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High throughput preparation of fly genomic DNA in 96-well format using a paint-shaker

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Keywords: DNA extraction, genotyping, high throughput, homogenizer, paint shaker, PCR, sample homogenization, 96-well format

Abbreviations: MSG, Multiplexed Shotgun Genotyping; PCR, Polymerase Chain Reaction.

Sample homogenization is an essential step for genomic DNA extraction, with multiple downstream applications in Molecular Biology. Genotyping hundreds or thousands of samples requires an automation of this homogenization step, and high throughput homogenizer equipment currently costs 7000 euros or more. We present an apparatus for homogenization of individual *Drosophila* adult flies in 96-well micro-titer dishes, which was built from a small portable paint-shaker (F5 portable paint-shaker, Ushake). Single flies are disrupted in each well that contains extraction buffer and a 4-mm metal ball. Our apparatus can hold up to five 96-well micro-titer plates. Construction of the homogenizer apparatus takes about 3–4 days, and all equipment can be obtained from a home improvement store. The total material cost is approximately 700 euros including the paint-shaker. We tested the performance of our apparatus using the ZR-96 Quick-gDNA™ kit (Zymo Research) homogenization buffer and achieved nearly complete tissue homogenization after 15 minutes of shaking. PCR tests did not detect any cross contamination between samples of neighboring wells. We obtained on average 138 ng of genomic DNA per fly, and DNA quality was adequate for standard PCR applications. In principle, our tissue homogenizer can be used for isolation of DNA suitable for library production and high throughput genotyping by Multiplexed Shotgun Genotyping (MSG), as well as RNA isolation from single flies. The sample adapter can also hold and shake other items, such as centrifuge tubes (15–50 mL) or small bottles.

Introduction

Modern technologies now allow the sequencing of hundreds or even thousands of samples at once. *Drosophila* genetics and genomics studies often examine hundreds of samples, whether obtained from adult flies sampled in the wild (population genetics and association mapping studies) or from controlled laboratory crosses (quantitative trait locus mapping). Preparing sequencing libraries always involves a nucleic acid extraction step, which starts with tissue homogenization. Homogenizing adult fly tissues for high throughput studies can be done manually in 1.5-mL tubes one-by-one to maximize the yield of extracted genomic DNA, but this procedure is extremely laborious. Consequently, laboratory items have been developed to handle samples in 24-well, 96-well or 392-well format, and corresponding commercial homogenizers for high-throughput *Drosophila* adult fly disruption are now available (Tissue Lyser II from QIAGEN, Geno Grinder from Ops

diagnosticsc or FastPrep 96 from MP Biomedicals), but are relatively expensive (around 7000 euros or more). We have designed and built a device based on a portable paint-shaker that can homogenize single flies in 96-well plates. Our device can fit on a bench and achieve complete tissue homogenization. It allowed us to obtain DNA whose quantity and quality is adequate for standard PCR applications. A comparable homogenizer built from a paint-shaker has been described for isolating DNA from *Arabidopsis* tissues.¹ Compared to this previously designed apparatus, our homogenizer is more economical and smaller: it does not need to be attached on a wall and it is portable. We describe here the construction of the apparatus, especially the plate adapter that fits on the paint-shaker. We also examine the performance of our homogenizer for tissue homogenization and subsequent applications. Our device should be of interest to the fly community because it is simple to build and it is a cost-effective and efficient alternative to commercially available machines.

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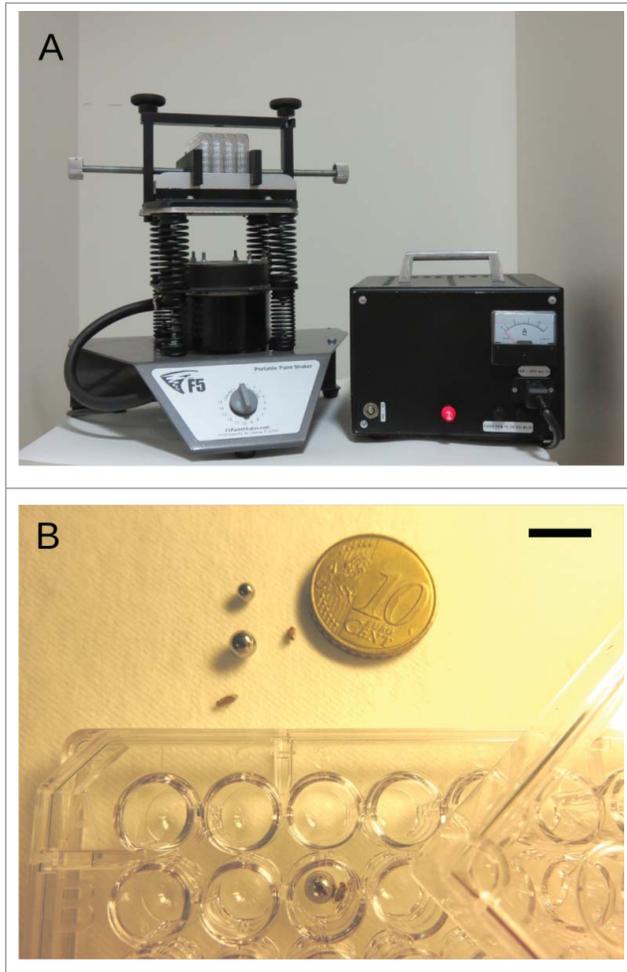


