Video Article

A Direct and Simple Method to Assess *Drosophila melanogaster*'s Viability from Embryo to Adult

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Abstract

In *Drosophila melanogaster*, viability assays are used to determine the fitness of certain genetic backgrounds. Allelic variations can result in partial or complete loss of viability at different stages of development. Our lab has developed a method to assess viability in *Drosophila* from embryo to fully mature adult. The method relies on quantifying the number of progeny present at different stages during development, starting with hatched embryos. After embryos have been quantified, additional stages are counted, including L1/L2, pupae, and mature adults. After all stages have been examined, a statistical analysis such as the chi-square test is used to determine if there is a significant difference between the starting number of progeny (hatched embryos) and later stages culminating in the observed number of adults, thus rejecting or accepting the null hypothesis (that the number of hatched embryos will be equal to the number of larvae, pupae, and adults recorded throughout the stages of development). The primary advantage of this assay is its simplicity and accuracy, as it does not require an embryo rinse to transfer them to the food vial, avoiding losses from technical errors. Although the protocol described here does not directly examine L2/L3 larvae, additional steps can be added to account for these. Comparing the number of hatched embryos, L1, pupae, and adults can help determine if viability was compromised during the L2/L3 stages for further studies (the use of colored food helps with visual identification of larvae). Overall, this method can help *Drosophila* researchers and educators determine when viability is compromised during the fly life cycle. Routine assessment of stocks using this assay can prevent accumulation of secondary mutations that may affect the phenotype of the originally isolated mutant, especially if the original mutations affect fitness. For this reason, our lab maintains multiple copies of each of our *Dm ime4* alleles and routinely checks the purity of each stock with this method in addition to other molecular analyses.

Video Link

The video component of this article can be found at https://www.jove.com/video/59996/

Introduction

Lifespan is affected by genetic and non-genetic factors. In standard lab growth conditions at room temperature, our lab has observed significant variation of fitness and viability among different *Dm ime4* alleles grown under identical conditions (**Figure 1** and **Supplemental Figures**). Viability studies are frequently done to investigate the effects of a certain allele combination or growth condition in population genetic studies[1,2,3,4]. However, detailed analyses of viability within a non-complementary group of mutations are hard to find in the scientific literature. An allele is usually labeled "non-essential" if the researcher finds a few individuals homozygous for that allele within the food vial that houses the balanced stock[5,6]. However, accurate Chi-square analyses to assess whether these homozygotes arise at the expected Mendelian ratios are not reported[5,6]. The most permissive temperature for any *Drosophila* stock is room temperature (22-23 °C) and, with appropriate nutrients, the life cycle of wild-type flies takes approximately twelve days to complete[7,8]. As the duration of each developmental stage of wild-type *Drosophila* is known[7,8], the method described in this report can be used to examine whether the *Drosophila* strain under study is fit at each stage in comparison to a control appropriate for the genetic background tested. In contrast to studies that focus on one specific aspect of development[9], this protocol provides a practical way to assess viability at different developmental stages.

In our lab, this protocol is used to assess the viability of stocks that are deficient for Drosophila Inducer of Meiosis 4 (*Dm ime4*). *Dm ime4* is an essential gene[10] that encodes an RNA methyltransferase with critical roles in RNA metabolism in *Drosophila* and other multicellular organisms[5,6,10,11,12,13,14]. To quickly evaluate novel alleles of *Dm ime4* generated via CRISPR/Cas9 (**Supplemental Figures**), an end-point viability assay was performed that only counted adult progeny produced within vials of balanced stocks (**Figure 1**). Some of the stocks used were described in previous *Dm ime4* reports[5,6]. Homozygous mutants emerged at sub-Mendelian levels, as determined by chi-square analyses (**Figure 1** and **Supplemental Materials**). To assess whether these lower-than-expected numbers were due to fewer embryos being laid, or fewer hatched embryos, or loss of viability in L1/L2 or pupae, we expanded the tracking to include counts at each of these developmental stages (**Figure 2**, **Figure 3**, **Figure 4**, **Figure 5**, **Figure 6**, **Figure 7**, **Figure 8**, and **Figure 9**).

