Set screw homogenization of murine ocular tissue, including the whole eye

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Purpose: To compare methods for homogenizing the mouse whole eye or retina for RNA extraction.
Methods: We tested five homogenization techniques for the whole eye and the retina. Two established shearing techniques were a version of the Potter-Elvehjem homogenizer, which uses a plastic pellet pestle in a microfuge tube, and a Dounce homogenizer. Two modern bead-beating methods used commercially manufactured devices, the Next Advance Bullet Blender and the Qiagen TissueLyser LT. The last method involved vortex mixing multiple samples simultaneously in a buffer containing a stainless-steel set screw, a novel approach. RNA was extracted from the tissue after each technique was used. Degradation of RNA was measured with the RNA integrity number (RIN score) after electrophoresis on an Agilent BioAnalyzer RNA LabChip. Nucleic acid yields were measured with ultraviolet (UV) spectroscopy in a BioTek Synergy H1 Hybrid plate reader. The purity of the nucleic acids was assessed with the mean absorbance ratio (A_{260}/A_{280}). The preparation time per sample was measured with a digital stopwatch. Costs of necessary consumables were calculated per ten samples.
Results: The RIN scores for all homogenization methods and both tissue types ranged from 7.75±0.64 to 8.78±0.18; none were statistically significantly different. The total RNA yield per whole eye from the bead-based methods ranged from 7,700 to 9,800 ng and from 3,000 to 4,600 ng for the pellet pestle and Dounce shearing methods, respectively. The total RNA yield per retina from the bead-based methods ranged from 4,600 to 8,400 ng and from 2,200 to 7,400 ng for the pellet pestle and Dounce shearing methods, respectively. Homogenization was faster using the bead-based methods (about 15 min for ten samples) because multiple samples could be run simultaneously compared to the shearing methods that require samples be homogenized individually (about 45–60 min per ten samples). The costs in consumables for the methods tested ranged from $2.60 to $14.70 per ten samples. The major differences in overall costs come in the form of one-time equipment purchases, which can range from one hundred to thousands of dollars. The bead-based methods required less technician involvement and had less potential for sample contamination than the shearing methods.
Conclusions: The purity and quality of RNA were similar across all methods for both tissue types. The novel set screw method and the two bead-based methods (bullet blender and TissueLyser) outperformed the two shearing methods (the pellet pestle and Dounce techniques) in total RNA yields for the whole eye. Although the bullet blender, TissueLyser, and set screw methods produced comparable levels of RNA yield, purity, and quality, the set screw method was less expensive. Researchers seeking the efficiency of sophisticated bead homogenization equipment without the high equipment costs might consider this novel method.

Preliminary steps for preparing tissue samples for subsequent analysis are often crucial in obtaining reliable, good-quality data. One such preliminary step in the biochemical analysis of a sample is homogenization to break down and disperse a heterogeneous tissue or organ into a uniform colloid or suspension. Homogenization is commonly done in conjunction with a lysis step to dissolve or solubilize cells and tissues using potent denaturants, detergents, or hypotonic conditions. This opens a cell and frees the contents of the subcellular compartments, preparing the sample for an extraction step to isolate various classes of molecules. Many devices and approaches are used for homogenization. The need for such variety is partly due to differences among tissues, the analytes that are to be measured, the size of the sample, and the number of samples to be processed.

Proper homogenization of samples can lead to uniform isolation of downstream analytes, such as protein or RNA. Additionally, automation of this step standardizes the time needed per sample, as well as negates differences in personnel training and technique, which, in turn, leads to accurate measurements and comparisons and increases reproducibility across experiments and laboratories. Although numerous techniques for homogenization exist, they vary in efficiency,

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ease, and cost and have not been systemically compared and evaluated.

