**Structured Tracking of Alcohol Reinforcement (STAR) SOP**

**Developed by the Siciliano Lab, Vanderbilt University, Department of Pharmacology**

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# Overview

This is the SOP for the Structured Tracking of Alcohol Reinforcement (STAR) procedure developed by the Siciliano Lab. The purpose is to capture individual differences in drinking behavior that develop over time with exposure to alcohol. As originally performed, the experiment consists of a training phase followed by a baseline, Pre-test. Animals then go through an event or series of events (in our case, a period of binge drinking through a two-bottle choice task) and then finally re-run the same test as in baseline as a Post-test comparison. Individual changes in animals’ consumption patterns from Pre- to Post-test are examined and used to determine how the test affects animals’ disposition to compulsive drinking behavior.

# General Experimental Guidelines

## Consistency

Consistency is the bedrock of the STAR. Animals should start behavior and fed at the same time every day to the best extent possible. Since every animal will move through the experiment at their own pace, it is not possible to control every factor in the experiment. However, since differences in individual animal’s behavior is the focus, giving every animal the most consistent day-to-day experience will result in the cleanest and most accurately comparable results.

## Timeline

Animals should be run ~2 hours into their dark (active) cycle each day. Bring animals to experimental space to acclimate for at least 30 minutes prior to running the experiment. The experiment runs on consecutive days for at least 21 days, though exact experimental timeline varies based on the speed at which animals acquire and the specific task and schedules being tested. Training lasts for at least 7 days, depending on animals’ speed of acquisition, while Pre- and Post-test last 7 days each, regardless of performance on the task. The test part can be changed around and so will vary based on what exactly is being tested and the methods employed. For a visual timeline of the experiment, see [here](STAR%20Timeline.pdf).

## Solution Making

Alcohol used throughout this experiment is 15% EtOH in water.190 proof (95%) undenatured ethanol was used (see Materials section for exact part number). Regardless of vendor, make sure to *not* use 200 proof ethanol as that [has impurities that make it unsafe for consumption](https://labproinc.com/blogs/chemicals-and-solvents/what-are-the-differences-between-denatured-and-non-denatured-ethanol). Similarly, denatured alcohol has additives that make it unsafe to consume, so ensure any ethanol for consumption is undenatured.

## Box Cleaning

Since alcohol is consumed during this experiment, boxes should be cleaned with acetic acid and not ethanol. 0.03% (v/v) acetic acid and double distilled water were used as cleaning solutions throughout this experiment.

## General SOP Notes

Some things will be marked in order of importance. See the following coloring guide for details:

**CRITICAL** – Part or step that is of utmost importance to the experiment running correctly

**Important** – Part or step that should be paid close attention to or will provide benefits in the future if done correctly

**Optional** – Not critical to experiment, though might have additional benefits further down the line if done

**Experimenter Note** – Thing that was done that is not critical to the experiment, but from experience has been found to be useful

# Materials

## Operant Box Parts

All operant behavior was run in operant boxes from Med Associates (St. Albans, VT). Since a Med Associates behavioral setup is already needed to run the experiment, parts will be broken down into sections based on their importance to STAR directly, vs. other parts of the experiment. Feel free to contact the authors for further details of their specific experimental setup.

### General Parts

Can be different based on setup specific to your lab/experimental environment. Some parts may have a “W” at the end of the part number, denoting they were originally made for the Extra Wide Modular Test Chamber. The same pieces may have different part numbers to fit the standard modular mouse chamber (ENV-307A). Always double check your setup to make sure parts are compatible.

* Standard Multi-Density Fiberboard (MDF) Sound Attenuating Chamber (SAC) – ENV-022SA
* Extra Wide Modular Test Chamber with Modified Top for Mouse – ENV-307W-CT
* Stainless Steel Grid Floor for Extra Wide Mouse Modular Mouse Chamber – ENV-307W-QD

### **CRITICAL** Parts

* Retractable Sipper w/ Graduated Pipette for Mouse – ENV-352AW
* Graduated Pipette (only) – MSUB-ENV-352A1
  + **Experimenter Note:** we purchased two sipper tubes per box being run, one for each solution being used (in our case, ethanol and quinine). Since each retractable motor moves a slightly different amount, this allowed us to keep using the same tubes per box each day and avoid any changes different sipper tubes might have as well as avoid any cross contamination of solutions
* Triple Contact Lickometer Controller – ENV-250C
* White Noise Amplifier with Cage Speaker for ENV-307W Wide Mouse Chamber – ENV-325SW
* Cage Speaker for ENV-307W Wide Mouse Chamber – ENV-324W

