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A High-Throughput Method for Measuring Alcohol Sedation Time of Individual Drosophila melanogaster

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cover letter

January 11, 2020

Phillip Steindel, Ph.D.
Review Editor
JoVE

Dear Dr. Steindel,

We are submitting a revised version of JoVE61108 with the slightly revised title "*A High-Throughput Method for Measuring Alcohol Sedation Time of Individual Drosophila melanogaster*" by Tatum N. Sass, Rebecca A. MacPherson, Trudy F. C. Mackay and Robert R. H. Anholt.

We have carefully considered the editor's and reviewers' comments and revised the manuscript accordingly.

I appreciate your efficient handling of this manuscript and trust that the revised submission will now be acceptable for publication in JoVE.

Sincerely, on behalf of all authors,

Robert R. H. Anholt, Ph.D.
Provost's Distinguished Professor of Genetics and Biochemistry
Director of Faculty Excellence Initiatives, College of Science
Corresponding Author

TITLE:

High-Throughput Method for Measuring Alcohol Sedation Time of Individual *Drosophila melanogaster*

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KEYWORDS:

behavior, genetics, ethanol, model organism, screening, Drosophila Genetic Reference Panel

SUMMARY:

Current methods to measure alcohol sensitivity in *Drosophila* are designed to test groups of flies. We present a simple, low-cost, high-throughput assay for assessing alcohol sedation sensitivity in large numbers of single flies. The method does not require specialized tools and can be performed in any laboratory using common materials.

ABSTRACT:

Drosophila melanogaster provides an excellent model to study the genetic underpinnings of alcohol sensitivity. In contrast to studies in human populations, the *Drosophila* model allows strict control over genetic background, and virtually unlimited numbers of individuals of the same genotype can be reared rapidly under well-controlled environmental conditions without regulatory restrictions and at relatively low cost. Flies exposed to ethanol undergo physiological and behavioral changes that resemble human alcohol intoxication, including loss of postural control, sedation, and development of tolerance. Here, we describe a simple, low-cost, high-throughput assay for assessing alcohol sedation sensitivity in large numbers of single flies. The assay is based on video recording of single flies introduced without anesthesia in 24 well cell culture plates in a set-up that enables synchronous initiation of alcohol exposure. The system enables a single person to collect individual ethanol sedation data on as many as 2,000 flies within an 8 h work period. The assay can, in principle, be extended to assess the effects of exposure to any volatile substance and applied to measure effects of acute toxicity of volatiles on other insects, including other fly species.

INTRODUCTION:

The National Institute on Alcohol Abuse and Alcoholism reports that in 2015 excessive alcohol consumption, designated as "alcohol use disorder", affected an estimated 16 million people in the United States. Alcohol abuse causes a wide range of adverse physiological effects and is a major cause of death in the U.S. In humans, decreased sensitivity, or a low level of response to alcohol, has a strong genetic component and is associated with a higher risk of developing alcohol use disorders[1-4]. Genetic risk studies on human populations are challenging because of population admixture, diverse developmental histories and environmental exposures, and reliance on self-reported questionnaires to quantify alcohol-related phenotypes, which are often confounded with other neuropsychiatric conditions.

Drosophila melanogaster provides an excellent model to study the genetic underpinnings of alcohol sensitivity[5-8]. The *Drosophila* model allows strict control over genetic background, and virtually unlimited numbers of individuals of the same genotype can be reared rapidly under well-controlled environmental conditions without regulatory restrictions and at relatively low cost. In addition to publicly available mutations and RNAi lines that target a majority of genes in the genome, the availability of the *Drosophila melanogaster* Genetic Reference Panel (DGRP), a population of 205 inbred wild-derived lines with complete genome sequences, has enabled genome-wide association studies[9,10]. Such studies have identified genetic networks associated with effects on development time and viability upon developmental exposure to ethanol[11,12]. Evolutionary conservation of fundamental biological processes enables translational inferences to be drawn by superimposing human orthologs on their fly counterparts.

Flies exposed to ethanol undergo physiological and behavioral changes that resemble human alcohol intoxication, including loss of postural control[8], sedation, and development of tolerance[13-15]. Alcohol induced sedation in *Drosophila* can be quantified using inebriometers. These are 122 cm long vertical glass columns with slanted mesh partitions to which flies can attach[16,17,18]. A group of at least 50 flies (sexes can be analyzed separately) are introduced in the top of the column and exposed to ethanol vapors. Flies that lose postural control fall through the column and are collected at 1 min intervals. The mean elution time serves as a measure of sensitivity to alcohol intoxication. When flies are exposed to alcohol a second time after recovering from the first exposure, they can develop tolerance, as evident from a shift in mean elution time[13,15,19,20]. Whereas inebriometer assays have led to identification of genes, genetic networks, and cellular pathways associated with alcohol sedation sensitivity and development of tolerance[12-14,21], the assay is time consuming, low-throughput, and ineffective for measuring alcohol sensitivity in single flies.

Alternative ethanol sedation assays that do not require the elaborate inebriometer set-up allow for more convenient measurements but are still limited in throughput and generally require analyses of groups of flies rather than individuals[21-25]. Assessing single flies minimizes the potential for confounding effects due to group interactions, such as those stemming from social behaviors. Here, we present a simple, low-cost, high-throughput assay for assessing alcohol sedation sensitivity in large numbers of single flies.

PROTOCOL:

1. Construction of the testing apparatus

1.1. Create a cardboard template the size of a 24 well cell culture plate by tracing around the plate on cardboard and cutting out the designated area.

1.2. Cut a piece of small insect screen mesh the size of the cell culture plate using the cardboard template from step 1.1.

1.3. Prepare a 24 well cell culture plate by placing a small line of hot glue around the perimeter of the top of the plate using a hot glue gun and affixing the screen mesh on top of the open wells.

1.4. Secure a wooden craft stick to each of three sides of the same cell culture plate from step 1.3 using a hot glue gun. The modified cell culture plate should now resemble the plate diagram shown in **Figure 1A** and the experimental setup shown in **Figure 2**.

[Place **Figure 1** here]

[Place **Figure 2** here]

NOTE: Prepare at least as many cell culture plates as will fit in the filming chambers (see below).

2. Construction of the filming chamber

2.1. Create a filming chamber by cutting a hole the size of the video camera lens on the side of a polystyrene box. Cut an additional slit the width of the illumination pad in the opposite side of the polystyrene box. The filming chamber should resemble the filming chamber shown in **Figure 1B** and **Figure 2**.

2.2. Prepare the filming chamber for use by inserting the illumination pad into the slit and positioning the camera in the lens hole above the illumination pad.

2.3. Place all materials and perform all subsequent testing in a controlled environment, preferably a behavioral chamber with approximately 30% humidity, 25 °C temperature, uniform airflow, and noise levels less than 65 dB.

3. Preparation of the testing apparatus and flies

3.1. Pipette 1 mL of 100% ethanol through the screen mesh into each well.

3.2. Dry the screen mesh with a piece of cheesecloth.

3.3. Cut two pieces of cheesecloth the dimensions of the cell culture plate using the cardboard template created in step 1.1. Place them on top of the dry screen mesh of the modified cell culture plate containing ethanol from step 3.2.