Here, we describe the method using wild-type (OreR) flies. To empirically test this method for use with other genetic backgrounds or Drosophila species, we recommend using OreR as a reference and adjust timepoints according to the experimental organism. The protocol was further

evaluated to assess viability of progeny generated by crossing virgin wild-type females with males from a heterozygous *Dm ime4* mutant stock[5,6] (**Figure 4**).

Protocol

1. Media Preparation

1. Prepare grape agar according to manufacturer instructions (see **Table of Materials**) and pour into a 35 mm Petri dish to half-full (**Figure 5**). Allow to solidify for approximately 1 h. After the grape agar solidifies, immediately use or store at 4 °C.
2. Gently make scratches across the agar plate using a small plastic knife (flies like to lay on uneven surfaces) leaving the middle of the plate without scratches (**Figure 5**). Place a small amount of yeast paste (made fresh; see **Table of Materials**) at the center of plate (**Figure 5**).

2. Embryo Collection Mini Cage Set Up

1. Set up a cross using two virgin females and one young male inside the embryo collection cage containing the grape agar plate supplemented with yeast paste (**Figure 5**).
2. After 24 h, inspect the cages for laid embryos without opening the cage by looking at the bottom of the agar plate. If embryos have been laid, remove the agar plate from the chamber and place it inside a humid chamber for microscopic observation (**Figure 6**). If several days of laying are scored (e.g., fertility/longevity assays), keep the breeding parents and replace the plate with a fresh one prepared as described. Early collections of less than 24 h can be done but, when using virgin females, be aware that they will not lay until 48 h after emerging from their pupa case.
3. Cover the agar plate containing embryos with the Petri dish lid to avoid dehydration and place it immediately inside a humid chamber (**Figure 7**). Observe under a dissecting microscope and record hatched embryos and L1. Replace lid and store at room temperature in humid chamber until all embryos had hatched and developed into L1 larvae (**Figure 6**).

3. Counting Embryos and Larvae

1. After 48 h, observe the plates under the dissecting microscope and record the numbers one last time before transfer of agar disc to food vial. Fertilized/viable embryos should become L1 by then. Longer incubation periods prior to transfer are possible but be aware that plates may start losing moisture and the agar may crack compromising a clean transfer to food vials (**Figure 7**). To ensure the plates remain hydrated and embryo viability is not compromised while counting, avoid using a direct gooseneck light over the agar surface (**Figure 7E** shows an appropriate distance).
2. After counting, cover the plate and store it in the humid chamber until ready to transfer. Record findings.

4. Transfer the Grape Agar Disc to a Food Vial to monitor Viability During Development

1. Once the numbers are recorded (hatched embryos/L1/L2), use a spatula to carefully transfer the grape agar disc to a vial large enough to accommodate a 35 mm disc containing Drosophila food media prepared according to manufacturer's instructions (refer to list of reagents). Place the grape agar disc L1-side down on the food (**Figure 7**). After transferring the agar disc with larvae to the food vial, carefully inspect the empty Petri dish for any larvae left behind (**Figure 7E**).
2. Set up a schedule to inspect food vials daily, at roughly the same time each day, to ensure L2/L3 larvae are observed making their way to the food in the vial (**Figure 8**).
3. Record the number of pupae and adult Drosophila (**Figure 9**). Keep counting until no more adults are observed and avoid counting the following generation (do not count past 9 days after observing the first adults).
4. Observe and record the findings. Adjust the time frame of embryo collection, counting, and recording accordingly to the mutant stock being used.
5. Perform a chi square analysis. The null hypothesis assumes 100% viability such that the number of adults will be equal to the number of hatched embryos and L1 originally recorded and transferred to the food vials

Representative Results

This method accurately and reproducibly allows one to gauge viability from embryos to emerged adults when coupled to chi square analyses.