### **Optional** Parts

* Illuminated Nose Poke response for Wide Mouse Modular Chamber – ENV-313W
* Shock / Lickometer grid switch - ENV-250S (only needed if doing shock and lickometer readings concurrently)
* Sound level meter – Model 732A, BK Precision (for normalizing volume of white noise in operant chambers)

### **Experimenter Note** Video Recording Parts

At the top of each sound attenuating chamber an infrared camera was set up and connected to an NVR for a bird’s eye view recording of each box. This is useful for a variety of reasons, mainly watching animals as they run the behavior to keep an eye out for any abnormalities and also a visual check to make sure the quantitative measurements (e.g. nose pokes and licks) are being recorded correctly. Additionally, videos can later be fed into programs, such as DeepLabCut, for tracking and scoring of behavior. Security camera setups are perfect for this task as they are all interconnected, and yet separate, allowing for both individual control and recording in each box as well as synchronous multi-box recording. Equipped with IR lights as well, they make running behavior in low light with good video quality still feasible. Though all security camera systems are similar, we went with Security Camera Warehouse (Asheville, NC - https://www.getscw.com/) which has proved incredibly robust and reliable.

* Admiral Pro 16 Channel 4K NVR – ADMP16P16
* Deputy 2.0 2MP Fixed Wide Lens Turret Dome Camera – 26DF2
* 10TB Surveillance Grade Hard Drive – 10TB-HD

## Two-Bottle Choice (2BC)

* 50mL Conical Tube – #22-170-199, Fisher Scientific
* Rubber (black) stopper, Size 6 – #6R, Ancare, Bellmore, NY

## Solutions/Chemicals

* Quinine Hydrochloride Dihydrate – CAS# 6119-47-7, Sigma-Aldrich, Q1125
* 190 Proof Undenatured Ethanol – Decon Labs, #2801, Ethyl Alcohol CAS# 64-17-5, Water CAS# 7732-18-5
* Acetic Acid, Glacial – CAS# 64-19-7, Fisher Scientific, A38-500

# Operant Behavior SOP

## Overview

Operant behavior is the majority of behavior run during STAR. It consists of a training/acquisition phase of 4 steps each with their own criteria needed to advance. This happens at each animal’s own pace depending how their performance each day. After learning the task, the animals run a Pre-test consisting of 3 days of baseline ethanol consumption followed by 4 days of escalating punishment of ethanol. After the Pre-test, animals will have another period in which they are exposed to different conditions (depends on experiment and what’s being tested) and then run the same 7-day experiment from Pre-test as a Post-test. How each animal’s behavior changes from the Pre- to Post-test can give us insights into how the middle exposure period can affect animal’s compulsive drinking behavior.

## Step-by-Step Guide

* **CRITICAL:** All animals will run each day in the same operant box – this should never change. Box cleaning will happen between animals to ensure that the conditions are as similar as possible from day to day
* **Important:** As mentioned in the experimental overview, animals should be run at the same time every day, starting ~2 hours into the dark (active) cycle. Feeding will take place at the end of the day when all animals have completed the experiment for the day, so that the time between feeding and start of operant behavior the following day will be consistent for each animal
* Bring animals to experimental area at least 30 minutes prior to when experiment should start to allow animals to acclimate to environment
* Set up boxes by screwing in flooring and changing any parts out as needed
* Clean all boxes and floors thoroughly with 0.03% acetic acid before any animals run. This will also be done between animals, but by wiping down first with acetic acid, all animals get the same conditions
  + **Important:** Avoid spraying acetic acid near/into the boxes as the aerosol can mess up the delicate electronics in the chambers. Instead, spray onto paper towel and then wipe down necessary areas
* Put a paper towel down in the bottom of each metal tray underneath the grid flooring to make for easier cleanup and more clear video backgrounds
* Fill up and test sipper tubes
  + See [lickometer filling guide](Lickometer%20Filling%20and%20Testing%20SOP.docx)for specifics on how to do this and additional notes
* Carefully put tubes onto retractable sipper tube and tighten the screw to ensure contact between tube and the lickometer wire
* **CRITICAL:** Load the program that will be run that day and start it. Test each nosepoke as well as the lickometer (once extended) using the [lickometer testing guide](Lickometer%20Filling%20and%20Testing%20SOP.docx). Make sure nose-pokes are illuminated and correctly counted and that contacts are counted accurately
* **Important:** After testing, read the volume of the lickometer to the nearest 0.05mL
* End program, reload it using proper information for each animal, and place animal in box to run. Once program is started, it will run for 1 hour (3600 seconds) or until the criteria is met for the day
* **Optional:** If using video recording, make sure to start the video(s) prior to animals going into the operant chamber. That will ensure that all behavior is captured on video and in case there is any mistake with the box operation, the animal’s behavior can still be observed and measured
* **Important:** When animal has completed experiment for the day, weigh immediately upon removing from operant box before returning to home cage. The animal’s weight will be used to normalize any alcohol intake and thus is critical to write down as close to when the animal ran as possible
* **Optional:** Stop video(s) once animals have been removed from the box
* **Important:** After the animal is done, but before retesting any of the tubes, write down the final volume of solution to the nearest 0.05mL. If volume isn’t recorded, total consumption for the day cannot be calculated
  + **Experimenter Note:** As a general rule of thumb, 100 licks is ~0.1mL consumption. If the amount consumed is very disproportionate to that (e.g. 100 licks and negligible volume difference OR 0.2mL volume change and only 50 licks) check that spout is working correctly
* Remove the paper towel from the tray and clean out box again using 0.03% acetic acid
* **CRITICAL:** Retest lickometer between animals to ensure that counting and flow are still working as expected