3.4. Create a small piece of thin, flexible plastic cutting board by tracing around the cardboard template created in step 1.1 as a general guide and expanding the traced area by 1–2 cm on one of the short sides. Cut out the expanded traced area from the thin, flexible plastic cutting board. After cutting, ensure that the plastic still fits between the three wooden craft sticks on the testing apparatus, but hangs off one end by 1–2 cm.

3.5. (Optional) If an aspirator needs to be created, assemble an aspirator like the one shown in **Figure 3** by first cutting a P1000 pipette tip in half. Insert the piece with a larger diameter into one end of a ~30 cm piece of flexible tubing to serve as a mouthpiece.
[Place **Figure 3** here]

3.6. (Optional) To complete the aspirator assembly, cover the wide end of a 10 cm piece of serological pipette with gauze to prevent flies from getting into the tubing and insert the pipette, gauze first, into the open end of the tubing to serve as a fly chamber. The aspirator should resemble that shown in **Figure 3**.

3.7. Using an aspirator (**Figure 3**, steps 3.5 and 3.6), aspirate one fly per well into a separate 24 well cell culture plate. Use the flexible plastic to cover any wells containing previously aspirated flies. Record the well position and any relevant genotype or phenotype information of each fly.

3.8. Hold the flexible plastic flush with the top of the cell culture plate containing the flies to prevent their escape and invert the plate onto the top of the modified cell culture plate with the ethanol. The sheet of flexible plastic should be resting on top of the sheets of cheesecloth. Align the inverted cell culture plate containing flies using the craft sticks to ensure each well with ethanol aligns with each well containing a fly.

3.9. The experimental setup should resemble **Figure 2**.

4. Testing the flies

4.1. Ensure the illumination pad is lit at full brightness for maximum visual contrast. Start recording with the video camera.

4.2. To expose the flies to ethanol, carefully remove the plastic from between the well plate and testing apparatus, taking care not to dislodge the cheesecloth.

4.3. Terminate the video recording once all flies have lost postural control. Once it is suspected that all flies have lost postural control, tap firmly in the center of the plate to ensure that all flies have complete loss of postural control. If there is movement, continue to record. Continue to tap periodically (every 1–2 min) until no movement occurs.

4.4. (Optional) To quickly recover the flies, remove only the top plate from the testing apparatus, revealing sedated flies resting on the cheesecloth. Aspirate individual flies into chosen containers for recovery.

4.5. Replace the ethanol in the modified cell culture plates with 1 mL of fresh 100% ethanol at least 1x every hour to control for evaporation and humidification of the ethanol and to maintain consistent ethanol exposure throughout the assay. Dry the screen mesh with cheesecloth.

4.6. Repeat for as many samples as desired.

NOTE: For highest throughput, aspirate the next round of flies into new cell culture plates during the video recording. The protocol can be paused here, as the video recording can be reviewed later.

5. Determination of fly sedation time

5.1. Record sedation time for each individual fly by watching the video recording. Sedation time is defined as the moment a fly loses complete postural control and locomotor ability. It is recommended to watch the film in reverse and record the time that the fly begins to move to ensure accuracy.

REPRESENTATIVE RESULTS:

Two 24 well microtiter plates could generate data simultaneously on 48 individual flies within as little as 10 min. **Table 1** lists measurements of ethanol sedation times for 48 individual flies, males and females separately, of two DGRP lines with different sensitivities to alcohol exposure on development time and viability[13]. Flies of line RAL_555 were less sensitive than line RAL_177 (**Figure 4**, **Table 2**; $p < 0.0001$, ANOVA). Males and females of RAL_177 showed no sexually dimorphic effect (**Figure 4**, **Table 2**; $p > 0.1$, ANOVA), whereas females of line RAL_555 were less sensitive to ethanol exposure than the males (**Figure 4**, **Table 2**; $p < 0.006$, ANOVA). The large number of flies that can be measured simultaneously and the ability to measure sexes and different lines contemporaneously can increase accuracy by reducing error due to environmental variation.

[Place **Table 1** here]

[Place **Figure 4** here]

[Place **Table 2** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of the testing apparatus and filming chamber. (**A**) Upper Diagrams. The top, side, and front views of the testing apparatus are shown, respectively. A screen mesh lays flat on top of a 24 well cell culture plate. The wooden craft sticks, represented by the arrowheads, are

attached to three adjacent sides for stability and alignment aid, two on the side of the well plate with six wells and one on the side of the plate with four wells. All attachments are hot glued onto the apparatus. (**B**) Lower Diagrams. The top, side, and front views of the assay set-up are shown, respectively. A slit is cut in the right side of the box, from the opening for the lid to the back of the opening, with the bottom of the slit level to the inner surface. The hole on the top of the box, the surface parallel to the ground, is centered for maximum video exposure. The shaded box represents the video camera.

Figure 2: Photograph of the assay system. The video camera is placed on top of the polystyrene chamber, with the lens inserted in the cut-out hole, illustrated in the diagrams of **Figure 1B**. Two sets of modified 24 well cell culture plates rest on top of an illumination pad that is inserted in a slit through the side of the chamber.

Figure 3: A fly aspirator in which flies are collected with an interchangeable mouthpiece attached to flexible tubing and a wide bore serological pipette with a cotton gauze stopper. The operator can aspirate a single fly into the pipette for transfer without anesthesia.

Figure 4: Alcohol sedation times of DGRP lines RAL_177 and RAL_555. The bars represent means and the error bars SEM (n = 48). Sedation times for RAL_177 flies were less than those for RAL_55 flies ($p < 0.0001$, ANOVA). Individual data points are indicated in **Table 1**. Additional statistically significant differences between sexes and lines are indicated in the text and in **Table 2**.

Table 1: Measurements of ethanol sedation times (**s**) **of individual flies of** (**A**) **DGRP lines RAL_177 and** (**B**) **RAL_555 for separate sexes** (**n = 48**)**.** See also **Table 2**, **Figure 4**.

Table 2: Analyses of variance for sedation time across sex and DGRP line. The model used was $Y = \mu + L + S + LxS + \varepsilon$, where μ is the overall mean, L is the fixed effect of the DGRP line (RAL_177, RAL_555), S is the fixed effect of sex (male, female), LxS is the interaction term (fixed), and ε is the error term. The models $Y = \mu + L + \varepsilon$ and $Y = \mu + S + \varepsilon$ were used for the reduced models. Line, Sex, and the Line x Sex interaction term were all significant in the full model at $\alpha < 0.05$. Reduced models by sex and DGRP line RAL_555 were also significant at $\alpha < 0.01$. See also **Table 1**, **Figure 4**. df = degrees of freedom, SS = Type I Sums of Squares.

DISCUSSION:

Here, we present a simple, inexpensive, and high-throughput method for assessing sedation time due to ethanol exposure in *Drosophila melanogaster*. Unlike many current methods, which require group analyses, this assay enables a single person to collect individual sedation time data for ~2,000 flies within an 8 h work period. We found that a single person can score 48 flies for sedation time in about 5 min. At this rate, 2,000 flies can be scored in approximately 4 h, though scoring can be conducted later. With our assay, the recorded sedation time for most flies ranges from 5–15 min at an exposure to 1 mL of 100% ethanol. Lower concentrations of ethanol or smaller delivery volumes will result in longer sedation times.

