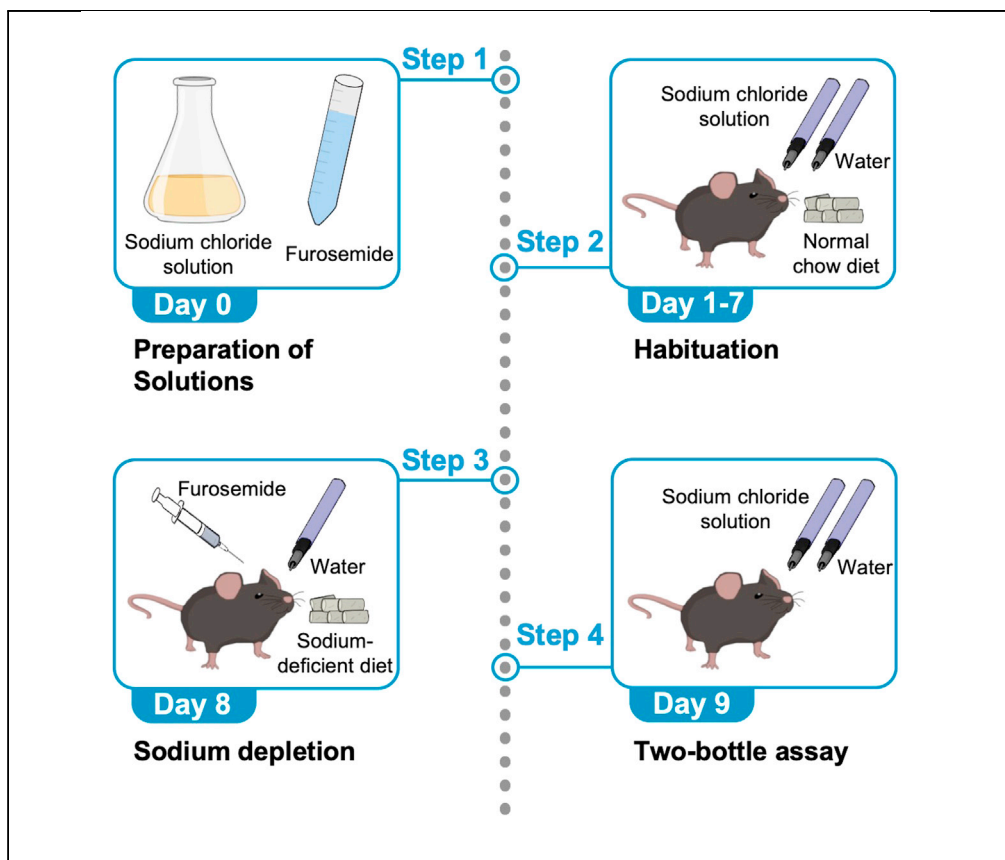


## Protocol

# Protocol for sodium depletion and measurement of sodium appetite in mice



Sodium appetite is a state that motivates animals to consume normally unappetizing concentrations of sodium. Here we describe a protocol to induce sodium appetite in mice by furosemide-induced diuresis and measure sodium intake using volumetric drinking tubes. This protocol induces sodium appetite rapidly and can be used to assess the effect of various treatments on sodium appetite. This protocol does not require electronic equipment and can be implemented easily.

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### Highlights

Protocol describes easy and affordable approaches to study sodium appetite in mice

Sodium appetite is induced by sodium-losing diuretics and sodium-deficient diet

Sodium appetite is assessed by two-bottle assay using volumetric drinking tubes

Protocol can be used to assess effects of various treatments on sodium appetite

## Protocol

## Protocol for sodium depletion and measurement of sodium appetite in mice

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## SUMMARY

Sodium appetite is a state that motivates animals to consume normally unappetizing concentrations of sodium. Here we describe a protocol to induce sodium appetite in mice by furosemide-induced diuresis and measure sodium intake using volumetric drinking tubes. This protocol induces sodium appetite rapidly and can be used to assess the effect of various treatments on sodium appetite. This protocol does not require electronic equipment and can be implemented easily. For complete details on the use and execution of this protocol, please refer to Park et al. (2020).

## BEFORE YOU BEGIN

## Preparation of sodium chloride solution

⌚ Timing: 1 h

1. Preparation of 300 mM sodium solutions
  - a. Weigh 17.53 g of sodium chloride.
  - b. Dissolve sodium chloride in 1,000 mL of distilled water.

