

Drinking in the Dark (DID): SOP for the Mouse

This document summarizes two variations of the “standard” Drinking in the Dark method as we use it for C57BL/6J mice. I append comments at the end that people may find useful for adapting the basic protocol to their specific questions (superscripted in the main text refer to these notes). I assume that you group-house your mice and that they are on the same 12:12 light/dark cycle you are, i.e., lights on about 6AM.

DID 2-Day Test (used for selective breeding on BEC at 4 hr on Day 2)

1. If purchased, mice should be acclimated to the facility for at least 2 weeks before testing, and testing should be when they are adult (at least 8 weeks old)¹. If you don't want to test them during your circadian night, mice should be brought directly into (or shifted to) a reversed light/dark cycle at least 2 weeks, ideally 3 weeks, before testing. We use, lights OFF at 9:30 AM and lights ON at 9:30 PM. Food is available *ad libitum* throughout rearing and testing. We use Purina 5001 and Bed-o-cob bedding (which avoids exposure to some chemicals emanating from wood chips). We maintain our colony and test rooms at 21 ± 1 °C. We test the animals in the colony room where they are adapted to reversed L:D.

2. 1 week (6-8 days) prior to testing, individually house animals and place them in cages on a flat rack (ours are double-sided and have six shelves and therefore hold 84 cages). If multiple passes are required to test all the animals in an experiment, then each pass should be individually housed for the same number of days prior to the start of testing.

3. Our animal facility uses square stoppered plastic water bottles on the cage top. A pinhole in the side allows mice to obtain water by licking. We replace these water bottles with standard sipper tube water bottles at the time of individual housing to acclimate mice to drinking from a sipper tube. These tubes have no ball bearings and have a 2/16 inch aperture. Our cages have stainless steel wire bar tops with a triangular shaped indentation down into the cage. There is a flat ring like a washer on one side of this triangular trough through which the sipper tube is introduced. The bottle area is separated from the rest of the indented triangle by a flat stainless partition, and this half of the area is for food pellets. We need to monitor our mice for a day or two to make certain they figure out how to drink from a tube.

4. All testing starts at 180 min after lights off².

5. After the 6-8 days acclimation to individual housing and drinking from a sipper tube, begin testing. On the first day, starting at three hours after lights out, replace the sipper tube water bottles with 10 mL drinking tubes containing 20% ethanol (v/v in tap water)³. Tubes are placed where the water bottle was. Secure these tubes with Acco brand (Lincolnshire, IL) Medium Binder Clips (1.587 cm capacity); clipping prevents loss of fluid as these very light tubes are otherwise knocked around by the mice. Record fluid levels by reading the meniscus. The recording of fluid levels begins as soon as each cage has an ethanol tube secured to the cage top. For 80 mice, securing all of the tubes takes around 5 minutes for 3 people⁴. All subsequent timing is with reference to reading the level of the first tube (T0).

6. Starting exactly two hours after reading the first drinking tube (T0), record the fluid levels a second time. After all tubes are read, remove the drinking tubes and replace with the water bottles⁵.

7. On Day 2, weigh animals at least one hour prior to lights out.
8. Starting exactly 3 hr after lights out, repeat step 5. Two hours later, read all tubes.
9. Starting exactly four hours after the tubes are first read, record the volumes for a third time.
10. As soon as the first cage's volume is read, remove this cage to another room (preferably), or under a fume hood (second best). Take a 20 μ L blood sample from the peri-orbital sinus with a capillary tube. Given the assay we use, we place the sample immediately into a microcentrifuge tube with 50 ml ZnSO₄ and then on ice.
11. We freeze processed blood samples and store at -20 °C until assay by gas chromatography. Our standard curve uses 0.2367, 0.4734, 0.9468, 1.578, 1.9725, 2.959, 3.945, and 4.932 mg/ml EtOH.

DID 4 day version

In this version, which we have used for most of the published studies and for most of the work ongoing, mice are given three days of exposure to 20% ethanol for 2 hr, and the fourth day they are given 4 hr access and a blood level is taken. All other aspects are the same.

For routine studies, we prefer the 4 day version for several reasons.

a) as reported in Rhodes et al (2005) and from a good deal of unpublished data, we found that Day 1 intake does not correlate very substantially with Day 2 intake. However, Day 2 intake correlates substantially with intake on all subsequent days (up to 12 days). Most of the data supporting this generalization are from individual differences in C57BL/6J mice. Because they are inbred, these differences are not allelic, and must represent other environmental differences (or, conceivably, differences in gene expression).

b) If a treatment is to be applied, or if multiple groups are being tested, Days 2 and 3 allow the experimenter to match subjects for initial intake (Day 1, or Days 1-3, for example). There are substantial individual differences, in all strains we have examined. Particularly if offering high ethanol concentrations, some mice clearly avoid drinking during the test, which may represent a conditioned taste aversion to the initial exposures (though we have not tested this hypothesis).

c) A 4 day test still fits easily into a work week, and we superstitiously believe that the extra days of experience with ethanol if anything lead mice to drift to a higher average intake, though these effects if real are very small.

Notes:

¹ We have tested mice as young as 39-42 days old using the procedures described above, and we obtained perfectly reasonable data. We have no information on mice older than about 14 weeks. Testing very young mice may require some adaptation to the procedures (lengthening the spout, for example).

² See Rhodes et al (2005) for the effects of other start times. We have looked at the effect of starting as late as 6 hr into the dark phase and saw no pronounced difference from 2-3 hr.