Figure 1. Picture of our homogenizer. (A) The paint-shaker, together with the plate adapter we built, is on the left and the transformer is on the right. Four 96-well plates are held within the paint-shaker in a vertical position. (B) Image of the 2 types of stainless steel balls that we used, next to a few *Drosophila* adult flies, within or outside the wells of a 96-well plate. Scale bar is 10 mm.

Results

Our device is composed of three elements: a paint-shaker, a transformer and a plate adapter. The plate adapter is firmly attached to the paint-shaker and can hold up to five 96-well plates (Fig. 1A). The transformer is necessary to lower the secondary voltage to 60 V. A complete description of the construction of the plate adapter and of the transformer can be found in the Materials and Methods section. For DNA extraction, we used adult flies that were kept at -20°C , and we used the ZR-96 Quick-gDNATM DNA extraction kit (Zymo Research). In each well of round bottom 96-well micro-titer dishes (3799 Corning Costar), we loaded extraction buffer and one 3.969-mm (5/32 inch) stainless steel ball (Fig. 1B). Single flies were then deposited in each well. The 96-well plates were sealed with a SMX-DW96 Silicon Sealing Mat (Phoenix) and were fitted inside our homogenizer device as indicated in Fig. 1A. While the

paint-shaker is in vibrating mode, stainless steel balls are expected to move rapidly within wells and thus disrupt fly tissues.

We observed that vertically oriented plates (as in Fig. 1A) yield better homogenates compared to horizontally placed plates. Indeed, when plates are oriented horizontally (with covers on top), the metal balls tend to rotate along the round walls in a horizontal orbit, and this has little effect on the fly, which remains at the bottom of the well. Thus, in all subsequent tests, plates were placed vertically within our homogenizer device (Fig. 1A).

To test for DNA extraction efficiency, we homogenized individual frozen *D. melanogaster* flies for 14 min in a 96-well plate that contained the lysis buffer and a single 3.969-mm ball. We then isolated genomic DNA according to the manufacturer protocol. The total amount of extracted DNA ranged between 10 ng and 318 ng, with an average of $138 \text{ ng} \pm 62 \text{ ng}$ (standard deviation, $n = 88$). The yield was lower but not significantly different from test extractions with flies homogenized manually one-by-one with the Quick-gDNATM DNA extraction kit (Zymo Research) (range: 90–280 ng, mean = $184 \text{ ng} \pm 66$ (standard deviation), $n = 10$, Welch Two Sample *t*-test, $t = -2.1027$, $df = 10.9$, $p > 0.059$).