*Optional:* Autoclave the solution.

- c. Allow the solution to cool back to room temperature before use.

**Note:** The concentration of sodium chloride may need to be adjusted according to the study. At 300 mM, sodium chloride is mildly unappetizing to B6 mice in sodium-replete conditions (Bachmanov et al., 2002). 500 mM sodium chloride, a more unappetizing concentration, has also been used in many other studies (e.g., [Jarvie and Palmiter., 2017](#); [Resch et al., 2017](#)).

## Preparation of furosemide

Furosemide, with low-sodium or sodium-deficient diet, has been widely used to induce sodium appetite since earlier studies in rats ([Jalowiec, 1974](#)) and mice ([Rowland and Fregly, 1988](#)). We have optimized the protocol for easy and affordable use in mice. For instance, experiments described in step 3 below for “habituation of mice” as well as steps 7 and 8 for “sodium depletion” and steps 11 and 12 for “two-bottle assay” have been optimized to reliably and accurately measure the volume of fluid intake using volumetric drinking tubes and Lixit valves.

⌚ Timing: 30 min



2. Preparation of 10 mL furosemide (10 mg/mL)
  - a. Weigh out 100 mg of furosemide.
  - b. Place a small beaker on a stirring plate and fill with 8 mL of sterile 0.9% saline and a stirrer.
  - c. Place a pH meter into solution.
  - d. Pour furosemide powder into saline. Furosemide will not dissolve at first.
  - e. Dissolve NaOH into saline until pH reaches 10.0–10.2.

**Note:** Take note of the volume of NaOH used. In our lab, this typically requires around 410  $\mu$ L of 1M NaOH.

**Note:** When 410  $\mu$ L of 1M NaOH is added directly to saline without furosemide, this typically brings the pH of the saline to 12.6–12.7 pH. The presence of furosemide lowers the pH of the entire solution.

**△ CRITICAL:** Solution must be at a basic pH, otherwise it will not dissolve furosemide.

- f. Start stirring to fully dissolve furosemide into solution.
- g. Slowly add HCl into solution, until pH reaches 8.2–8.5.

**Note:** In our lab, this typically requires around 100  $\mu$ L of 1M HCl.

**△ CRITICAL:** Take note of the volume of HCl used added into solution, so that the final volume of the solution can be accurately calculated for the next step.

- h. Fill the remaining solution to 10 mL with sterile 0.9% saline.
- i. Filter furosemide solution through a 0.45  $\mu$ m filter.

**Note:** The pH of furosemide solutions may lower slightly when injected into an animal, thus avoid bringing the solution to completely neutral pH. Pharmaceutical grade furosemide (10 mg/mL) can be used as a substitute. Some institutions may require use of pharmaceutical grade furosemide for animal experiments.