Current methods for assessing sedation time require testing large numbers of flies without readily enabling measurements on single individuals[15-26]. Many current sedation and sensitivity assays rely upon ST50[22-24], the timepoint at which 50% of the flies are sedated as a result of ethanol exposure. Although obtaining the ST50 for groups of flies was not the primary motivation for developing this assay, the video recordings demonstrate higher utility compared to current methods, as the recordings can be used to ascertain the ST50 for groups of individually tested flies and to measure the percentage of flies that satisfy a given criterion (e.g., loss of postural control) at any time point. It should be noted that such video analyses would require additional time.

Unlike current inebriometer assays, the method we describe does not require specialized tools to set up and can be performed in any laboratory using common materials. Using this method, we have obtained reliable and consistent sedation times for individual flies. The assay can, in principle, be extended to assess the effects of exposure to any volatile substance. The assay can also be applied to measure effects of acute toxicity of volatiles on other insects, including other fly species. Individual sedation time data can be used to assess the extent of phenotypic variation within a population, such as the DGRP.

We used small insect screen mesh to prevent direct contact with the ethanol solution while allowing adequate quantities of ethanol vapors to reach the fly. The layer of white cheesecloth on top of the screen mesh provides visual contrast between the fly and the surface below and ensures that flies do not get caught in the screen mesh, which could lead to ambiguous determination of loss of postural control. Commercially available membranes that are porous to water and air gave inconsistent results and were insufficiently penetrable to ethanol vapors. We intentionally used small insect screen mesh because it is a uniformly porous material that minimizes variation in ethanol exposure as a result of fly position within a well. Modifications can be made to this protocol based on available materials, although we recommend a controlled behavioral chamber, access to 90%–100% ethanol close to the fly, and uniform ethanol exposure.

Fly position within the cell culture plates should be randomized between replicates to avoid positional bias. For larger experiments that require use of this assay across multiple days and are therefore subject to environmental variation that could influence assay results (e.g., changes in barometric pressure)[27], we strongly recommend that flies be tested at the same time each day and randomized both within and across days, especially if different lines and/or sexes are to be compared against one another.

The method we developed is best suited for measuring the effect of acute alcohol exposure but is not suitable for obtaining consumption data or modeling addiction. Alcohol sedation sensitivity data obtained from this assay can, however, be integrated with other measures of alcohol-related phenotypes. One limitation of the system is that the vertical height of standard cell culture plates allows for vertical fly movement that cannot be readily tracked by video for detailed assessment of overall activity or locomotion. However, this limitation does not affect accurate assessment of sedation time. When using flies of different genotypes (e.g., in DGRP-derived outbred populations[28]), this assay also enables retrieval of individual flies to collect pools

of flies with contrasting phenotypes for bulk DNA sequencing and extreme QTL mapping[29,30]. Overall, this assay permits rapid, inexpensive collection of alcohol sedation data on large numbers of single flies.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