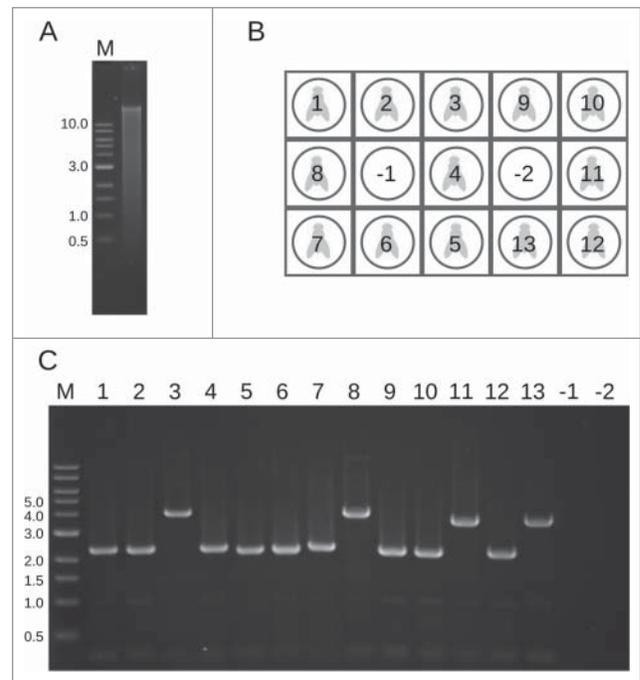


Figure 2. Quality control and cross contamination test. (A) Genomic DNA extracted with our homogenizer. M: 1 kb DNA Ladder (NEB) 0.5 $\mu\text{g}/\text{lane}$. (B) Schematic drawing of the arrangement of the samples in the 96-well plate. 1–13 indicate wells containing single fly specimen, –1 and –2 indicate wells devoid of flies. (C) PCR amplification products of *GAL4* inserts. PCR products are of expected size. 1: VT054796-*GAL4* 2275 bp, 2: VT054833-*GAL4* 2270 bp, 3: *GMR15E09-GAL4* 4029 bp, 4: VT054820-*GAL4* 2219 bp, 5: VT054795-*GAL4* 2188 bp, 6: VT054841-*GAL4* 2184 bp, 7: VT054829-*GAL4* 2288 bp, 8: *GMR15E09-GAL4* 4029 bp, 9: VT054824-*GAL4* 2202 bp, 10: VT054838-*GAL4* 2177 bp, 11: *GMR13B12-GAL4* 3586 bp, 12: VT054839-*GAL4* 2212 bp, 13: *GMR13B12-GAL4* 3586 bp. M: 1 kb DNA Ladder (NEB) 0.5 $\mu\text{g}/\text{lane}$.

We pooled ten single fly extractions and examined the size distribution of genomic DNA fragments on an agarose gel (Fig. 2A). We observed that genomic DNA was partially degraded into fragments that were smaller than 10 kb but that a substantial fraction of DNA was larger than 10 kb.

To evaluate the quality of our DNA isolated from single flies and to test for possible cross-contamination between samples during homogenization, we performed the following experiment. We placed *D. melanogaster* flies in the wells of a 96 well plate according to the arrangement depicted in Fig. 2B, with two wells devoid of adult flies as negative controls surrounded by wells containing a fly. Each individual fly carried a different enhancer-*GAL4* construct inserted at the same *attP2* site. We used oligonucleotides that annealed to the flanking regions of the inserted enhancers, such that PCR products should yield fragments of different size that range from 2 to 4 kb. Homogenization and DNA extraction was carried out as above. We then tested for the presence of *D. melanogaster* genomic DNA in each sample by PCR amplification of the *GAL4* enhancer fragment. We did not detect any amplification product in the negative controls (Fig. 2C). PCR amplification from each single fly DNA sample produced DNA fragments at the expected size and no double bands were detected. We conclude that no cross contamination occurred between wells and that our homogenizer device can generate DNA templates that are appropriate for PCR amplifications up to at least 4 kb.

Discussion

We constructed a tissue homogenizer that allows isolation of high molecular weight genomic DNA from single flies in 96-well format. We used oligonucleotides that annealed to the flanking regions of the inserted enhancers, such that PCR products should yield fragments of different size that range from 2 to 4 kb. Homogenization and DNA extraction was carried out as above. We then tested for the presence of *D. melanogaster* genomic DNA in each sample by PCR amplification of the *GAL4* enhancer fragment.

We did not detect any amplification product in the negative controls (Fig. 2C). PCR amplification from each single fly DNA sample produced DNA fragments at the expected size and no double bands were detected. We found that the amount of DNA obtained is satisfying and that the quality of the DNA is appropriate for PCR amplifications up to at least 3.9 kb. In principle, our homogenizer can also be used for other applications, such as