In initial studies, after counting embryos and larvae, the grape agar was placed inside the vial upright on the side of the vial. Unfortunately, when the agar was placed on the side of the vial many of the embryos and larvae did not mature to adults. This was likely due to the grape agar disc drying out (**Figure 3**). This placement introduced an environmental variable (hydration of agar disc) that could confound the results. Approximately 39% of the progeny was lost between embryos to adult as only 61% of adults emerged. The biggest losses occurred between the L1 larvae (counted on surface of grape agar plates) and pupae (counted on walls of food vials) counts. However, when the grape agar was placed faced down inside the vial in direct contact with the food surface, less than 6% of the progeny was lost (**Figure 2**). The agar remained hydrated as the entire grape agar disc remained in contact with the moisture of the instant food inside the vial (**Figure 7**, **Figure 8**). These results indicate that the position of the agar inside the vial is important for obtaining reliable data and minimize contribution of environmental variables. Further data to support the effectiveness of the method was collected by comparing wild-type progeny used in initial method validation and progeny produced by crossing virgin wild-type females to males from a balanced *Dm ime4* mutant stock (**Supplemental Figure 2**, *ime4Δnull/TM3*^{Sb}). Approximately 91% of the progeny matured to adulthood compared to 94% of the progeny from wildtype (**Figure 4**). This difference was not statistically significant.

Homozygous *Dm ime4* mutant males[5] were also used to provide further validation of the method. However, the homozygous mutants were too sick to reproduce and died before embryos were deposited. Therefore, one limitation of the experiment is that males need to be healthy enough to reproduce to generate a starting embryo population to track.

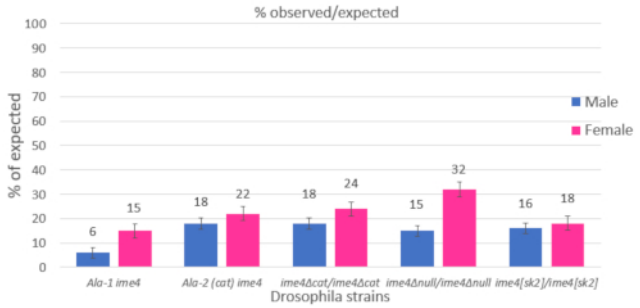


Figure 1: *Dm ime4* mutant stocks emerge at sub-Mendelian levels. Stocks in which portions of the catalytic domain or Ado-Met binding domain were mutated are shown (refer to **Supplemental Figure 1** for details). Some stocks had portions of either domain replaced with Ala (alanine-scanning mutagenesis), while other stocks had either or both domains deleted. The ratio of observed number of homozygotes to the expected numbers is represented in percentages (compared to heterozygous sibling controls, refer to **Supplemental Figure 3** for sorting scheme and chi square analysis). Chi square analyses were performed to determine if the difference between observed and expected was statistically significant and not due to chance alone (p-values < 0.01). Error bars represent standard error of the mean (minimum of three trials per cross indicated). A spreadsheet with all the data can be found in **Supplemental Materials**. Please click here to view a larger version of this figure.

