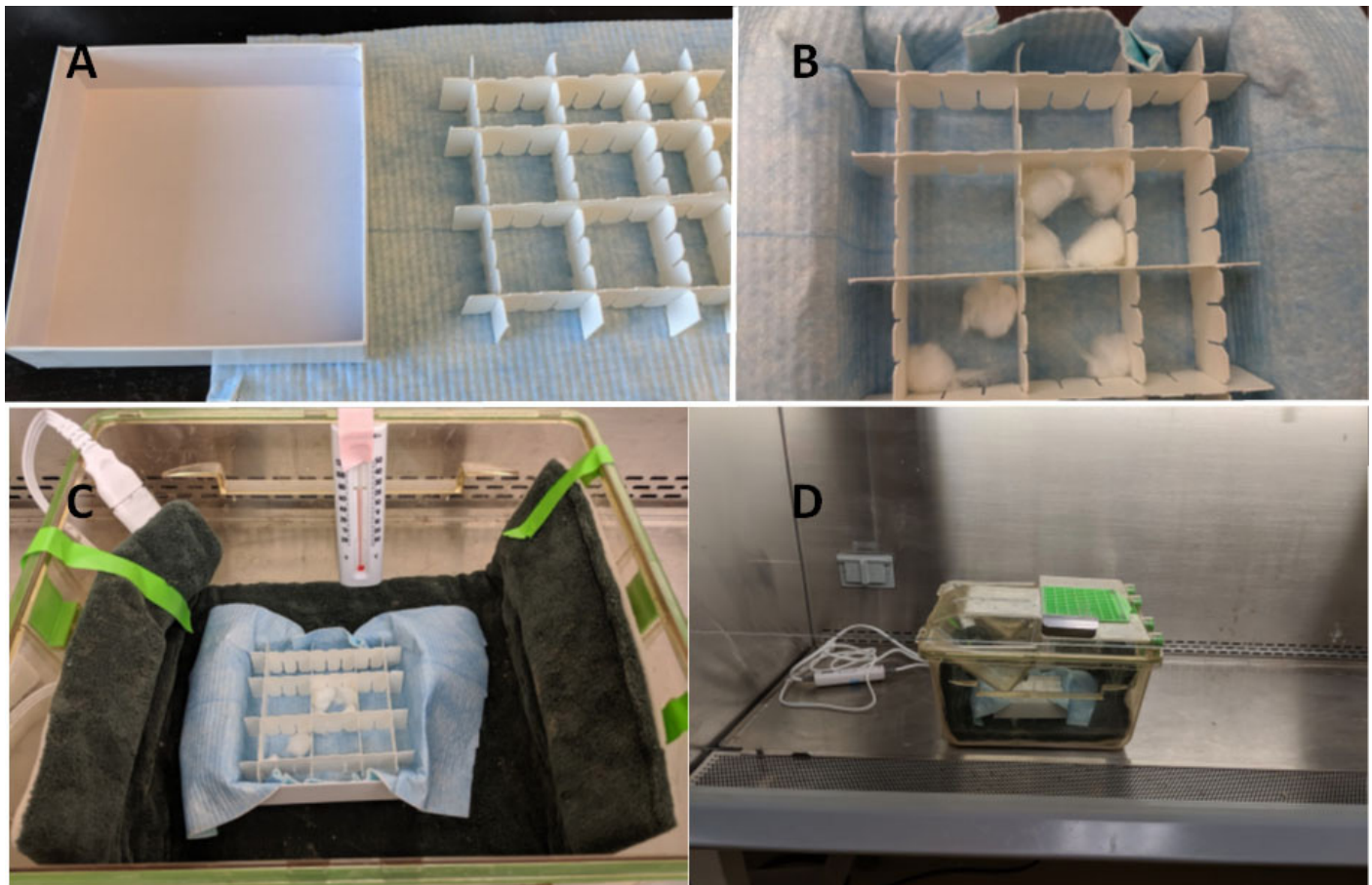


Figure 1. Diagram of setting up apparatus for neonates. **A.** A box, bench protector, and cardboard separator. The box is $13 \times 13 \times 3$ cm³ in dimension. The size of the grids is based on application. **B.** Place soaked cotton balls in the box. Usually all corners are placed with balls. **C.** Place the box into a large microisolator cage for rats with heating pad and thermometer in place. **D.** Close the lid in a BSC hood.

14. Close the cage lid, change the cotton balls twice a day. The pad may be changed every day if necessary.
15. Manipulate and dispose the neonatal mice for proper experiments following approved protocols for desired times.

CAUTION: Because of the wet cotton balls and frequent contacts, ink marks on neonates could not stay for certain time. As neonates are usually housed as a group of two and if identification of the members is a necessity, we recommend use other established labelling methods to differentiate individuals in compliance with regulations and research protocol requirements.

Clean-up

16. Spray the mouse cages with 10% bleach and/or 70% ethanol. Discard all the materials/objects into a biosafety waste container.
17. Remove the waste liquid container from the hood and add additional 10% bleach, wait for 10 min, and pour the waste liquid down the sink. Autoclave the plastic and other solid waste in a biohazard container and then dispose them as instructed by your institute’s biosafety officer.
18. Clean any equipment or surfaces that came in contact with the procedures with 10% bleach and then 70% ethanol.
19. Discard lab bench paper into biohazard bin and wipe down all surfaces and instruments that may have come in contact with the baby mice. Discard gloves.