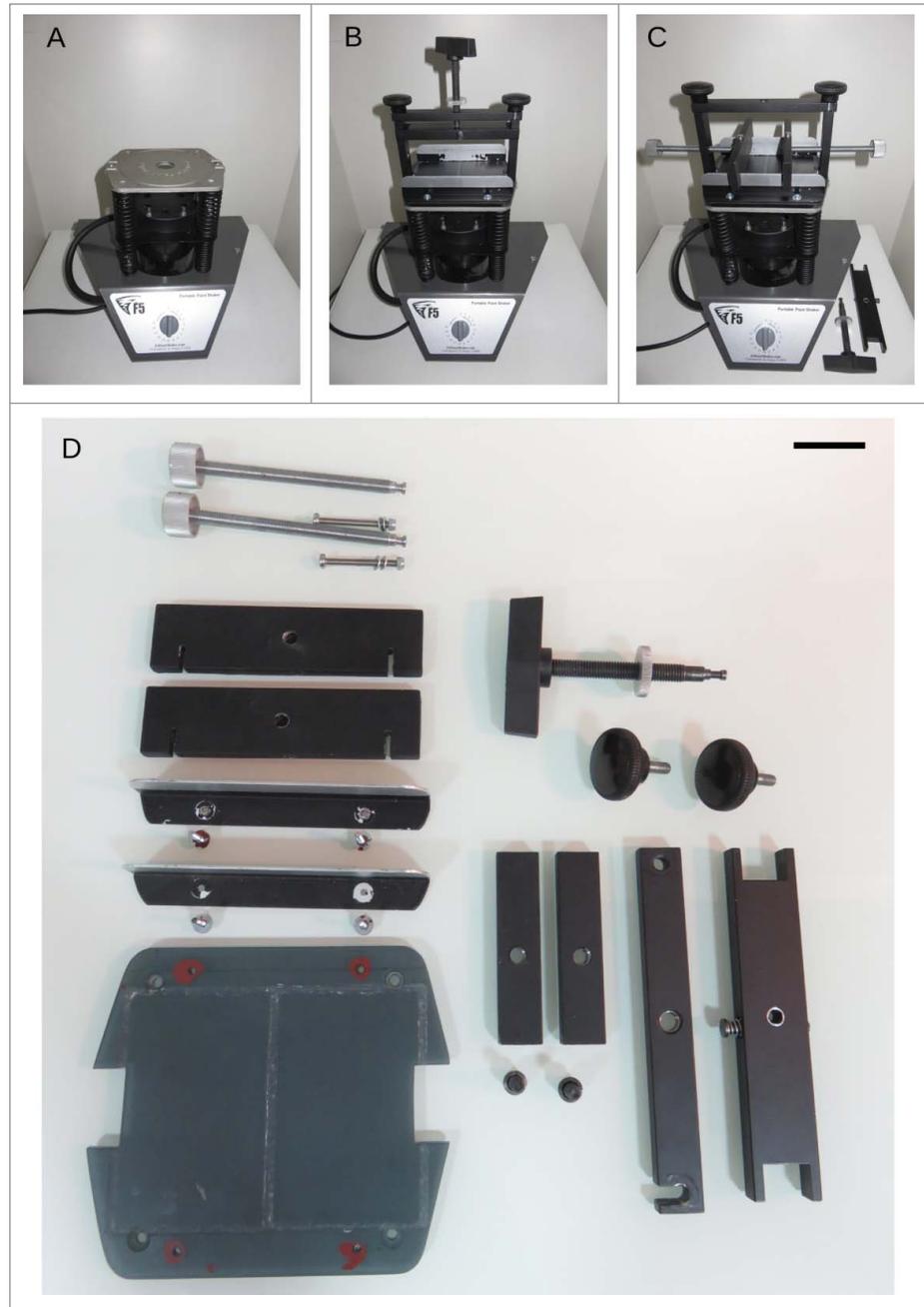


Figure 3. Pictures of the step-by-step construction of the homogenizer. (A-C) Pictures of the paint-shaker at 3 successive stages during the building process: (A) Paint Shaker F5 (Ushake) without the plate adapter, (B) with the horizontal clamp and (C) with the 2 vertical clamps assembled, and parts of the horizontal clamp on the right side on the table. (D) Pictures of the different parts of the plate adapter (See Fig. 5 for details). Scale bar is 5 cm.

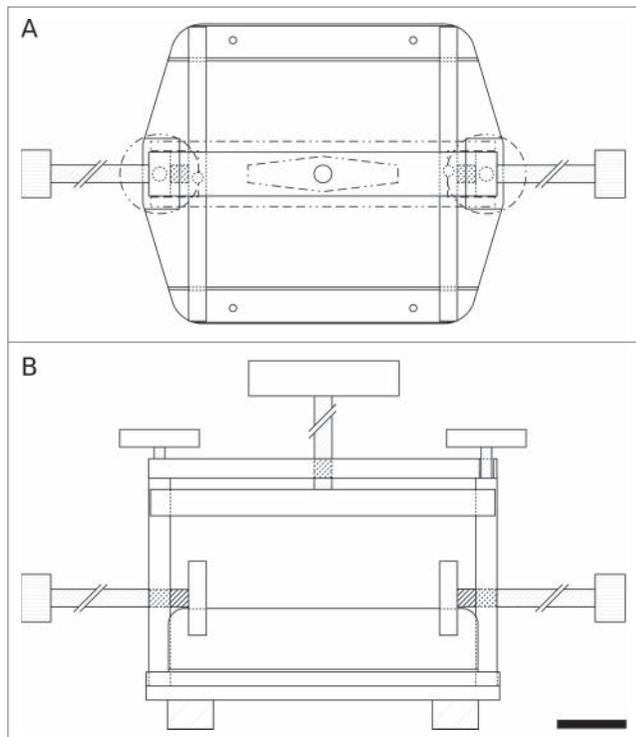


Figure 4. Engineering drawing of the assembled plate adapter. (A) Top view. (B) Side view. Scale bar is 40 mm. Straight lines indicate visible objects. Fine-dashed lines represent outlines of hidden objects. Coarse-dashed lines outline the upper clamp that can optionally be removed from the holder.