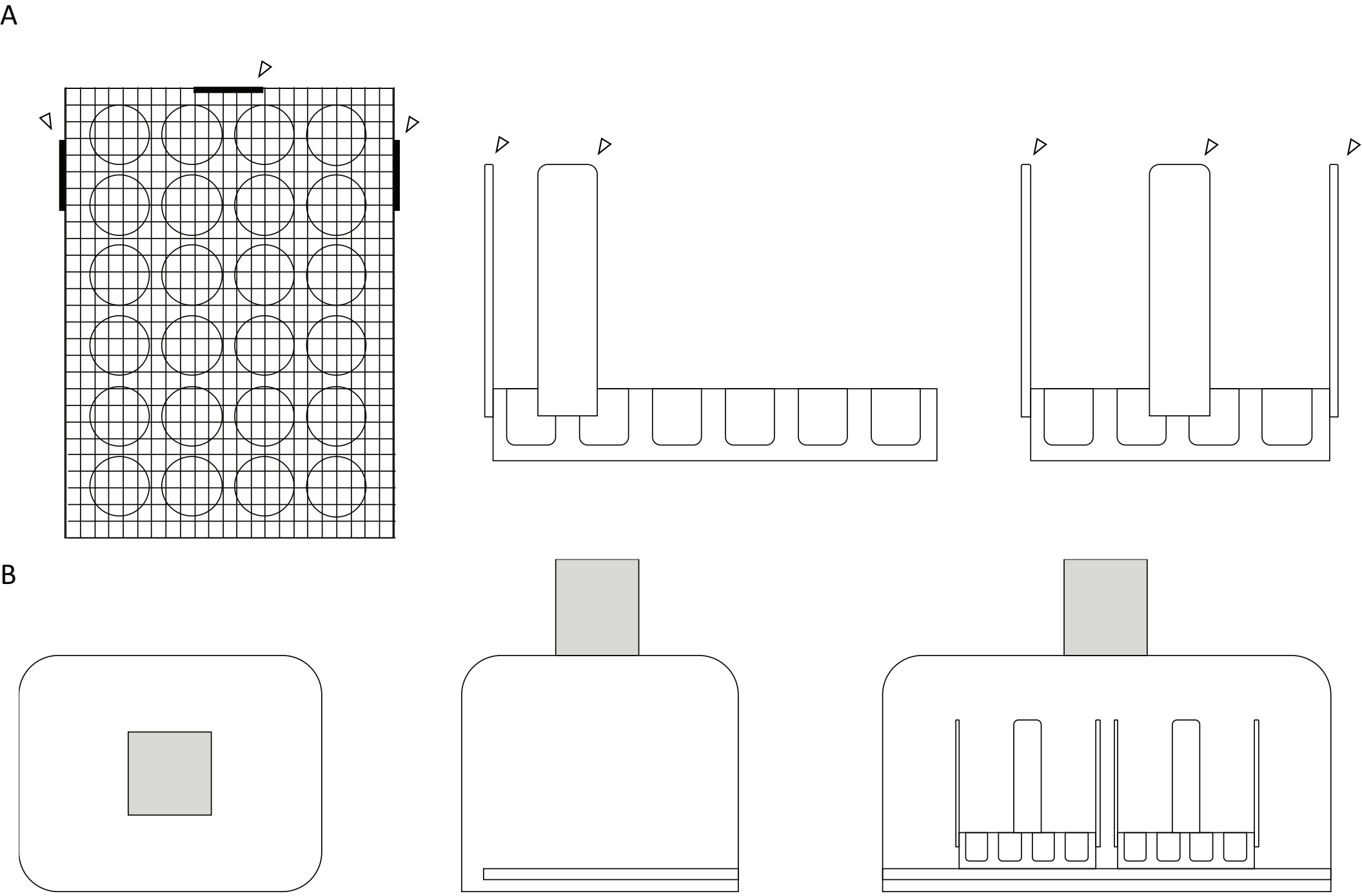

Figure 2

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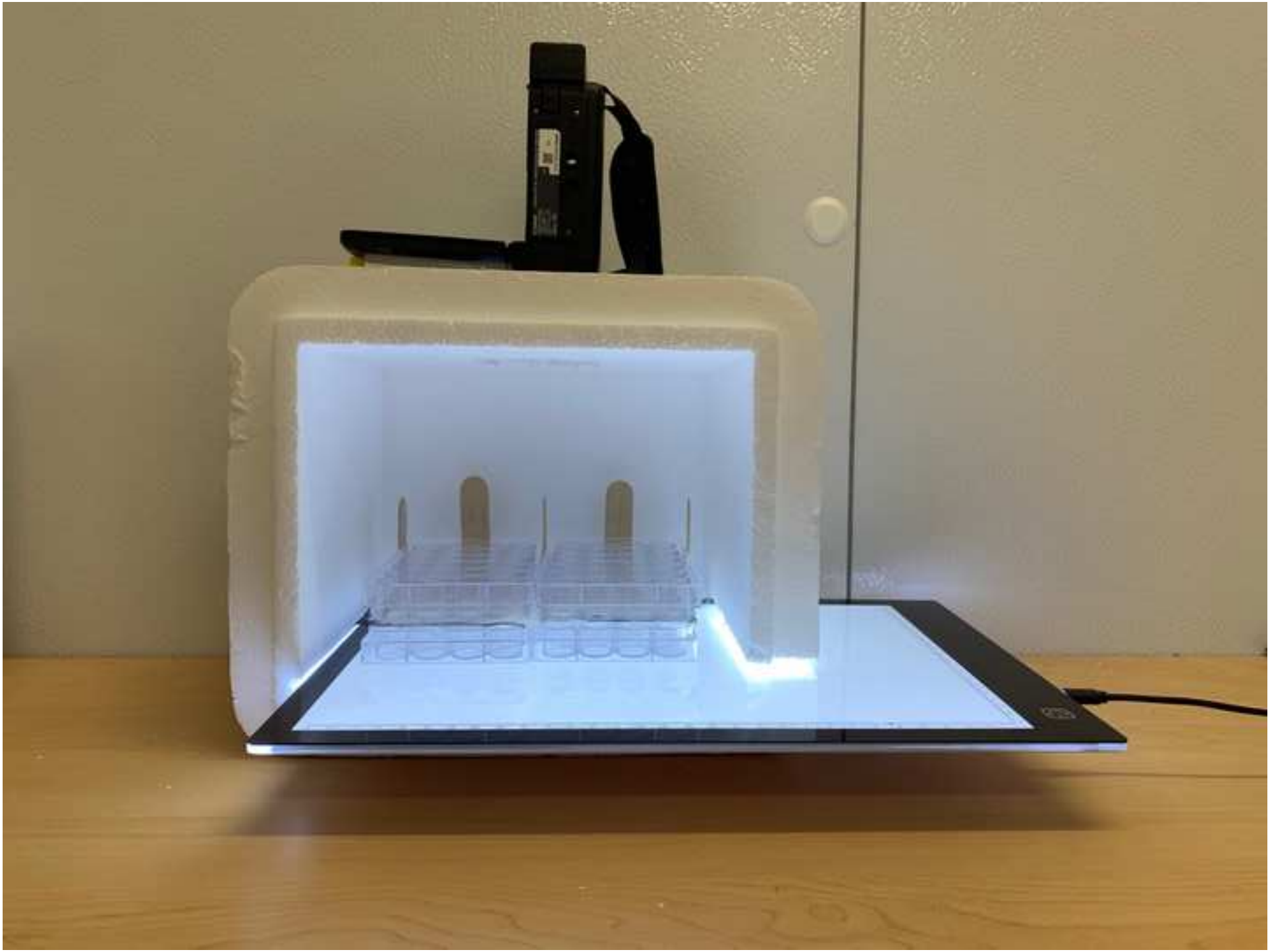