## Overview of Phases, Criteria, and Programs

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Magazine Training** | **Operant Training 1** | **Operant Training 2** | **Operant Discrimination** | **STAR Test** |
| **Trial Structure** | Sipper constantly extended, nose-pokes have no consequence | FR1-30s schedule; 1 active nose poke 🡪 30s extension of tube containing 15% EtOH | FR1-10s schedule; 1 active nose poke 🡪 10s extension of tube containing 15% EtOH | FR5-10s schedule; 5 active nose pokes 🡪 30s extension of tube containing 15% EtOH | FR10-10s schedule; 10 active nose pokes 🡪 10s extension of tube containing 15% EtOH or in water or 250-1000uM Quinine |
| **Session End Criteria** | 100 licks or 1 hour | 100 licks or 1 hour | 100 licks or 1 hour | 100 licks and ≥ 70% nose pokes on active side | 1 hour |
| **Phase End Criteria** | Reaches lick criteria for 1 day | Reaches lick criteria for 2 consecutive days | Reaches lick criteria for 2 consecutive days | Reaches lick and discrimination criteria for 2 consecutive days | Moves on after each day, regardless of performance |
| **Program Run** | 00\_STAR\_ MAGTRAINING\_ CAPPED | 01\_STAR\_ACQ\_ FR1\_30s\_CAPPED\_ LEFT/RIGHT | 02\_STAR\_ACQ\_ FR1\_10s\_CAPPED\_ LEFT/RIGHT | 03\_STAR\_ACQ\_ FR5\_10s\_CAPPED\_ LEFT/RIGHT | 04\_STAR\_TASK\_ FR10\_10s\_NOCAP\_ LEFT/RIGHT |

# Two-Bottle Choice (2BC) Behavior SOP

## Overview

2BC is run as the middle part of STAR to allow animals to have intermittent access to alcohol and water for a few hours a day. By comparing how each animal drinks during the Pre- and Post-binge operant experiments that are run, we can see how this middle period, in this case intermittent free ethanol access, changes animal’s behavior to alcohol being punished in the Pre- and Post-tests.

## Step-by-Step Guide

* Set up a cage for each animal who will be running this part of the experiment
  + **Optional:** a control cage was also run to estimate the normal change in bottle weight due to dripping, evaporation, and other processes which might cause the bottles to change in weight over the course of a few hours
* Place animals in individual cage to acclimate to environment while bottles are being prepared
  + Animals in this phase will have 2BC access for 2 hours, 4 hours, (checking in 2 hours into experiment) or be in abstinence (4 days of 2-hour access, 1 day of 4-hour access, 2 days of abstinence; repeat cycle twice for a total of two weeks)
  + If animal is in abstinence for the day, leave them in their homecage
* Fill up bottles ~80% with solution and tightly secure the stopper
  + **Important:** Turn tube ~45° downwards and using gloved hand, tap on ball bearing in spout – solution should come out, releasing bubbles into the tube and leaving solution on glove, showing it’s flowing. Should not drip freely at all
* **CRITICAL:** Weigh each pair of tubes and write down the weights before gently placing them on the cages
* **CRITICAL:**  After 2 hours, remove and weigh all tubes. Replace any back on cage if animal is running for 4 hours that day. After 4 hours, remove and reweigh those tubes as well.
  + Once animal is done with 2 hour access, weigh the animal and then give them a normal water bottle for the cage while the others finish up the 4 hour access period. All animals will be fed at the end of the day once returned to homecage
  + The volume of each solution consumed is inferred from the change in weight of the bottles between the beginning and end of the session
* Empty all tubes at end of day and triple rinse them and the rubber stoppers with water. Make sure tubes are labeled with the solution they contained so that cross contamination is eliminated as much as possible