Multiplexed Shotgun Genotyping (MSG)^{2,3} or RNA extraction. A homogenizer built from a relatively voluminous paint-shaker (13×14×25," 45 kg) has already been reported for *Arabidopsis* plant tissue.¹ We show here that a smaller paint-shaker (16×13×13," 10 kg) can be used to construct a homogenizer for *Drosophila* flies. The total material cost was approximately 700 euros, including the paint-shaker. Our device is thus more affordable than commercial alternatives. Its construction takes approximately three to four days and requires fine mechanics equipment that can be obtained at a conventional home improvement store. In principle, any similar paint-shaker apparatus could be used to construct a high-throughput tissue grinder. Although we have only used our device for *Drosophila* adult flies, it should be easily adapted for homogenization of other types of tissues. The sample adapter can also hold other items such as 200 mL bottles or 50 mL centrifuge tubes to mix any liquids or suspensions. It is thus a versatile tool for a variety of applications in the laboratory.

Materials and Methods

Construction of the plate adapter

The F5 Paint Shaker (Ushake) has an adjustable horizontal clamp that can fit a wide variety of containers both in size and

shape. However, the clamp had to be modified so that it could hold 96-well micro-titer plates securely in place during shaking (Fig. 3A-C).

For holding plates horizontally, the two vertical aluminum bars that hold the 37 mm × 195 mm × 15 mm upper horizontal clamp were shortened to a height of 110 mm. New 7-mm threaded holes were then drilled in the center of each bar so that bars could be reconnected to the aluminum ground plate with 7-mm screws (Figs. 3, 4 and 5). A 8-mm thick Plexiglas plate (Fig. 5C) was also fitted and screwed onto the aluminum platform to provide a flat surface. For this, the screws that were holding the container-platform were replaced by longer 40 mm × 4 mm counter-sunk screws that locked in place the Plexiglas plate and the aluminum platform onto the springs of the shaker with self-locking screw-nuts. All screws were set in place with UHU Schrauben Sicher tamper screw seal. The outlines of two adjacent 96-well plates were carved at 1 mm depth into the Plexiglas surface using a Dremel 3000 Rotary Tool Work Station (Dremel) with a 5 mm round grinding head (item 7 in Fig. 5C). The resulting adapter (Fig. 4A and B) can hold up to 10 96-well plates (two stacks of five plates) in horizontal orientation.

A support for vertically oriented plates was also built (Figs. 1, 4A and B). Two aluminum L-profiles (175 mm × 35 mm × 20 mm, 2 mm thick, item 6 in Fig. 5A) were screwed onto the Plexiglas ground plate (item 7 in Fig. 5C) at a distance of 128 mm, with the 20 mm × 175 mm sides facing down and outwards (Figs. 1, 5A and B). This provided a lateral boundary for holding the 96-well plates tightly in place. To fix the L-profiles, two 3-mm holes were bored into each L-profile and at corresponding positions into the Plexiglas plate. A 3-mm thread was also bored into the aluminum platform to hold the 3 mm × 20 mm screws fixing the L-profiles. The edges of the L-profiles were rounded to avoid potential accidents during shaking. Again, all screws were secured with tamper screw seal.

The L-profiles also support two adjustable vertical clamps that can hold the 96-well plates in vertical orientation from both sides (Figs. 1, 4A and B). To build the two adjustable vertical clamps, we used 167 × 42 × 10 mm aluminum bars (item 5 in Fig. 5A). Two symmetric 5 mm × 15 mm cuts were sawed into the bottom of the aluminum bars at a distance of 128 mm from each other and a 10-mm hole was drilled into the center of the flat 42 mm × 167 mm side. Another 4-mm hole was then drilled vertically into the 10 mm × 176 mm side, in the center at 87.5 mm distance from one end (dashed lines on item 5 in Fig. 5A). These holes were used to connect the aluminum bar to a 10 mm × 150 mm threaded rod (item 2 in Fig. 5A) that allows the distance of the clamp to be adjusted along the horizontal axis (through rotation of the threaded rod). These vertical clamps (items 1–5 in Fig. 5A and item 11 in Fig. 5D) were built in a configuration similar to the one of the horizontal upper clamp provided by Ushake (items 8–14 in Fig. 5D). First, a 10 mm threaded hole was bored into each vertical bar of the clamp holder (item 11 in Fig. 5D) centrally and at a 50-mm distance from the aluminum ground-plate. Two threaded rods (item 2 in Fig. 5A) were later screwed through these 2 holes. One tip

of both threaded rods (item 2 in Fig. 5A), of length of 10 mm, was thinned using an ordinary metal file and a drill press until it reached a diameter of 8 mm (item 5 in Fig. 5A). In addition, part of the 8-mm tip (4.5 mm long) was thinned further to a final diameter of 5 mm, at a distance of 2 mm from the distal end (item 2 in Fig. 5A). This form serves to lock the threaded rod into the aluminum bar of the vertical clamp. It was locked within the bar with a 4 mm × 50 mm screw, while enabling its free rotation. Finally, a knob (in our case we used buttons of an old Revox tuner) was added to the end of both rods and a rubber mat was glued onto the vertical clamps on the side that contacts the 96-well plates.