³ We use 10 mL Falcon disposable clear polystyrene serological pipets from Fisher Scientific. These are cut off at both ends, leaving a cylinder with the original graduated markings from about 2 mL to 9 mL (see Note 5). We purchase stainless steel sipper tubes (2.5", Ancare Corp.).

We use sipper tubes with ball bearings and a 5/16 inch aperture because high ethanol concentrations have a tendency to leak from sipper tubes without ball bearings. (One could argue that the noise from the ball bearings might influence the behavior of adjacent mice. We don't know of data on this point). The sipper tubes are inserted (jammed, actually) into the end of the plastic tube (it is a tight fit) and secured/sealed with heat shrink tubing (we purchase this at a local electronic store, it is primarily used to hold bunches of wire together; the tubing we use is green and has a diameter of 1/2" and shrinks down to at least 5/16"). After filling with alcohol solution, a silicone stopper (Fisher, European size 10D) is placed in the upper end. The mice like to chew on the stopper if they can reach it. This system holds about 5 mL in the tube (the stopper extends nearly 1mL in) plus 2 mL in the tip. We use corn-derived ethanol - for all you ever wanted to know about the actual contents of different kinds of ethanol, contact Dick Deitrich!

Depending on the goals of an experiment, and the genotype tested, you may wish to try higher or lower ethanol concentrations. High-preference genotypes such as C57BL/6J will self-administer 30% ethanol [when there is concurrent water available - see Blednov et al (2005)]. C57BL/6J mice drank 30% ethanol in a DID test, but we did not collect blood ethanol levels in that experiment [Rhodes et al., (2005)]. In the genetically heterogeneous stock of HS/Npt mice from which we are breeding HDID selected lines, few animals drink substantial amounts of 20% ethanol, but were we to use 10%, we would see many more animals drinking.

⁴ Note that this is occurring in the darkened colony room. Our colony is lit during the "dark" phase with a red bulb (2 lumen/square foot, about 21.5 lux). We use a standard AA mini-maglite with a 3 LED aftermarket conversion for reading the tubes (<http://www.thinkgeek.com/clearance/852c/>). During all phases of reading tubes, we try to disturb the mice a little as possible - talking only *sotto voce*, for example. Mice are given their bottles in the same order every day, and bottles are removed in the same order, in batches. Often we have to move the binder clips to find the meniscus. For small batches of mice, one person can perform the readings. For larger batches, we will use two - one reading and the other recording - in order to move through the mice rapidly.

⁵ Our tubes were made purposefully short to fit under the cage rack shelf above, when we were delivering fluid at a 90° angle to the cage bottom. We now allow them to assume the angle of the triangular cage top cavity that holds food and water bottle. According to the readings, some mice will 'drink' more than 5 mL in 4 hr. This amounts to a g/kg alcohol intake of 40 or more g/kg, which we believe to be impossible (as these mice are not grossly intoxicated, either by behavioral observation or by BEC). This may represent leakage, or the animal's 'playing' with the ball bearing, or a combination of the two. While this is not a major problem, it is seen in one or two animals in almost every experiment. For example, in our most recent selected generation of HDID-1 female mice, we tested 68 females. Their average intake was 7.1 ± 0.7 g/kg. One mouse 'drank' 47g/kg in 4 hr (5.2 mL fluid) and reached a BEC of only 0.92 mg/ml. Another 'drank' 21.7 g/kg to reach 0.83 mg/ml. One 'drank' 17.5 g/kg for a BEC of 1.54 (a believable pair of values), and a fourth 'drank' 16.7 to reach only 0.53. All other mice drank 10.8 g/kg or less.

This is one reason we elected to breed selectively on blood ethanol level attained rather than intake in g/kg, and why we believe this to be a more appropriate end point for behavioral studies of DID in general. In the case of the animal with a 47 g/kg intake, the research assistant noted the exceptionally high intake at the 2 hr reading and replaced the tube with one that was full. For 20% solutions, the potential that a mouse might drink the tube dry could be avoided entirely by cutting the pipetting tubes as long as possible. For such limited access drinking studies, we do not recommend using tubes with much larger volumes of fluid, nor do we

recommend weighing rather than reading meniscus levels (weighing works fine for 24 hr access for rats, where volumes are much higher).

References reporting DID data

- Blednov YA, Metten P, Finn DA, Rhodes JS, Bergeson SE, Harris RA, Crabbe JC. (2005) Hybrid C57BL/6J x FVB/NJ mice drink more alcohol than do C57BL/6J mice. *Alcoholism Clinical and Experimental Research* 29(11):1949-58
- Rhodes JS, Best K, Belknap JK, Finn DA, Crabbe JC (2005) Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiology and Behavior* 84:53-63
- Rhodes JS, Ford MM, Yu C-H, Brown LL, Finn DA, Garland Jr. T, Crabbe JC (2006). Mouse inbred strain differences in ethanol drinking to intoxication. *Genes Brain and Behavior* 6(1):1-18.
- Kamdar NK, Miller SA, Syed YM, Bhayana R, Gupta T, Rhodes JS (2007) Acute effects of naltrexone and GBR 12909 on ethanol drinking-in-the-dark in C57BL/6J mice. *Psychopharmacology (Berl)*. In press, On-Line early.

Help

For scientific questions, contact John Crabbe (crabbe@ohsu.edu)

For methodological/practical questions, contact Lauren Brown (brolaure@ohsu.edu)