Figure 3

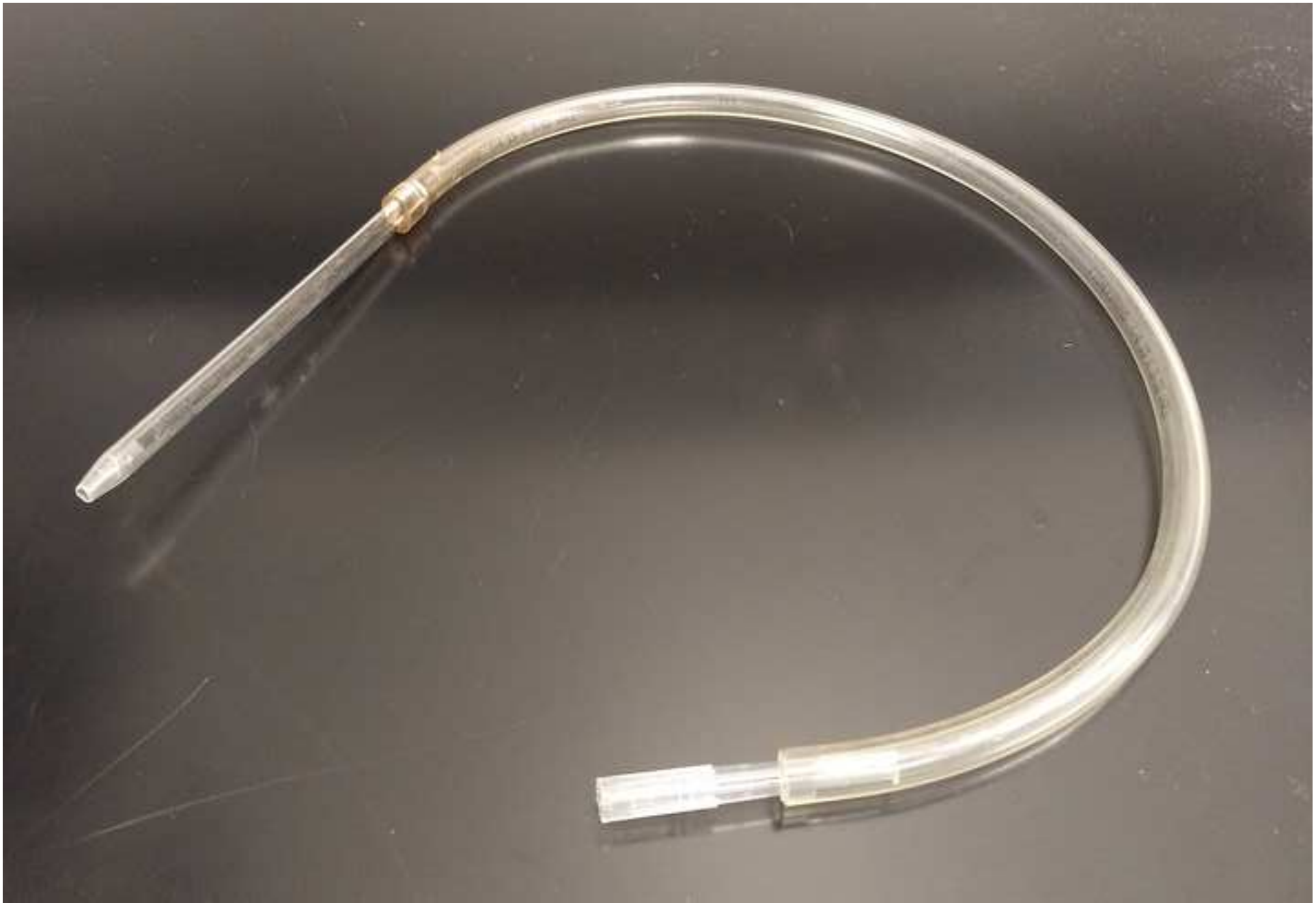

Figure 4

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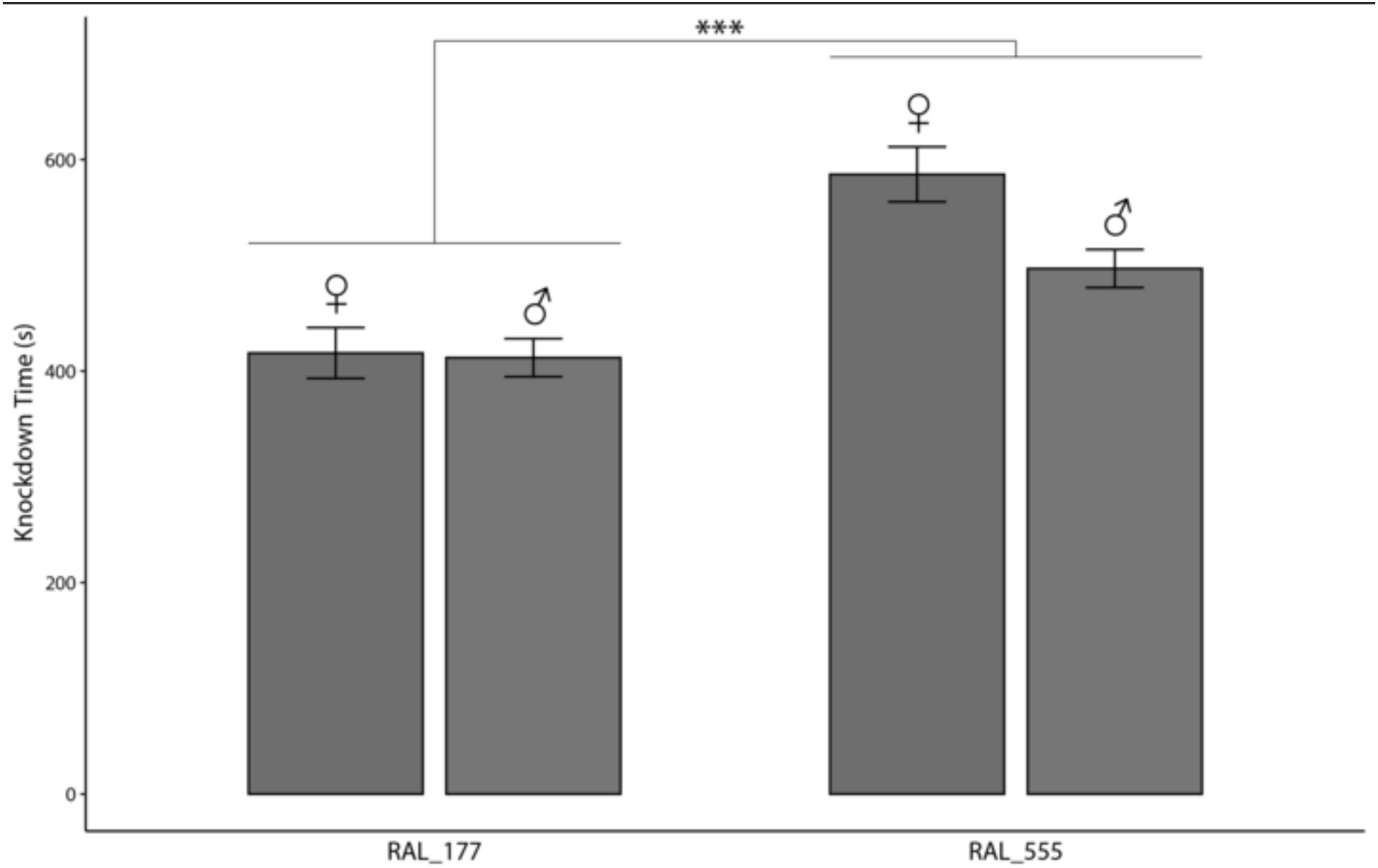

Table 1

A.

Ethanol Sedation Time (s)					
Females			Males		
414	365	477	423	568	309
201	384	498	411	523	626
228	364	333	440	403	267
440	416	404	408	422	384
888	283	285	322	369	287
1079	519	315	393	376	284
718	287	432	275	206	411
598	337	398	279	631	372
241	398	364	347	374	808
229	423	534	386	396	628
388	488	451	523	322	533
252	529	375	427	330	540
674	401	303	401	307	311
303	453	351	429	525	262
258	483	302	389	562	319
346	426	385	416	596	287

B.

937
791
504
970
595
418
366
437
665
312
682
1045
394
540
356
626

Ethanol Sedation Time (s)				
Females		**Males**		
742	622	460	331	498
619	197	467	455	562
744	513	570	582	506
540	369	865	533	492
550	606	392	544	345
709	553	308	477	388
564	558	385	576	377
692	578	460	511	412
729	484	532	425	354
576	305	334	531	506
638	420	560	548	379
741	708	832	509	472
675	381	477	449	784
690	520	556	495	226
615	336	454	524	590
678	840	634	677	509

Table 2

Analysis	Source of Variation	df	SS	F-Value	*P* -value
Full Model Pooled	Line	1	769627	34.869	<0.0001
	Sex	1	105001	4.757	0.0304
	Line x Sex	1	86021	3.897	0.0498
	Error	188	4149491		
Reduced Model Females	Line	1	685126	23.58	<0.0001
	Error	94	2730718		
Reduced Model Males	Line	1	170522	11.3	0.0011
	Error	94	1418774		
Reduced Model RAL_177	Sex	1	473	0.023	0.8800
	Error	94	1943741		
Reduced Model RAL_555	Sex	1	190549	8.12	0.0054
	Error	94	2205751		

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
24-well Cell Culture Plates	Corning	3526
Aspirator		
Cheesecloth	Genesee Scientific	53-100
Ethanol	Decon Labs	V1001
Flexible Plastic Cutting Board (Plate Cover)	Walmart	550098612
Gauze (for aspirator)	Honeywell North	67622
Illumination Pad	Amazon (AGPtek)	ASIN B00YA9GP0G
Jumbo Craft Sticks	Michaels	10334892
P1000 Pipette Tip (for aspirator)	Genesee Scientific	24-165RL
Serological Pipette (for aspirator)	Genesee Scientific	12-104
Small Insect Screen Mesh	Lowe's (Saint-Gobain ADFORS)	89322
Testing Chamber		
Tygon Tubing (for aspirator)	Grainger	9CUG7
Video Camera	Canon	1959C001AA

Comments/Description

Flat-bottomed; will house flies throughout assay

Widely available.
Widely available.

Any flat plastic that can slide easily and cover a 24-well plate completely. Flexible plastic cutting board works well.
Widely available.

Any light pad to provide contrast is suitable.
Any craft stick at least 7 cm long is suitable.
Any P1000 pipette tip is suitable.

Any small insect screen mesh is suitable.
Interior space dimension big enough to encompass light pad. Can be constructed from a polystyrene box.
Widely available.
Any video camera is suitable.

Response to Reviewers

We appreciate the time the reviewers spent carefully reading and reviewing our work. We are thankful for their constructive and insightful comments and have revised the manuscript the manuscript, as indicated below.

Editorial comments:

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully proofread our manuscript.

Protocol:
1. For each protocol step/substep, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have reviewed our protocol steps.

Figures:
1. Figures 1-3 do not appear to be cited correctly.

We have ensured that the Figures are correctly numbered.

References:
1. Please do not abbreviate journal titles.

We provide unabbreviated journal titles in our reference section.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have updated the Table of Materials to include more information on all materials used.