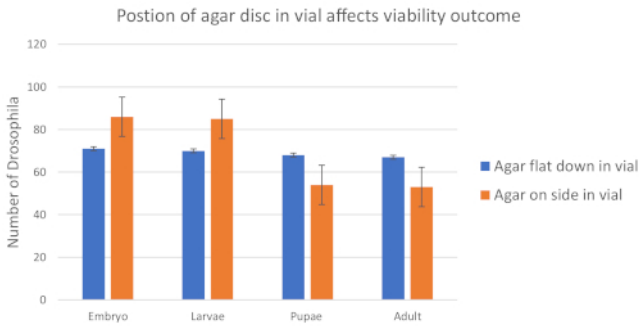


Figure 2: Agar position inside the vial can introduce unintended environmental variables. Histogram of the number of individuals at different stages of development counted from grape agar disc embryo side down in food vial or agar on the side of the food vial. Placing the agar disc embryo-side down in the vial resulted in 94% (SD ± 0.02) of initial embryos surviving until adulthood. Placing the agar on the side of the food vial resulted in only 61% (SD ± 0.07) of the originally counted embryos reaching adulthood. A spreadsheet with all the data can be found in the **Supplemental Materials**. The expected numbers at each stage is set as the initial number of embryos/L1 counted on the grape agar disc prior to transfer to food vial (Null hypothesis: 100% of hatched embryos develop). The observed numbers are the actual number of individuals counted for the developmental stage indicated. Thus, each stage is compared to the initial number of hatched embryos counted on the grape agar plate. Chi square analyses were performed to determine if the difference between observed and expected was statistically significant and not due to chance alone using a p-value threshold of 0.05. The difference was significant for embryos grown with agar on the side of the food vial, but not for those grown in a disc embryo side down. Errors bars represent standard error (minimum of three trials per cross indicated). Please click here to view a larger version of this figure.

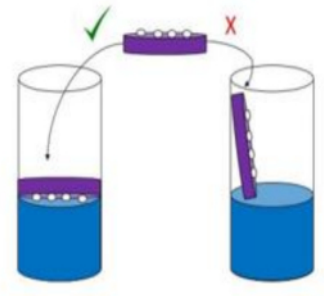


Figure 3: Cartoon showing two ways grape agar discs carrying embryos/L1s can be placed inside the food vial. There was a significant difference between the total number of embryos and adult progeny based on the position of the agar in the food vial. The difference stems from an environmental variable (hydration) created by the difference in disc placement inside the food vial: there is a statistically significant difference between expected and observed numbers in the larvae and pupae counts when the agar disc was placed on the side of the vial versus no significant difference in expected versus observed numbers when the agar disc was in contact with the food. Please click here to view a larger version of this figure.

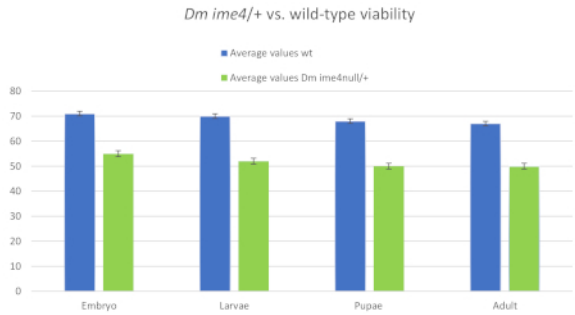


Figure 4: Histogram showing the number of individuals at different stages of development from wild-type males versus males from a *Dm ime4* mutant balanced stock. Balanced males crossed to virgin wild-type females as described in 2.1. Agar was transferred embryo-side down into food vials. The average ratio of adults emerging from the original embryos counted in grape agar discs from wild-type X wild-type was 94%, SD ± 0.02, whilst that ratio for *Dm ime4* mutant/+ X wild-type was 91%, SD ± 0.01. Statistical analysis was performed as described for **Figure 2**; differences were not significant for either group. Please click here to view a larger version of this figure.