We sought an inexpensive, fast, and easy method for simultaneously homogenizing individual mouse eyes or retinas. Whole eye samples are easy to collect and ensure that an entire organ with complete sampling that includes tissues that are difficult to dissect (e.g., complete RPE sheet, trabecular meshwork, and ciliary body) is collected and offers a complete, if global, isolation of RNA. We chose five different techniques to test based on several criteria. These criteria excluded many types of homogenization methods and devices (some are listed in Table 1). All of the techniques were previously used in our laboratory; thus, they were readily available and were suitable for whole mouse eyes and small volumes of liquids. Two classical methods are the glass-on-glass manual-powered Dounce homogenizer [1,2] and the Potter-Elvehjem [3] homogenizer, which employs a motor-driven, disposable, polypropylene pellet pestle or a bead or ball mill (often called a bead beater). Two other methods included commercial bead homogenization equipment that were available to us: the Bullet Blender from Next Advance and the TissueLyser LT from Qiagen. Additionally, we developed a potential alternative technique that used the same concept as the bead beating approach. This method homogenizes a sample in a single screw cap plastic tube using a commercially available adaptor for a vortex mixer designed for hands-free tube shaking and a single set screw as a heavy bead. The set screw is massive and dense compared to a mouse eye, and we predicted that the threaded surface would macerate, gouge, and cut tissue during vortex mixing, resulting in homogenization.

METHODS

Mouse care: Mouse care and manipulations were conducted according to the ARVO Statement for Use of Animals in Research and were approved by the Emory University Institutional Animal Care and Use Committee (IACUC). The whole-eye experiments were conducted using mice from our breeding program of mixed strains and genotypes. Experiments in which isolated retinas alone were analyzed used BALB/c mice. Ages ranged from postnatal day (P) 22 to 120. Mouse housing conditions and diet were described in Johnson et al. [4]. Briefly, mice were housed at 23 °C in facilities managed by the Emory University Division of Animal Resources and given standard mouse chow (Lab Diet 5001; PMI Nutrition Inc., LLC, Brentwood, MO) and water ad libitum. They were maintained on a 12 h:12 h light-dark cycle, with daytime lighting ranging 200–750 lm outside the cage depending on lower, middle, or top shelf position of the cage rack.

Collection and storage: Whole eyes were collected and frozen on several different days. Retinas were collected and frozen all on one day. All samples were stored at −80 °C.

Dissection: Mice were killed by asphyxiation via exposure to CO_2 for 5 min before the eyes were harvested and homogenized. Whole eyes were enucleated and submerged in 50 µl of 160 U/ml RiboLock RNase inhibitor in 1X HBSS (EO0381; ThermoFisher Scientific, Waltham, MA), immediately frozen in dry ice, and stored at −80 °C. The retinas were immediately dissected out of the eye [5] and frozen in 50 µl of RiboLock. The tubes used for collection were chosen based on the method of homogenization being tested (described below). Both eyes or retinas were harvested from each mouse and collected in separate tubes. No experimental group contained two eyes or retinas from the same mouse. Unless otherwise noted, before homogenization, the samples were thawed, and 350 µl of RLT Lysis Buffer from a Qiagen RNeasy mini kit was added to each tube (Cat. no. 74,106; Qiagen, Hilden, Germany). The composition of RLT Buffer is proprietary. No β-mercaptoethanol was added as RNase inhibition was already accomplished after the eyes were harvested with the use of RiboLock. After the buffer was added, the samples were homogenized according to one of the methods described below, and then the homogenate was frozen and stored at −80 °C unless otherwise noted.

Dounce homogenization: After harvesting, the sample was placed in a 2.0 ml screw cap microfuge tube. Eyes or retinas were thawed with 350 µl RLT buffer and poured into a clean 2 ml Dounce homogenizer (Kontes Glass Co, Vineland, NJ). The tissue was sheared with even strokes of the B pestle for 60 s for the retinas or 3 min for the whole eyes. These times were determined based on the amount of time required for the sample to be either visibly uniform or maximally dispersed without concern about splashing, spills, or glass breakage due to speed. The sample was then removed and returned to its original tube using a pipette with a gel-loading tip to reach the bottom of the homogenizer tube and not touch the pipet to the side.

Plastic pestle method: A Kimble-Kontes plastic pestle kit (K749520−0500; ThermoFisher), which included pestles and tubes, was used. After collection, the thawed sample was placed in the Kimble-Kontes microcentrifuge tube, and a plastic pestle driven with a stationary laboratory mixer (Omni-Mixer 17,150; Sorvall, Inc., Newtown, CT) was used to homogenize the tissues. The homogenization time was 30−60 s per retina and 90 s per whole eye at a speed setting of 2.5.