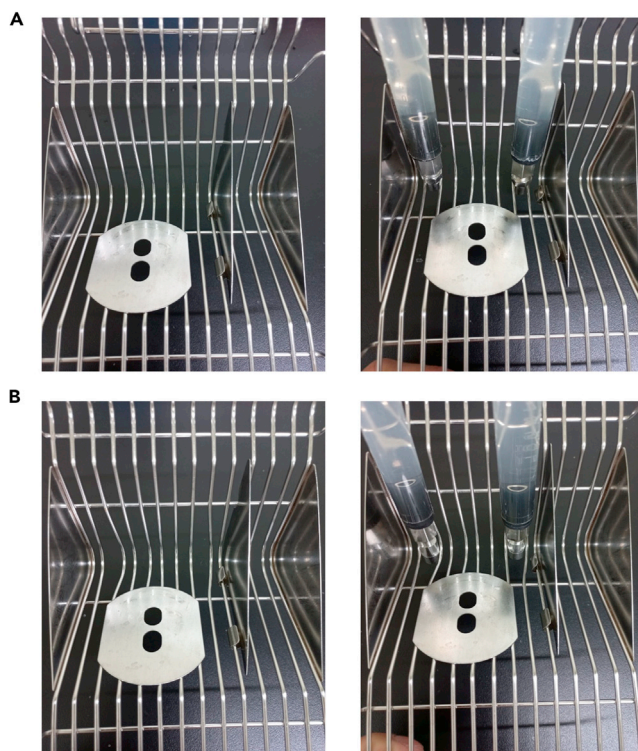
## Habituation of mice

⌚ **Timing:** 7 days

3. Habituate mice to volumetric drinking tubes. We have used both MedAssociates Volumetric Drinking Tube w/Standard Lixit for mouse and Volumetric Drinking Tube w/Deluxe Lixit for mouse. Ensure that the type of volumetric drinking tubes (Standard Lixit or Deluxe Lixit) for sodium solution and water are the same for each mouse.
  - a. Place drinking tubes into mice home cages. We achieve this in our lab by inserting the ends of the tubes between the metal rails on the lids of the home cages. We use physical force to adjust the width between the metal rails on the lids, if required, to ensure the ends of the tubes are tightly fastened between the rails (Figure 1). This is to ensure a “plug and play” type of connection.
  - b. Fill one tube with distilled water and the other tube with 300 mM NaCl solution.

**Optional:** Mice can be handled lightly during this period to reduce stress during future experiments.

- c. During this time we replace both tubes every 3 days to clean and refill. Furthermore, we clean sodium chloride containing tubes whenever we notice rust forming. We find that MedAssociates Volumetric Drinking Tubes are able to hold enough water to keep mice hydrated for 3 days when filled to their maximum capacity. We clean the tubes using the methods explained in steps 7 and 8 of this protocol.



**Figure 1. Adjusting the width between the metal rails on the lids**

(A) (Left) Lid for home cage. (Right) Inserting volumetric drinking tubes (PHM-127-15, MedAssociates) to the lids. (B) (Left) Widened gaps between the metal rails of the lid. (Right) Inserting deluxe volumetric drinking tubes (PHM-127A-15, MedAssociates) into the lids.

△ **CRITICAL:** Experimental procedures should be performed in compliance with institutional animal welfare guidelines. These procedures were conducted according to the Korean Advanced Institute of Science and Technology (KAIST) Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (protocol no. KA2018-80).

**Note:** When using volumetric drinking tubes with Lixit, researchers should carefully check the instructions for the equipment as well as guidelines from their animal facility to ensure a proper operation.

**Note:** During this time, mice will drink the sodium solutions in small amounts (Need-free sodium intake). The typical amount of need-free sodium intake differs according to the strain of mice, concentration of solution and sex of the mice (Tordoff et al., 2007). A study from Bachmanov et al. (2002) measured the typical need-free sodium preference of B6 mice to be around 30%, when presented with 300 mM NaCl vs water.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Furosemide	Sigma-Aldrich	F4381
Sodium chloride	Sigma-Aldrich	S7653

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sterile 0.9% saline	JW Pharmaceutical	Cleancle (1,000 mL)
Hydrochloric acid, 35.0–37.0%	SAMCHUN CHEMICALS	00H0255
Sodium hydroxide, bead, 98.0%	SAMCHUN CHEMICALS	00S0610
<b>Experimental models: Organisms/strains</b>		
Mice (C57BL/6J, male, 9–16 weeks old)	The Jackson Laboratory	000664
<b>Other</b>		
Stock solutions of furosemide (10 mg/mL)	Sigma-Aldrich	F4381
Stock solutions of sodium chloride (300 mM)	Sigma-Aldrich	S7653
Volumetric Drinking Tube w/Standard Lixit for mouse	Med Associates	PHM-127-15
Volumetric Drinking Tube w/Deluxe Lixit for mouse	Med Associates	PHM-127A-15
pH meter	Thermo Fisher Scientific	STARA2115
Analytical balance	OHAUS	SPX223KR
Analytical balance	OHAUS	AX223KR
Analytical balance	OHAUS	PAG214C
Syringe	Koreavaccine	1 mL, 30 mL
Needle for syringe	Koreavaccine	21G, 26G
Syringe filter (0.45 µm)	Wonkang Bio	SY25VG
Normal chow diet	ENVIGO	2018S
Sodium-deficient diet	Research Diets	D08070603

**Alternatives:** Reagents can be ordered from different manufacturers. The sodium-deficient diet listed above can be replaced with an equivalent diet.

**Note:** According to the manufacturer, “D08070603 may contain 150 ppm sodium”. The particular sodium-deficient diet used in this protocol is a modified AIN-93G diet, which should be used as a control diet if needed. Researchers using different sodium-deficient diets should ensure that their control diets are chosen appropriately. We have used both MedAssociates standard lixits and deluxe lixits to success.