Since the vertical clamps block the adjustment of the horizontal clamp, either they must be removed or a stack of up to four empty 96-well plates should be used in the bottom part of the plate adapter when using the upper horizontal clamp (for plates in the horizontal orientation). The vertical clamps (for plates in the vertical orientation) can be used without removing the upper horizontal clamp.

Optimization of the paint-shaker power supply and heat reduction

The F5 portable paint-shaker (Ushake) is made for 120V/60Hz power supply. We used a 230V/120V (500VA, 2,17A) transformer (ELMA TT, #cat IZ 76) to connect the paint-shaker to the 230V/50Hz European mains supply (Figs. 1 and 6). Since we noted a significant heating of the motor after 10 min of use, we first reduced the secondary potential to approximately 60 Volts with 2 16 Ohm adjustable resistances connected in series. Finally, we replaced the transformer and the resistances by a toroidal 230V/60V (225VA, 3,75A) transformer (Nuvotem Talema 0225P1-2-030) (Fig. 6). In addition, eight 10-mm holes were drilled into the lid of the paint-shaker motor case to enhance heat release. This allows the apparatus to shake for at least 30 min without

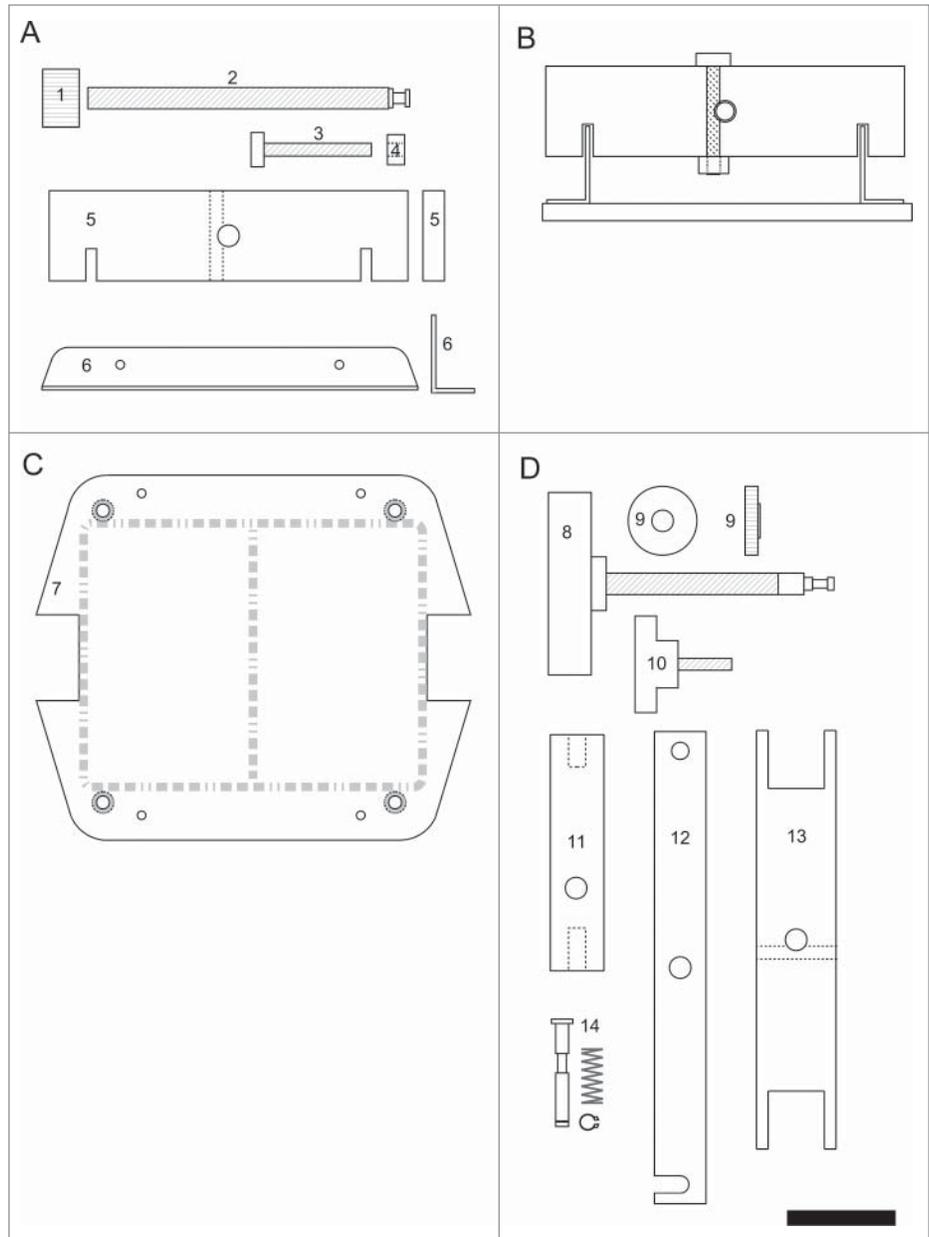


Figure 5. Engineering drawing of the different parts of the plate adapter. (A) Lateral clamp. Two of each represented item were prepared. 1: aluminum knob from a Revox tuner, 2: 10 mm × 150 mm threaded rod with a modified tip (see Material and Methods), 3: 4 mm × 50 mm screw to lock the threaded rod (2) into the vertical clamp (5), 4: Screw-nut 4 mm, 5: 167 × 42 × 10 mm aluminum bar that is part of the lateral vertical clamp (lateral profile is shown on the right), 6: Aluminum L-profile 175 mm × 35 mm × 20 mm, 2 mm thick (lateral profile is shown on the right). (B) Pieces of the lateral vertical clamp assembled on the shaker. (C) 7: Plexiglas ground-plate, 8-mm thick. Gray dashed lines indicate the outline of 2 sample plates that are carved into the upper surface of the plate. (D) The modified container holder support (provided by Ushake). 