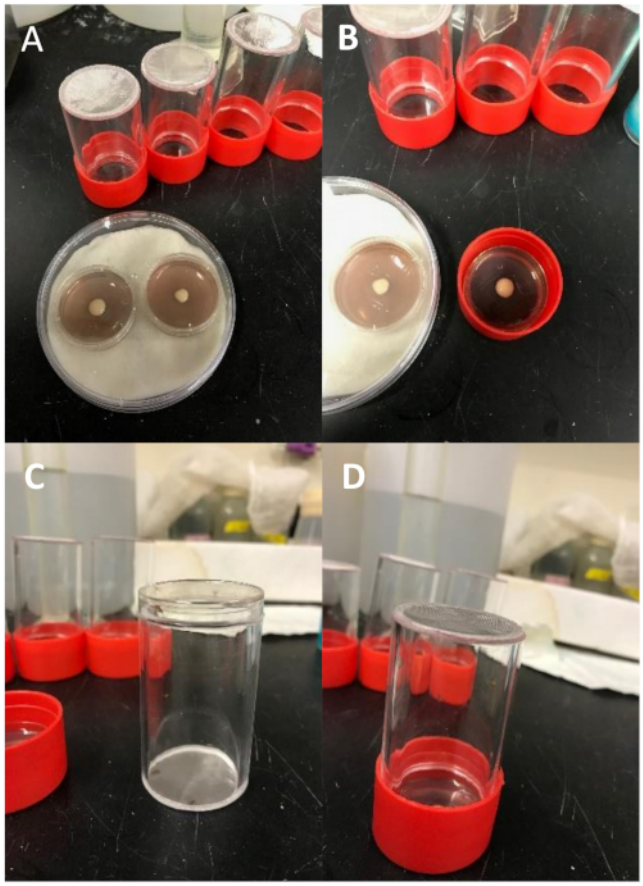
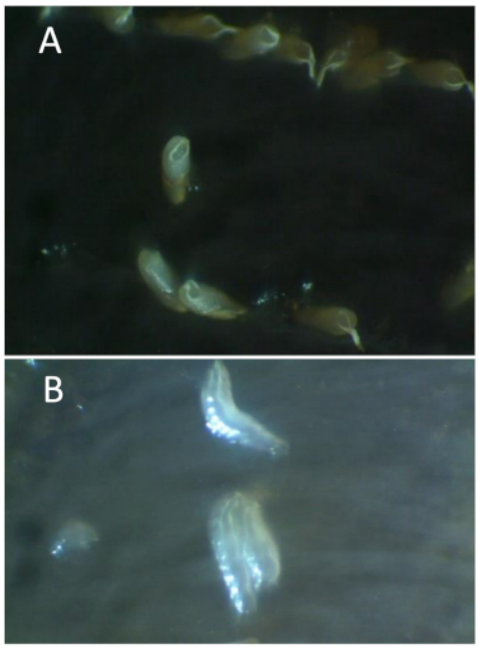


Figure 5: Experimental setup. (**A**) Embryo collection mini-cages and grape-agar mini-Petri dishes. Plates are placed inside a humid chamber (standard Petri dish with absorbent paper disc saturated with water) to prevent dehydration. A small amount of yeast paste was placed at the center of each grape-agar plate. (**B**) Detach the embryo collection plate holder lid (red) and place a prepared plate, yeast side up. (**C**) Transfer the cross to the embryo cage and immediately place a Petri dish lid to hold flies inside. (**D**) Quickly remove cover and flip the embryo cage so opening contacts grape-agar plate. Incubate for 24-48 h, inspecting daily. Please click here to view a larger version of this figure.



C.

	Embryos	L1	
Plate 1	64	62	Expected number=62
Plate 2	69	69	Expected number=69

Figure 6: Embryo development on grape-agar plates. (**A**) Wildtype embryos deposited on the grape-agar mini-Petri dishes are counted under a dissecting microscope. Keep plates inside humid chamber when not visualizing and avoid direct light to prevent dehydration. (**B**) Grape-agar plates are kept in a humid chamber and hatched wildtype embryos are recorded. This photo shows three wildtype L1 larvae that developed from embryos shown in A. (**C**) Example of recorded numbers of embryos (day 1) and L1 larvae (day 2) for the two plates shown in **Figure 5**. The "expected number" for the null hypothesis is determined by the number of embryos that hatched and became L1 larvae at the time of transfer to the food vial (table below). For wildtype flies, almost all embryos hatch and develop into L1 larvae. This may not be the case for other genetic backgrounds and this method is used to determine these differences in viability. Please click here to view a larger version of this figure.