Qiagen TissueLyser LT: Samples were collected in Safe-Lock microcentrifuge tubes (EP-022363433; Eppendorf, Hamburg,

TABLE 1. CLASSIC COMMON HOMOGENIZATION METHODS.

Method	References	Principle	Volume	Cell types	Comments	Equipment cost
Ottawa Sand	[6]; patent by DuPont for sand milling US 2855156 A	Sand milling and grinding	ml	Mammalian tissues	Particulates may carry through; messy	Negligible
Thomas or Potter-Elvehjem	[3]	Shearing by motor-ized pestle	ml	Mammalian tissues	Slow, one sample at a time; heating	Homogenizers $100-$200; Motor, $thousands
Aminco-French pressure cells	[7]	High shear by passing through a narrow orifice	50+ ml	Even suspensions of dilute bacteria	Complicated, expensive, large volumes, susceptible to clogging and fouling, one sample at a time	$tens of thousands
Stomacher	[8]	Kneading	100+ ml to liters	Detaching bacteria from biofilms, plant matter, pathologic tissue	One large sample at a time, slow	$thousands
Waring blender	[1]	Chopping with high speed rotating blades	30 ml to liters	Mammalian tissues	One sample at a time, large volume, heating	200
Freeze-thawing or freeze-crush, also called cryo-impacting	[9,10]	Pulverization; shattering on freeze planes	< 1 gram per sample	Mammalian tissues	Good for multiple and small samples; slow, tedious; requires much dry ice or LN_2; difficult to keep the samples dry; difficult recovery of crushed tissues; risk of spillage; messy	negligible
Bead-beating	[11,12]	Tissues are squashed and pulverized between much more massive and hard beads	< 1 gram per sample	Mammalian tissues	Multiple beads can be damaged in colliding with each other fracturing the beads often glass, zirconium, or silica. Ceramic and stainless steel are more durable. Temperature climbs rapidly with bead beating up to 10 °C per minute	$thousands
Sonication	Weaver, C.E. US patent: US2163650 -1939	Ultrasound generates microcavitation bubbles that explode in tissues dispersing them	< 1 gram per sample	Mammalian tissues	efficient, but slow; one or few samples at a time; cross contamination, oxidation; heating a major problem	$thousands
Double ended syringe needles (micro-emulsifying needles)		High shear by passing through a narrow orifice	< 1 gram per sample	Only suspensions of cells	Sclera will not pass through an 18 gauge needle. Clogging and fouling, slow, difficult, but manual	100
Centrifugation through succes-sively smaller holes	[13]	High shear by passing through a narrow orifice	< 1 gram per sample	Soft tissues (brain, liver); suspensions cultured cells; blood cells.	Prone to clogs	$Negligible

Method	References	Principle	Volume	Cell types	Comments	Equipment cost
The Willems polytron with a rotor-stator and similar devices	US patents 2789800 A, 2541221 A	Chopping with high speed rotating blades	1-10 grams per sample	Mammalian tissues	Very efficient, difficult to clean	$Thousands
Dissolved Nitrogen gas decompression Aka the Parr Bomb	[14,15].	Dissolved N_2 produces gas bubbles on rapid decompression that explode in tissues dispersing them	< 1 gram per sample	Mammalian tissues	Efficient; difficult to clean; Slow	$Thousands
Dounce	[1,2]	High shear by passing between a glass pestle and a glass cylinder	<10 grams	Soft tissues	difficult to clean; Slow; messy; manual	100

Germany). One 4.7 mm ferric ball bearing (4RJH8; Grainger, Lake Forrest, IL) was placed in each tube containing the thawed samples. The tubes were put in the tube adaptor of the Qiagen TissueLyser LT. The samples were homogenized at 50 Hz for 5 min (retina) or 10 min (whole eye).

Next advance bullet blender: After harvesting, the samples were placed in Axygen 1.5 ml conical screw cap tubes (SCT150OS; ThermoFisher). The bullet blender requires specifically shaped tubes. Improper tubes shatter in the machine. One approximate 0.02 ml scoop of 0.9–2.0 mm stainless steel beads (SSB14B-RNA; Next Advance, Averill Park, NY) was added to each tube containing thawed samples. The tubes were placed in the bullet blender. Samples were homogenized on speed 8 for 5 min (retinas) or 10 min (whole eyes).