8: Threaded rod with knob, unmodified (Ushake); 9: Flat knurled nut, unmodified (Ushake), to block the rod of the upper clamp; 10: Threaded rod 7 mm × 20 mm, unmodified (Ushake) to assemble the horizontal clamp frame; 11: Lateral vertical bars (Ushake) shortened to 110 mm. 12: Upper horizontal support that holds the upper clamp, unmodified (Ushake), 13: Upper horizontal bar of the clamp, unmodified (Ushake). 14: Locking device that connects the threaded rod (8) with the upper bar of the clamp (13), unmodified (Ushake). It consists of a cylinder, a 25 mm × 10 mm spring and a 7 mm snap ring. Scale bar is 50 mm.

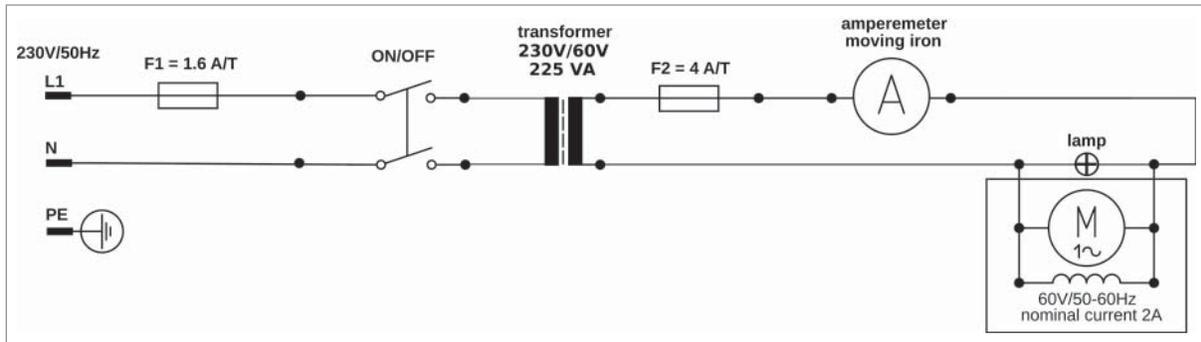


Figure 6. Electric circuit of the transformer, designed for 230V/50Hz mains supply. F1: fuse 1.6 A (T = slow); transformer 230 V / 60 V secondary potential, 225 VA, 375 A. F2: fuse 4 A (T = slow); M: motor of the paint-shaker; L1: Phase; N: Null; PE: Grounding.

overheating. We recommend for future designs to use a 230V/60V transformer instead of the resistances and the 230V/110V transformer. Please note that there is no possibility to adjust the speed of the F5 portable paint-shaker. Our movie recordings suggest that this paint-shaker rotates at about 200 rotations per minute.

Fly stocks

Transgenic lines *GMR15E09-GAL4* (FBsf0000162107) and *GMR13B12-GAL4* (FBsf0000161892) were obtained from Janelia Farm. Transgenic lines *VT054796-GAL4* (FBsf0000447620), *VT054833-GAL4* (FBsf0000447639), *VT054820-GAL4* (FBsf0000450570), *VT054795-GAL4* (FBsf0000453628), *VT054841-GAL4* (FBsf0000447646), *VT054829-GAL4* (FBsf0000447638), *VT054824-GAL4* (FBsf0000447633), *VT054838-GAL4* (FBsf0000447643) and *VT054839-GAL4* (FBsf0000447644) were obtained from the Vienna Drosophila Research Center. These lines harbor a *pBPGUw GAL4* enhancer construct inserted at a $\Phi 31$ integration site at position 3L:68A4.⁴⁻⁶