**Note:** Furosemide solutions should be stored away from light at room temperature (15°C–30°C). We have successfully stored furosemide for two weeks in these conditions. Expired furosemide solutions are slightly yellow. Sodium chloride solutions can be stored at room temperature (15°C–30°C). We have successfully used sodium chloride solutions for 4 weeks in these conditions.

## STEP-BY-STEP METHOD DETAILS

### Sodium depletion

⌚ Timing: 24 h

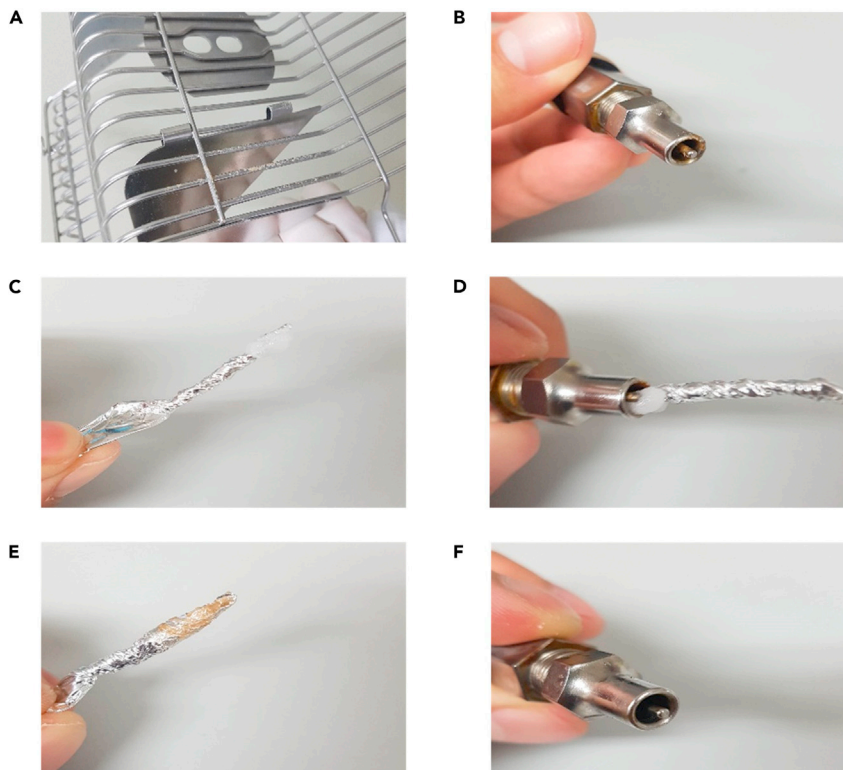
This step will use furosemide to induce diuresis. Mice are then allowed to rehydrate and given access to sodium-deficient diets for 24 h. This protocol should induce sodium appetite in mice, while minimally affecting thirst or hunger.

**Note:** The time of sodium deprivation may need to be altered according to the study.

1. Weigh each mouse.

**Note:** We typically perform this step at 19:30 (ZG 12.5).

2. Provide a fresh cage with a clean lid.



**Figure 2. Cleaning lids and valves**

- (A) Chow encrusted on the lid of the home cage.  
 (B) Rust formation inside the Lixit valve.  
 (C) Aluminium foil coated in sodium chloride.  
 (D) Scraping the inside of the Lixit valve with aluminium foil and sodium chloride.  
 (E) Discolouration of sodium chloride as rust is removed from the Lixit valve.  
 (F) Lixit valve with rust removed.

3. If a researcher chooses to replace bedding in the home cage, ensure that cage lids do not have any chow encrusted on them (Figure 2A).
  - a. Wipe down cage lids with a water dampened paper towel to remove encrusted chow.

**Note:** It is recommended to provide a fresh cage. Either way, the protocol should be adapted to follow the local guidance from animal facilities.

△ **CRITICAL:** The two previous steps ensure that remaining chow hidden inside the bedding or encrusted on the lid are removed and prevented from being used as a source of sodium.