ANTICIPATED RESULTS

CD1 mice were kept under standard laboratory conditions: artificial 14-h light/10-h dark cycle, with an ambient temperature of $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$; they were housed in filter-topped transparent cages and given standard diet and water ad libitum [10] Health monitoring was performed according to the guidelines confirming the specified pathogen free health status of sentinel animals maintained in the same animal room. Day

of birth was noted by daily visual inspections of the animals and considered as postnatal day 0 (P0). P1 or P7 neonates were transferred to new cages for experiments as described in the protocol for seven days, and the surviving pups were counted once a day. The results from two independent experiments are shown in **Figure 2**. Other than CD-1 mice, this method was also successfully implemented in C57BL/6 mice and its derivatives (data not shown).

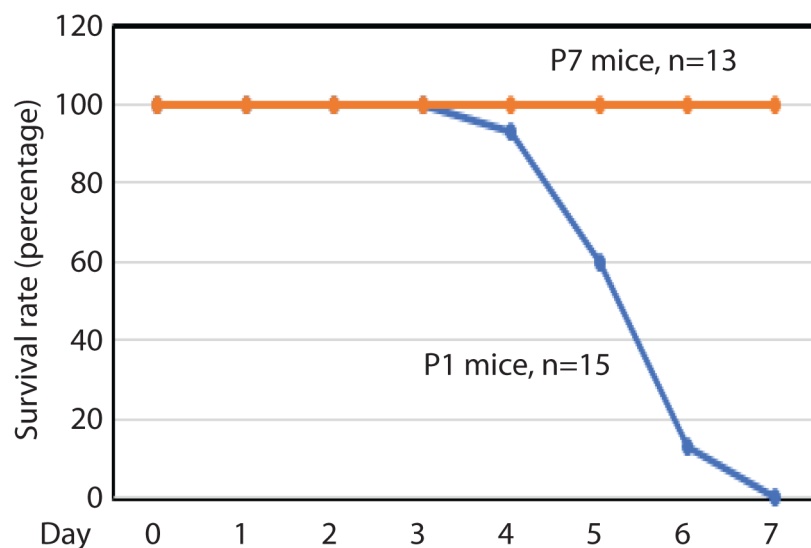


Figure 2. Surviving curve of neonates with the maintenance method. Newborn mice were maintained in the BSC2 in an apparatus as described in the protocol. The survival of the baby mice was monitored for seven days. Orange line: P7 mice, $n = 13$; blue line: P1 mice, $n = 15$.

DISCUSSION AND TROUBLESHOOTING

There is apparently no well-established method for the maintenance of neonates without foster mothers. Based on recommendations from the animal care staffs, there is a need to feed the neonates every two hours (<http://www.afrma.org/orphanrm.htm>; <http://www.rmca.org/Articles/orphans.htm>; <https://www.wikihow.com/Care-for-Baby-Mice>). Because certain biological experiments need just days to finish, we have established a protocol for maintaining neonatal mice without their mothers and the need for every two-hour feeding.

While this technique is quite straight forward, several technical details need careful considerations. First, one of the most critical factors for success might be the sterilization of all materials involved in

the maintenance. Because the temperature is set up at a fixed range (29°C – 31°C) and the humidity is fairly high with wet cotton balls present all the times, any contamination would be disastrous for the colonies. We recommend to use a BSC to handle all the materials.

Second, no more than two mice in a grid, especially for younger mice (e.g., P1 neonates), is critical. Fewer mice there would prevent them from huddling to each other to form a ball. The huddling instinct seems to be stronger in P1 mice than P7 ones. It is apparent that the older (P7) mice are easier to raise than younger ones (**Fig. 2**). It is also apparent that the older neonates have stronger instinct to hide under a cover. However, it is interesting to note that mother-pup interactions have a huge impact on fetal development [11]. For example, the better survival of the P7 neonates might be due to the few days of mother-neonate interactions

which generated a suckling habit [12]. The exact mechanism for the better survival of older neonates needs further investigations.

Third, minimum the neonates' stress might be another factor for success. Stress is clearly linked to the mortality and healthy of rodent neonates [13,14]. If manual feeding is a necessity, it is recommended to use a pipette to drop 50 µl milk into the mouth of mice. Try to avoid the noses as neonates might be suffocated. It is not recommended to feed the newborn mice in researcher's hands. Stress and distress generated in the process may outweigh the benefits of hand feedings. Of note, the dropping of milk into neonate's mouth may be a very effective way to keep them alive for days.

Fourth, we attempted to weight the neonates' body weights every day to follow their overall health. However, the higher mortality rates were observed (data not shown). It was presumed that the stress generated might be responsible [13,14]. Therefore, this simple protocol has omitted the weighting step. Although P7 mice can survive for seven days, they are not very healthy after 5 d based on plain observation.

In sum, we have established a simple method for neonatal studies for a short period of time, up to 3 d for P1 mice and 7 d for P7 mice without foster mothers' present. In addition, P4 mice can survive for four days without problems (data not shown). One of the shortcomings of the method is the long-term maintenance of neonates is not available with the current format. It would be useful for microbe infection and drug testing experiments that need short periods of time, especially for those experiments testing survival rates.

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