Sample homogenization and DNA extraction

For homogenization tests, flies stored in ethanol were used and re-hydrated for 10 min in H₂O prior to homogenization. We tested stainless steel balls which had a diameter of 2.8 mm (440C, Ops Diagnostics, cat#GBSS089-1000-07) and 3.969 mm (NO I 5/32 inch, Mat. 1.4401, Kugel-Winnie, cat#010402016-0007). The latter are bearing balls of non-hardened, non-magnetic but corrosive resistant steel. We obtained complete homogenization after 14 min of shaking with addition of one 3.969-mm (5/32 inch) ball per well and with addition of two 2.8-mm balls per well. In contrast, we found that with a single 2.8-mm ball flies remained largely intact, even after 20 min of shaking.

For fly genomic DNA extraction, we used flies stored at -20°C . One 3.969-mm (5/32 inch) ball was added into each well of a 96-well micro-titer plate 3799 (Corning Costar), followed by addition of 100 μL of ZR-96 Quick-gDNATM DNA extraction kit lysis Buffer (Zymo Research, cat#D3011). A fly was added to each well and plates were sealed with a SMX-DW96 Sealing Mat (Phoenix, cat#SMX-

DW96) or an adhesive PCR sealing film (Platemax, cat#PCR-TS). During tests we observed that the Lysis buffer can dissolve the glue of adhesive sealing films, and thus cause leaking and cross-contamination. It is therefore recommendable to use SMX-DW96 Sealing Mats. The plate was mounted in vertical orientation and grinding was carried out for 2×7 min. We added an empty 96-well micro-titer plate on each side of our sample plate in order to better distribute the mechanical pressure of the vertical clamps. The sample plate was then centrifuged for 30 sec at 1000g, the sealing mat was removed and the homogenate of each well was transferred to a 96-well Silicon-ATM Plate of the ZR-96 Quick-gDNATM DNA extraction kit (Zymo Research, cat#D3011). Genomic DNA extraction was then carried out following the manufacturer's instructions. Sample DNA was recovered from the plate with 50 μL of DNA Elution Buffer. The amount of genomic DNA was quantified with the Qubit 2.0 Fluorometer (Life Technologies) and the HS detection kit (Life Technologies).

Examination of genomic DNA quality

To visualize genomic DNA on an agarose gel, we pooled and concentrated 10 single fly extractions by addition of 0.1 volumes of 3M Na Acetate (pH 5.2) and overnight precipitation of DNA at -20°C in 2.2 volumes of 100% ethanol. DNA was pelleted by centrifugation for 20 min at 20,000g, at 4°C . Pellets were washed in 500 μL of 70% ethanol, followed by an additional centrifugation step at 20,000g for 5 min. Pellets were dried and re-suspended in 20 μL of TE Buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Samples were treated with 0.5 μL of 10 mg/mL RNase A (Roche) for 10 min at room temperature. DNA concentrations were estimated using the Qubit 2.0 Fluorometer (life technologies) and DNA was run on a 0.8% agarose electrophoresis gel. DNA was stained by bathing the gel for 30 min in 0.01 μL / mL of Sight DNA Stain (Euromedex). Gels were then destained by incubation for 1 h in distilled water at 50 rpm and DNA was examined on a UV-transilluminator Ebox VX2 (Vilber-Lourmat).

PCR tests

For amplification of inserts from the enhancer-*GAL4* lines we used primers that matched the flanking regions of the construct vector *pBPGUw*. An oligonucleotide was designed with Primer3 (Hosted Tools, www.justbio.com)⁷ in proximity of the vector attR2 site 5'-ATACCGCTGCGCTCGATC-3' and was paired with oligonucleotide attB1 5'-ACAAGTTTGTACAAAAAGCAGGCT-3'⁴ that is specific to the attR site flanking the opposite site of the insert. The amplicon was expected to be approximately 3.1 – 4 kb depending on the enhancer-*GAL4* line. PCR was performed in 25 μ L of reaction volume using 0.5 Units of Q5[®] High Fidelity Polymerase (NEB), 2 μ L of gDNA, 1X Q5 Reaction Buffer (NEB), 0.2 mM of each dNTP (NEB) and 0.5 μ M of each oligonucleotide. Thermocycling conditions were set to a hot start at 98°C for 30 sec, followed by 35 cycles of 10 sec at 98°C, 30 sec at 66°C, and 3 min at 72°C, with a final extension step of 8 min at 72°C. PCR products were run on a 0.8% agarose gel in 1X TAE Buffer stained with Sight DNA Stain (Euromedex) and visualized with a UV-transilluminator Ebox VX2 (Vilber-Lourmat) as described above.

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Disclosure of Potential Conflicts of Interest

The authors have no conflict of interest. The described apparatus has not been commercialized and no income has been generated.

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