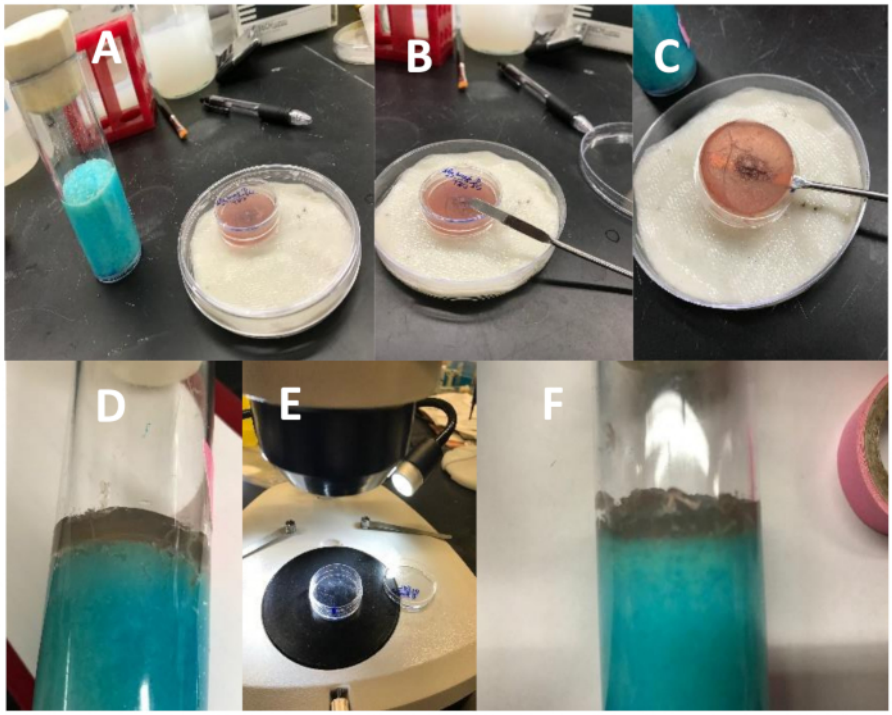


Figure 7: Transferring larvae. (**A**) After recording L1 counts, the grape-agar discs are carefully removed from the Petri dish using a clean (ethanol-wiped) spatula (**B**,**C**) and transferred L1 side-down to contact the food in the vials as shown in **D**. (**E**) The empty Petri dish is carefully inspected to detect any larvae left behind. If larvaee left behind are alive and can be transferred to the food vial, do so carefully and immediately (**F**). If larvae left behind are dead or cannot be transferred, record this fact to correct the "expected number" for subsequent calculations. Please click here to view a larger version of this figure.

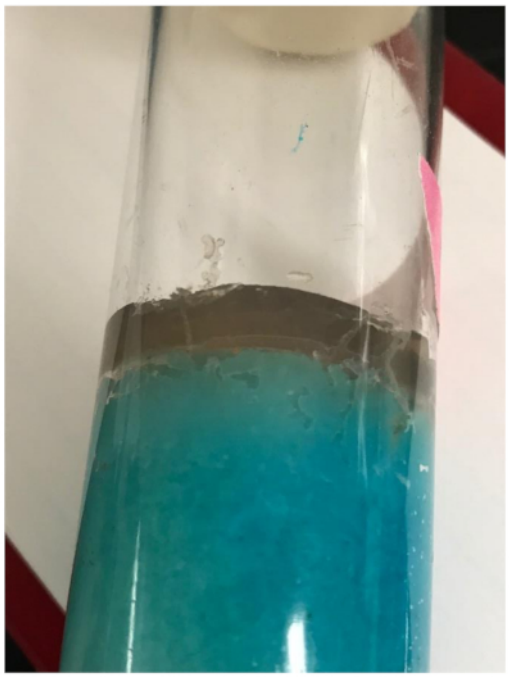


Figure 8: L2 larvae make their way into the food. See crevices and grooves between the grape-agar disc and the blue food. Set up a schedule to observe the vials daily, preferably at the same time of day. Please click here to view a larger version of this figure.