4. Remove the 300 mM NaCl solution containing drinking tube from the home cage.
5. Inject mice subcutaneously with 50 mg/kg of furosemide (10 mg/mL) in a 1 mL syringe with a 26G needle.
  - a. Volume of furosemide to be injected can be calculated by mouse weight in grams  $\times$  5  $\mu$ L.
6. Replace food from the home cage with sodium-deficient diet.
7. Clean the plastic part of the volumetric drinking tubes by scrubbing them gently under distilled water.
8. If necessary, remove rust from valves (Figure 2B).
  - a. Twist a piece of aluminium foil into a shape that can fit inside the valve.

- b. Dip the aluminium foil into water and then into sodium chloride to coat the foil in sodium chloride (Figure 2C).
  - c. Scrub the insides of the valve with the sodium chloride-coated aluminium foil; sodium chloride will become discoloured as rust comes off the valves. Remove all rust from the inside of the valves (Figures 2D–2F).
  - d. Soak the valves under copious amounts of distilled water to remove all traces of sodium.
  - e. Dry valves.
9. Fill the volumetric drinking tube with small amount of water and assemble with dried valve. Ensure the valve of the water tube is flowing properly by pressing on it. Fill the remaining water drinking tube with water and measure the volume.

### Two-bottle assay

⌚ Timing: 4 h

This step will measure sodium appetite in the sodium-depleted mice.

10. 23 h and 15 min later; weigh mice and remove distilled water from home cages.
  - a. Weigh mice. Mice should have decreased body weight.
  - b. Measure water intake. Mice typically drink at least 3 mL of water during this period.
  - c. Pharmacological manipulations can be performed at this step if required.

**Note:** We typically perform this step at 18:45 (ZG 11.75). For pharmacological experiments, this time may need to be adjusted accordingly for different drugs.

11. Clean the insides of the plastic tubes of the water-containing volumetric drinking tubes, by scrubbing gently under distilled water.
12. Fill volumetric drinking tubes with distilled water and sodium chloride solutions.
  - a. Ensure that all air bubbles are removed from the inside of the valves by tapping on them lightly.
  - b. Press on the Lixit valve lever to ensure that liquid flows from the valve.
  - c. Drain out any remaining liquid inside the valves with a dry paper towel (Figures 3A and 3B).

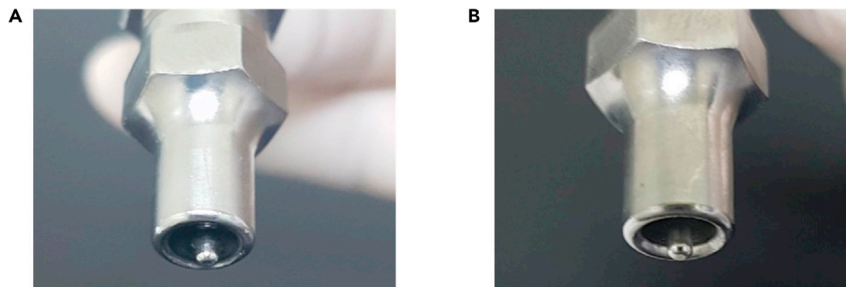
**⚠ CRITICAL:** Liquid remaining inside the valves can substantially alter measurements, leading to inconsistent results.

- d. Backfill the volumetric drinking tubes to full capacity using a 30 mL syringe with a 21G needle.

**Note:** We perform steps 11 and 12 away from mice, in a different room, to avoid disturbing them.

13. At 24 h after sodium depletion, replace both distilled water and sodium chloride solution containing volumetric drinking tubes back to the home cage of mice.
14. Measure water and sodium chloride intake every hour for 4 h.

**Note:** The duration of the test and frequency of measurements may need to be adjusted according to the study. We have found that a 4-hour assay is sufficient to measure elevated sodium intake in mice (Park et al., 2020), but other investigators also have used variable time intervals from as short as 1–2 h to as long as 6–24 h (Jarvie and Palmiter, 2017; Matusda et al., 2017; Resch et al., 2017; Rowland and Morian, 1999; Ryan et al., 2017).



**Figure 3. Removal of remaining liquid inside valves**

(A) Remaining liquid inside the Lixit valve.

(B) Remaining liquid removed from the inside of the Lixit valve.

### EXPECTED OUTCOMES

24 h after diuresis, followed by rehydration and access to sodium-deficient diets, mice typically lose on average around  $7 \pm 2$  % of their body weight, ranging from 3% to 13% and drink at least 3 mL of water.