Novel set screw method: Upon harvest, the sample was placed into a 2.0 ml screw cap microfuge tube (16466-042 VWR

Scientific, Radnor, PA). One stainless steel set screw with 10/24 threads, ~0.19 inch diameter, and 1/4 inch long (Cat no. 5MMT1, Grainger, Lake Forest, IL) was added to tubes containing the thawed samples. Tubes were placed on a vortex adapter that holds 1.5-2.0 ml tubes (13000-V1-24 MO BIO Laboratories Inc., Carlsbad, CA) and the vortex mixer was turned on. The orientation of the tubes in the vortex adapter is important; please refer to Figure 1 for correct setup. Samples were homogenized on the vortex mixer at maximum speed for 5 min (retina) or 10 min (whole eye).

Isolation of RNA from homogenates: The RNA extractions were performed by experimental group using the automated QIAcube (Cat number: 9001292; Qiagen) with an RNeasy Mini Kit (Cat number: 74,116; Qiagen). Homogenates were stored at −80 °C until processing (no more than 4 days between freezing and extraction). The samples were thawed and centrifuged at 10,000 ×*g* for 30 s to remove
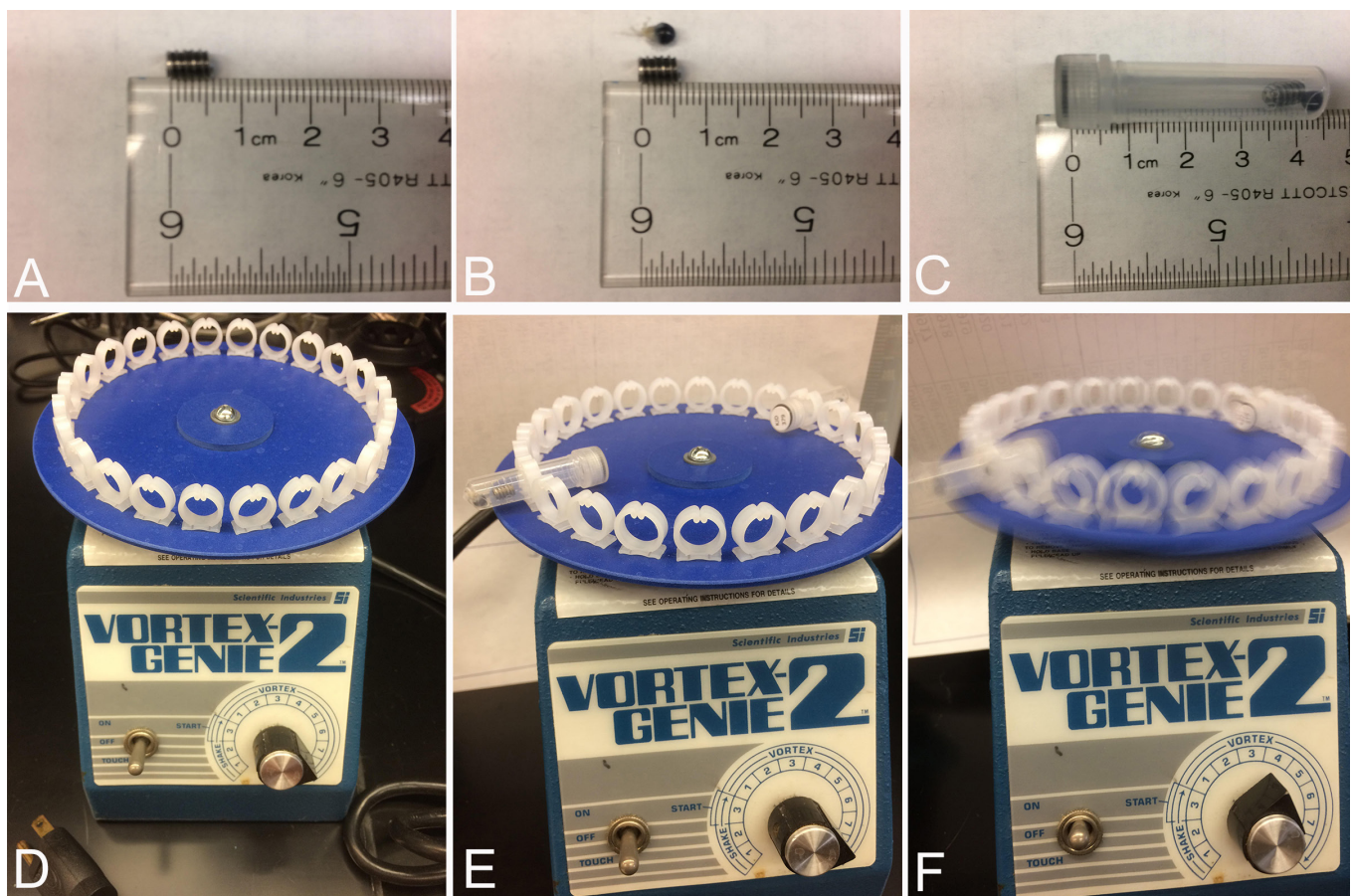