Reviewers' comments:

Reviewer #1:

Major Concerns:
1. Regarding Figure 4, the use of a t test to compare RAL_555 and RAL_177 is dubious because (i) the data are presented in two different figures suggesting they were from two different experiments, (ii) there is an effect of sex in one of the lines so the one group has mixed performance and (iii) t tests are used to compare males vs females in the same data. A much better approach would be to use a two-way ANOVA (for strain, sex and interaction) followed by

multiple comparisons tests (e.g. Sidak) to assess the specific comparisons. Also, the authors should determine whether the data from this method are normally distributed (i.e. conform to a Gaussian distribution) which will dictate whether paramatric or nonparametric statistical tests are appropriate.

We have changed Figure 4 according to the above suggestions. We removed panels A and B and created one figure on one set of axes, as all data were collected together as part of the same experiment. We have also changed Figure 4 to reflect mean values, as opposed to median values. Additionally, we have performed full and reduced model ANOVAs on the data, which are now presented in Table 2. The t-test results from the Representative Results section have been replaced by the appropriate ANOVA significance values. The data are approximately normally distributed.

2. Regarding the method as a whole, I think the authors should determine the internal alcohol levels in flies in this paradigm (i.e. males and females in RAL_555 and RAL_177). Doing so would (presumably) demonstrate that flies are in fact getting detectable levels of alcohol in this method and also allow another level of comparison between this and the other methods cited.

We note that we control both the genotype and environmental exposure precisely. Any variation in actual alcohol intake will be reflected in the error term of the ANOVA. Determining internal alcohol levels of flies is a separate assay outside the scope of the present manuscript.

1. In Figure 1, can the authors add a real photograph of the plate? The design/structure of the plate was not fully apparent in the graphic.

We added text directing the reader to Figure 2 to assist in visualizing the design of the plate: “The modified cell culture plate should now resemble the plate diagram shown in Figure 1 and *the experimental setup shown in Figure 2*.” Together with the graphic in Figure 1 and the photograph of experimental setup in Figure 2, we believe that the video published alongside this manuscript will make the design and structure of the plate fully apparent.

Minor Concerns:
1. Throughout the manuscript, the authors should refer to 'sedation sensitivity' as opposed to 'sensitivity' since the former is more specific and what they are measuring while the latter can have numerous meanings.

Where appropriate, we have changed “sensitivity” to “sedation sensitivity”.

2. It is not clear that the method is high-throughput. I was on board with this until I came to lines 160-165 (watching the video tape). The authors should comment on how long that essential step takes and include the time required for that step in their estimates for throughput.

We have added text incorporating the time required for one person to score flies: “We found that a single person can score 48 flies for sedation time in about five minutes. At this rate, 2000 flies can be scored in approximately 4 hours, though scoring can be conducted at a later time.”

3. In lines 46-57, (i) the description of developmental problems due to alcohol exposure do not

seem relevant to the main thrust of the manuscript (behavioral responses to acute alcohol exposure) and (ii) the description of the studies in humans being 'problematic' is less than ideal. 'Challenging' is a far better descriptor of the types of human studies noted, but even then this text does not add meaningfully to the manuscript so could be removed.

We agree with the reviewer. We have removed the lines of text from the introduction that discuss FASD and developmental ethanol exposure. In addition we have modified our description of studies in humans as follows: “Genetic risk studies on human populations are *challenging*, because of population admixture, diverse developmental histories and environmental exposures, and reliance on self-reported questionnaires to quantify alcohol-related phenotypes, which are often confounded with other neuropsychiatric conditions.”

4. In line 60, a single citation doesn't really demonstrate that the fly is an 'excellent' model to study the developmental effects of alcohol.

We have removed this sentence from the manuscript following the recommendation of Reviewer 1 in Minor Concern 3.

5. In line 64, suggest replacing 'virtually every' with 'the majority of' or something similar.

We changed ‘virtually every gene’ to ‘the majority of genes’.

6. In line 73, the term inebriometers should not be in quotes.

We removed the quotes from ‘inebriometers’.

7. It is worth mentioning in the Introduction or the Discussion or both the advantages of a method that uses single flies. For example, it is possible that there could be physical or other interactions between flies in the group-based methods that would be eliminated in this single-fly method.

The reviewer is correct that single-fly methods minimize the role that social interactions and other group-related phenomena may play in assessing ethanol sedation sensitivity for groups of flies. We have added the sentence “Assessing single flies minimizes the potential for confounding effects due to group interactions, such as those stemming from social behaviors.” in the Introduction to elaborate on this point within the manuscript.

8. It's worth mentioning in the Introduction or Discussion or both that there are 2 fundamental measures that cut across the various methods cited and used in this manuscript. The inebriometer and this single fly method measure time to criterion as the primary data, whereas other methods measure the % of flies meeting a criterion at several time-points, even if the data are transformed in aggregate to time to criterion. There is a bit more analytical power in the latter approaches because they can see nuanced changes in the time-courses of sedation that the simpler time-to-criterion methods would miss.

We agree with the reviewer that time-courses of sedation can be powerful tools to resolve small changes in alcohol sedation sensitivity. However, our assay still allows for creation of a sedation

time-course, as our assay results in video recordings that can be used for data collection well after the assay is complete. From videos created in our assay, one can collect ST50 times, percentages of flies that meet a criterion at any given time point(s), as well as individual sedation times. We have indicated this in the manuscript. See also Reviewer 2, Minor Concern 1.

9. Do the bars in Figure 4 truly represent the medians (line 200) or do they represent means (which is more typical)?

While the bars in Figure 4 from the previous submission did represent the medians, Figure 4 has been changed to reflect the means. See Reviewer 1, Major concern 1.

Reviewer #2:

Minor Concerns:
A common way to measure sensitivity to ethanol is to measure ST50 (time at which 50% of flies in a chamber become sedated. The authors, do not directly mention this techniques but they are obviously aware of it and indeed they quote the relevant references. However, it would be useful to discuss the difference between their method and the traditional ST50 with respect to the following:
1) One of the advantage of the ST50 measurement is that it eliminates the behaviour of out-layers i.e. flies that are sedated very quickly or very late. Early sedation of could be due to injury or other random defect of the individual fly . Late sedation could be due to flies staying further away from the source of ethanol (possibly less likely in the method presented) It would be interesting for the author to comment on this point

We agree with the reviewer with regards to the presence of individual variation between flies, and the outside possibility for outliers. However, our assay is compatible with the concept of ST50 and any noted outliers in raw data or on the video footage can always be excluded prior to analyses. We have added text to expand on the versatility of our assay: “Although obtaining the ST50 for groups of flies was not the primary motivation for developing this assay, the video recordings can be used to ascertain the ST50 for groups of individually tested flies and to measure the percentage of flies that satisfy a given criterion (e.g. loss of postural control) at a given time point. It should be noted that these video analyses would require additional time.”.

2) In the ST50 procedure the vials are tapped at regular intervals to ensure that the flies are indeed sedated. The authors do indicate that they tap the plates, but from the description alone it is difficult to imagine how this can be done with sufficient vigour without affecting the positioning of the plates under the camera, and the author may also recommend the frequency of tapping to ensure correct interpretation of individual flies being sedated.(among the 48 being observed) .

We have added the following clarifications regarding plate tapping: “Once it is suspected that all flies have lost postural control, tap firmly in the center of the plate to ensure that all flies have complete loss of postural control. If there is movement, continue to record. Continue to tap periodically (every 1-2 minutes) until no movement occurs.”. Although we have added text to elaborate on the frequency of tapping the plates, we believe that the video that will accompany

our manuscript will be indicative of the vigor needed. Additionally, we have not encountered issues with tapping and movement of the plates underneath the camera.