During a 4-h session, we find that mice will typically drink around  $0.9 \pm 0.3$  mL (mean  $\pm$  stdev) of 300 mM sodium chloride solution,  $0.4 \pm 0.2$  mL (mean  $\pm$  stdev) of distilled water and have a preference ratio (total NaCl solution consumed/(total water consumed + total NaCl solution consumed)) of  $0.7 \pm 0.15$ .

If non sodium-depleted mice are required as controls, then sodium-containing diet can be introduced at step 6, ensuring that the diet composition differs only in sodium content with the sodium-deficient diet. Mice with access to sodium-containing diets should have little decrease in body weight 24 h after furosemide injections and show little sodium appetite.

Potassium chloride (300 mM) and sodium bicarbonate solutions (300 mM) can also be introduced as negative and positive controls to test the specificity of behaviour towards the sodium ion.

### LIMITATIONS

Furosemide-induced sodium depletion does not mimic sodium depletion in a natural context. This protocol may engage other pathways that are not normally involved in regulating sodium appetite as a response to a chronic deprivation of dietary sodium (Geerling and Loewy, 2008). Sodium depletion through maintenance on a sodium-deficient diet may be more suitable, depending on the goals of the researcher.

Real-time data acquisition is not possible with volumetric drinking tubes and measurements introduce a degree of disturbance for the animals. Researchers that require real-time data and/or less disturbance of animals may consider electronic alternatives such as those used in Jarvie and Palmiter (2017) or lickometer-installed cages (Park et al., 2020; Resch et al., 2017) for measuring fluid intake.

Protocols provided here have been validated using male mice. Experiments using female mice may require somewhat modified protocols.

### TROUBLESHOOTING

#### Problem 1

Mice do not drink at least 3 mL of water during sodium depletion (in step 10 of “two-bottle assay”).



#### Potential solution

If mice have not drunk enough water, furosemide solution is likely to have been made incorrectly or expired. Also check that Lixit valves are not blocked by air bubbles by pressing on the valve lever and ensuring that liquid flows (step 9 of “[sodium depletion](#)”).

#### Problem 2

Mice do not lose weight after diuresis (in step 10 of “[two-bottle assay](#)”).

#### Potential solution

If mice have drunk water but have not lost weight, sodium is likely to have been present in the home cage during sodium depletion. Ensure that cage bedding is replaced and that lids are cleaned, as small amounts of chow may be hidden inside bedding or encrusted on lids. Ensure that sodium chloride solutions are taken out of home cages. Ensure that sodium-deficient diet is being provided during the depletion step and not sodium-containing diet (steps 2–6 of “[sodium depletion](#)”).

#### Problem 3

Mice drink too much water or sodium chloride (>4 mL within 4 h) during the two-bottle assay (in step 14 of “[two-bottle assay](#)”).

#### Potential solution

Drinking tubes are likely to be leaking. Check to see that the plastic tubes do not have any cracks or leaks. Check to see valves are not clogged. Bedding can sometimes get stuck inside the Lixit valve, thus jamming the lever and causing liquid to continuously leak out. If this continues to be a problem, using volumetric drinking tubes with deluxe Lixit valves (PHM-127A-15, MedAssociates) can ameliorate this issue.

#### Problem 4

Mice do not drink enough fluid or do not drink any sodium chloride solution at all during the two-bottle assay (in step 14 of “[two-bottle assay](#)”).

#### Potential solution

Drinking tube valves may be blocked. Check that all air bubbles have been removed and ensure that liquid flows from the valve when levers are pushed on (step 12a of “[two bottle assay](#)”). Valves may have become too rusty; ensure that valves are clean by removing rust before proceeding with two bottle assays. Mice may not be accustomed to the sodium chloride solution-containing tube in their home cage. Ensure that the mice are properly habituated to the tubes before testing. Mice may not be accustomed to furosemide-induced sodium appetite and may require an additional trial to observe robust sodium ingestion.

Although we have not personally conducted these experiments, furosemide-induced diuresis causes changes increased urinary volume and urinary sodium concentrations, which may also be used to confirm diuresis ([Jalowiec, 1974](#)).

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, J.-W.S. ([jwsohn@kaist.ac.kr](mailto:jwsohn@kaist.ac.kr)).

### Materials availability

This study did not generate new unique materials.

### Data and code availability

Data is available upon request.

### ACKNOWLEDGMENTS

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### AUTHOR CONTRIBUTIONS

S.P. and J.-W.S. wrote and edited the article.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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