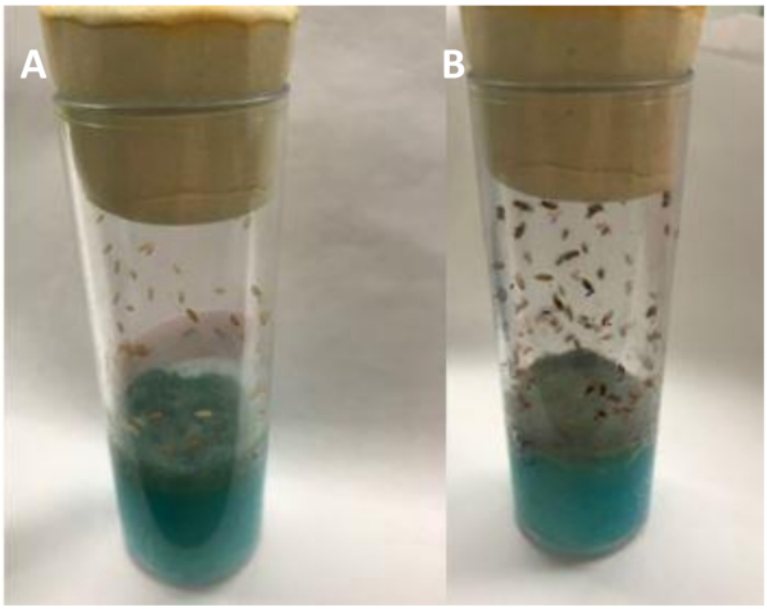


Figure 9: Adult emergence. (**A**) L3 larvae make their way out of the food to become pupae. (**B**) Adults start to emerge on day 11. Set up a schedule to observe the vials daily, preferably at the same time of day and carefully record your observations. Stop counting when all the pupae have emerged (count empty pupae cases) and avoid counting the next generation of flies by stopping the experiment on day 15 and count dead pupae if any (black/dried pupae). Mutants with lengthier life cycles may need longer periods of time, which need to be determined empirically. Please click here to view a larger version of this figure.

Supplemental Figures. Please click here to download these files.

Supplemental Materials. Please click here to download these files.

Discussion

In summary, this method provides an accurate and simple assessment of viability in *Drosophila*. The entire protocol takes approximately 14 days to complete. The procedure does not require expert technical skills; however, proper timing, a schedule of daily observations, and careful agar transferring is important for accuracy and reproducibility.

In addition to the placement of the grape-agar disc embryo-side down in the food vial, another crucial step in the procedure is transferring the agar disc to a food vial no later than 48 h after removing the grape agar plate from the embryo collection mini cages. Transferring the agar after 48 h resulted in embryo and larvae loss, likely due to dehydration of the agar disc. To count L2/L3 transitions, the plate needs to incubate for 72 h. If this stage is crucial, grape agar plates should be poured thicker and a humid chamber must be used to prevent desiccation. The crosses were set up in embryo collection cages that accommodate 35 mm plates; however, this procedure can be performed using larger embryo collection cages as well, such as one that accommodates 60 mm or 100 mm Petri plates. Food bottles must then be big enough to accommodate those sizes.

There are steps that can be added to this protocol. As mentioned above, L2/L3 transitions are challenging to quantify on plates due to the time required and the potential dehydration of the agar. Additionally, larvae become highly mobile on the surface of the agar, posing a challenge to accurately count them. Placing the plates in the refrigerator for 30 min or adding a few drops of a mild anesthetic (lidocaine solution) on the surface of the agar prior to counting L2/L3 larvae can help slow down their movements to count them more accurately. A caveat to these modifications is that they can introduce variables (cold sensitivity, anesthetic sensitivity) and confound the viability results. Even without these steps, by using colored food, researchers can quantify wandering L3s/prepupae as they settle on the side of the food vials to initiate pupation. A limitation to this method is that it requires the adults used in the crosses to survive being anesthetized with CO_2 for phenotyping to set up the crosses in the embryo collection cages. *Dm ime4* homozygous mutant males do not recover well and die a few hours after waking up from CO_2 treatment. Other methods to immobilize adults for sorting can be explored, such as placing vials in the refrigerator for a few minutes and then transfer vials to crushed ice to slow movement and sort quickly and efficiently.

Apart from comparing allelic strengths and their effects on viability, this method can be used to screen sensitivity or resistance to defined pharmaceutical compounds. Unlike other methods that screen compound toxicity in cell culture[15,16,17,18], this method uses whole organisms, making assessment of developmental effects easier to analyze. In sum, using this protocol and modifications therein, will allow to measure allelic strengths as well as the effects of environmental factors and chemical compounds on *Drosophila* viability, fecundity, fertility, lifespan, and duration of developmental cycle.

Disclosures

There are no conflicts of interest.

Acknowledgments

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