It would also be useful if the author gave advice on how to recover the flies after they are sedated. Presumably the plastic film can be reinserted below the cheese cloth and the plate flipped, and the flies transferred to vials while still sedated. If the authors have some data on recovery and re-exposure of the flies that would also be useful

We appreciate the comment and agree that adding advice on fly recovery could increase the utility of our assay. We have added an additional step in the protocol (Step 4.4) as follows: “(Optional) To quickly recover the flies, remove only the top plate from the testing apparatus, revealing sedated flies resting on cheesecloth. Aspirate individual flies into containers of the experimenter’s choice for recovery.”. We did not explore further extensions of our assay such as re-exposure and recovery times.

Given that the author wait until all the flies are sedated (while a ST50 experiment is stopped when half of the flies are sedated) it would be interesting to know what mortality rates they experience .

Mortality was not apparent and was not investigated in our experiments.

Reviewer #3:

Major Concerns:
1. The use of the term "alcohol knockdown" in the title and repeatedly within the manuscript text is atypical. The fly alcohol sensitivity studies often use the ethanol mean elution or sedation time as a measure and term rather than "alcohol knockdown". This needs to be clarified to prevent any possible misunderstanding and confusion. The phrasing at its current state is suggesting the breakdown of alcohol into its metabolites rather than sensitivity to alcohol. Also, the use of "knockdown" is more often associated with "genetic knockdown", thus it may lead to muddled terminology.

We acknowledge the lack of clarity and appreciate the suggestion. We have changed all relevant occurrences of “knockdown” to “sedation” or “sedation time”, including a change to the title of the manuscript.

2. The first two paragraphs in the "Introduction section" heavily refers to FASDs (P1 L46-50 and P2 L54-57), however, the method proposed does not model FASDs but only measures individual fly sensitivity to alcohol. These statements can appear to be misleading and the authors need to clarify these sections of the text.

See Reviewer 1, Minor Concern 3. We have omitted these portions of the manuscript.

3. In the protocol step 2.1, the authors refer to the wrong figure Figure 2 but it should be Figure 3 (L107-108) and should also refer to the chamber diagram Figure 1B.

We have corrected the numbering of Figures 2 and Figure 3 and included a reference to Figure 1B in protocol step 2.1.

4. In the protocol step 3.5, the authors refer to a fly aspirator depicted in Figure 3 (L128), this is incorrect and should be Figure 2.

We have corrected the numbering of Figures 2 and Figure 3.

5. In the protocol step 3.7, the authors refer to the wrong figure, which is Figure 2 but it should be Figure 3 (L139).

We have corrected the numbering of Figures 2 and Figure 3.

6. In the protocol step 4.4, the rationale for the replacement of 100% ethanol at least once every hour is unclear. The authors need to clarify how long it takes to finish a single experimental set, how many can be done in an hour, and does the ethanol vapor experienced by flies uniformly maintained throughout the hour timeframe?

Protocol step 4.4 is now Protocol step 4.5. We have included rationale for ethanol replacement by stating in the protocol: “Replace the ethanol in the modified cell culture plates with 1mL of fresh 100% ethanol at least once every hour to control for evaporation and humidification of the ethanol and to maintain consistent ethanol exposure throughout the assay.”. We stated in the manuscript that “recorded sedation time for most flies ranges from 5-15 min”, “Two 24-well microtiter plates can generate data simultaneously on 48 individual flies within as little as 10 min” and have also added “We found that a single person can score 48 flies for sedation time in about five minutes.”, thereby allowing the readers to determine the length of time that this assay would require for their own experiments.

7. In the "Representative results" section, the reference to the figure (Figure 4A) and the corresponding statistics information is missing for the comparison between sexes in the RAL_177 genotype (L173). Also, in L174 should refer to Figure 4B specifically.

We combined Figure 4A and Figure 4B and placed them on one set of axes, thereby eliminating portions A and B. We have corrected the text to reflect this change to Figure 4. We have also added the corresponding statistical information for the comparison of sexes for genotype RAL_177. See Reviewer 1, Major Concern 1.

8. In the figure legend 4 (L199-200), the authors state "bars represent medians" yet the statistical analyses done, as referred to in the text (L172 and L174), were Student's t-test that compares the means. The authors need to address this.

We have modified our analysis and performed ANOVAs. Figure 4 has been changed to reflect the means. See Reviewer 1, Major Concern 1.

9. The "Materials table" lack some information such as the catalog numbers for the cheesecloth, illumination pad and the small insect screen mesh. Also, it lacks specific information on the materials needed to assemble the fly aspirator. It would also be helpful to depict the assembly either in the video or a set of pictures in Figure 2 rather than just the final product.

Many of our materials, such as cheesecloth, illumination pad, and small insect screen mesh are available from many retailers. We have provided a company and catalog number for these items as well as other materials. We also expanded the materials list to include the aspirator objects. We have also updated the protocol by adding steps 3.5 and 3.6, which indicate aspirator assembly, and hope to detail this more in the accompanying video.

10. The "Discussion" section should briefly discuss the significance of the described alcohol exposure system compared to other existing methods.

Although the significance of this system is compared to current methods within the introduction, we have modified the discussion to highlight the significance of our method compared to other methods.

Minor Concerns:
1. The introduction section may benefit with the addition of information regarding the need for assays to measure individual animal's sensitivity to alcohol and why is it advantageous over group exposure.

We have added text to the introduction addressing the benefits of data collection on single flies. See Reviewer 1, Minor Concern 7.

2. In the protocol step 1.3, the authors should specifically refer to the Figure 1A not just Figure 1 (L99), since they are only explaining the testing apparatus assembly and not the filming chamber.

We have changed the text from “Figure 1” to “Figure 1A”.

3. In the protocol step 2.2, the authors refer to a controlled environment in a behavioral chamber, yet, they fail to specify the range values for humidity, temperature, airflow and noise level (L113). They need to add these details.

We have added details of our testing environment as noted by the reviewer.

4. In protocol step 3.3 (L121-123), how do they securely attach the cheesecloth to the modified plate? This information is missing and may be an important step to highlight.

We do not attach the cheesecloth to the modified plate. The friction between the cheesecloth and the screen mesh is enough to secure the cheesecloth.

5. In the protocol step 4.1, the authors refer to "full brightness" (L143), this is vague and should be clarified with a specific measure (e.g. range of lux).

Although the light pad is commercially available, brightness information for this illumination pad is not available to us. We have indicated the exact illumination pad used in the Table of Materials to account for this issue.

6. In the protocol step 4.3, the way this step is written almost implies that "loss of postural control" is equivalent to "immobility" - need to clarify.

We have clarified this as follows: “To ensure that all flies *have complete loss of postural control*…”

7. In the protocol step 5.1, it would be helpful if the authors have a depiction of their criteria for behavioral scoring (either in the video or an image in a figure). Also, the authors need to clarify why do they not just measure immobility rather than complete loss of postural control along with it.

We agree that behavioral scoring is difficult to portray within a manuscript. We anticipate that the video published alongside this manuscript will elucidate the behavioral scoring criteria.

8. In the figure legend 1, the short title only refers to the assay chamber (L182). It should refer to both testing apparatus and filming chamber.

We have modified the title of Figure 1 to include references to testing apparatus and filming chamber.

9. In the figure legend 2, there is reference to "Tygon tubing" (L192) but this is not reflected in the table of materials and should be added.

We have added Tygon tubing to the Table of Materials.

10. In the figure legend 3, it should specifically refer to Figure 1B not just Figure 1 (L196).

We have changed the reference from Figure 1 to Figure 1B.

Reviewer #4:

Major Concerns:
Lines 227 - 228. At this point the authors are talking about replicates and positional bias in the chamber and how to account for such bias. However, I think that they omit the most important source of variation in Drosophila behavioral assays and how to account for it. This source is day-to-day variation that is likely due to changes in barometric pressure. A good example of this is in Pohl et al (2013) Alcohol Clin Exp Res 37:1862-1871 Figure 1, Panel I (eye). In Pohl, the authors show substantial behavioral variation at the same internal blood alcohol concentration in the same behavioral chamber. The best solution to such a problem in the current assay would be to include replicates of an internal control (lab's wild type stock?) in each dish and to then normalize all data against this internal control. Or if the authors don't like normalization then at the internal control could at least be used as a reporter for when a lab is experiencing such day-to-day variation. I think that this is important to suggest that this might be necessary because it could take a person new to Drosophila behavior quite some time to realize that this is an important consideration. Perhaps the authors do not see this in their labs, but certainly a number of other investigators have noted such variability over the years.

We are aware of the pitfalls of changes in the environment and agree with the reviewer that these variations can have large effects on assays. We used randomization to account for this. The two DGRP lines were always tested simultaneously and flies from each line and each sex were randomly placed within plates and across time slots both within and across days of testing. Flies were tested within a 2-hour time frame and always at the same time of day. We anticipate that these measures will account for variation. Additionally, in our ANOVA analysis (Table 2) we have included an error term that captures this variation.

To encourage future readers of this manuscript to account for these sources of day-to-day variation, we have included text advising the readers to randomize their experiments: “For larger experiments that require use of this assay across multiple days and are therefore subject to environmental variation, such as changes in barometric pression, that could influence assay results[27], we strongly recommend that flies be tested at the same time each day and randomized both within and across days, especially if different lines and/or sexes are to be compared against one another”.

27. Pohl, J.B., Ghezzi, A., Lew, L.K., Robles, R.B., Cormack, L., Atkinson, N.S. Circadian genes differentially affect tolerance to ethanol in Drosophila. *Alcoholism: Clinical and Experimental Research*. **37** (11), 1862-1871 (2013).

Minor Concerns:
Lines 73 and 74, the sentences: "Alcohol sensitivity in D. melanogaster can be quantified using "inebriometers". These are 122 cm long vertical glass columns with slanted mesh partitions to which flies can attach17,18."

The citations given (17 and 18) are very early citations for this device but the authors should also include the original description for this device. It is: Cohan FM, Graf J-D (1985) Latitudinal Cline in Drosophila melanogaster for Knockdown Resistance to Ethanol Fumes and for Rates of Response to Selection for Further Resistance. Evolution 39:278-293. In this paper the authors say that they used it to measure ethanol tolerance but in their field this meant sensitivity.

Thank you for this information. We have included this citation in our manuscript.

Lines 96 - 97. The sentence is: "Prepare a 24-well cell culture plate by affixing the screen mesh on top of the open wells with a hot glue gun."

I would like a clarification concerning the gluing of the mesh to the 24 well plate. Does the glue go around the perimeter of the plate or around the perimeter of each well?

We have expanded the description of hot glue application prior to affixing the screen mesh to include “Prepare a 24-well cell culture plate by placing a small line of hot glue around the perimeter of the top of the plate using a hot glue gun and affixing the screen mesh on top of the open wells”.

Lines 110 - 111. The sentence is: "2.2 Prepare the filming chamber for use by inserting the illumination pad into the slit and positioning the camera in the lens hole above the illumination pad."

I thought it might be nice for the authors to tell us how they generated uniform illumination from the PAD. Is it displaying a photo of a white surface, a drawing of a white box, or a blank Keynote or Powerpoint slide?

The illumination pad that we used is commercially available and is now noted in the Table of Materials. Illumination pads have a series of small lights equally distributed throughout the pad area and therefore provide constant illumination directly from a light source, as opposed to a blank slide or white photo.

Lines 121 - 123. The sentence is "Cut two pieces of cheesecloth the dimensions of the 24-well cell culture plate using the cardboard template created in step 1.1 and place them on top of the screen mesh of the modified cell culture plate."

After the reading the entire protocol, it seems to me that it is important that the cheese cloth is larger than the plate so that when the plastic is pulled out one can easily grip the cheesecloth to keep it from moving. If it is is the same size (which is what is described) then won't it be difficult to make sure that it stays in place? I think that the protocol should be a changed here to account for this.

We agree with the Reviewer that movement of the cheesecloth could be problematic for the protocol. It has been our experience that the friction between the cheesecloth and the screen mesh prevents slippage and is not an issue for a careful experimenter. Additionally, were cheesecloth to be cut larger than the size of the cell culture plate, the cheesecloth would not lay flat between the craft sticks on the sides of the plate that serve as alignment guides.

Lines 126-127. "3.4 Cut a piece of thin, flexible plastic cutting board using the cardboard template created in step 1.1."

Once again, the protocol describes a plastic plate that is cut to the same size as the 24-well culture dish. But isn't it important for the plastic to be longer than the culture dish so that the investigator has something to grip when pulling it out of the dish:dish sandwich? I think that the protocol should be a changed here to account for this.

Thank you for this suggestion. We agree that it would be easier to slightly lengthen the piece of plastic on one end (while still allowing it to fit between the craft sticks). We have modified step 3.4 to reflect this.

Line 139. The sentence is: "The experimental setup should resemble Figure 2."

The authors have referred to the wrong figure. Figure 2 is the fly aspirator. The setup is in Figure 3.

We have corrected this mistake.

Lines 163 - 164. Watching the recorded video in reverse. Just wanted to say that this is clever and probably quite a time saver.

Thank you for the comment – we agree that this is a time saver!

Lines 187 - 188. The sentence: "The box is oriented with the opening for the lid perpendicular to the ground."
I really don't understand what this sentence means. Can you find a clearer way to describe this?

We have deleted this sentence.

Lines 229 - 230. The sentence: "Alcohol sensitivity data obtained from this assay can, however, be integrated with other measures of alcohol related phenotypes."

I think that the authors should mention that in humans ethanol sensitivity is a risk factor (negative correlation) for acquiring an AUD. Otherwise why should anyone care about a fly ethanol-sensitivity assay? This could also be brought up in the Introduction.

A good reference for this is:

Schuckit MA, Smith TL (2011) Onset and course of alcoholism over 25 years in middle class men. Drug Alcohol Depend 113:21-28.

We agree with the Reviewer. We have the following sentence to the Introduction with appropriate citations: In humans, decreased sensitivity, or a low level of response to alcohol, has a strong genetic component and is associated with a higher risk of developing alcohol use disorders[1-4].

Table of Materials - It would be nice if the authors included a web address and part number when possible. The catalog number is included for the first two items but not for the others. For the small insect screen mesh this might be a time saver. In any case, it is customary to be very specific in protocols.

We have added additional details to the Table of Materials, including catalog numbers for all relevant items.