Figure 1. Setup of the set screw homogenization technique. The upper row of panels depicts the relative sizes of the set screw alone (**A**), the set screw compared to an adult mouse eye (**B**), and a 1.5 ml tube containing a screw set and an adult mouse eye (sample). The lower row of panels depicts the vortex mixer and the adaptor alone (**D**), with two samples not running (**E**), and with two samples running (**F**). Note the orientation of the sample tube in the vortex mixer adaptor. The lid of the 1.5 ml tube should face the center of the adaptor to ensure that the sample remains in the adaptor.

TABLE 2. YIELD OF RNA BY DIFFERENT HOMOGENIZATION METHODS.					
Tissue type	Set screw	TissueLyser	Bullet blender	Pellet pestle	Dounce
Retina	4605 ± 1076	8345 ± 1450	5600 ± 481	2245 ± 452	7380 ± 994
Whole eye	7724 ± 2753	9784 ± 1779	8347 ± 834	2982 ± 1001	4584 ± 2103

Values are mean total ng in 40 µl eluent ± SD, n=8 for all retina measurements, n=10 for whole eyes except the pellet pestle (n=9). ANOVA multiple comparison analysis compared the mean of each technique within a tissue type (retina or whole eye) with the mean of every other technique for the same tissue. A full list of comparisons with p values is given in Appendix 1 and Appendix 2.

any homogenate from the lid. The samples from the bullet blender or pellet pestle groups were transferred into Safe-Lock microcentrifuge tubes (Cat. no. EP-022363433; Eppendorf) for compatibility with the QIAcube. Set screws or ball bearings for the TissueLyser were then removed with a magnet. The QIAcube extracted RNA simultaneously from up to 12 samples on each run. Before the quality analysis, a DNase digest was performed on all the samples using the TURBO DNA-*free*™ kit (AM1907; Invitrogen, Carlsbad, CA) according to the kit's included protocol.

RNA was analyzed for the mean absorbance ratio (A_{260}/A_{280}) with ultraviolet (UV) spectroscopy using a Synergy H1 plate reader (BioTek, Winooski, VT). A 1 µg aliquot was subjected to electrophoresis on an Agilent 2100 Bioanalyzer (G2940CA; Agilent Technologies, Santa Clara, CA), and electropherograms and RIN scores were obtained. Bioanalyzer analysis was performed by the Emory Integrated Genomics Core.

Statistical analysis: Data from each group are summarized as the mean ± standard deviation (SD) from at least eight different animals, as indicated in each table. Analysis was conducted with ANOVA with a post hoc Student–Newman–Keuls test (GraphPad Prism software, version 7; San Diego, CA). A p value of less than 0.05 was considered statistically significant. We interpreted the results as higher recovery indicating more complete and thorough homogenization, and higher RIN scores indicated better-quality RNA (i.e., fewer contaminants and less RNA degradation during homogenization).

RESULTS

The retinas were easily homogenized with every technique tested. Whole eyes were homogenized uniformly with the three bead methods (set screw, TissueLyser, or bullet blender), but homogenization was never complete with the shear methods (pellet pestle or Dounce homogenizer).

The RNA produced by the various homogenization methods was analyzed for yield, purity, and quality. RNA was obtained at acceptable and expected quantities for the whole eyes and the retinas and is presented in nanograms plus or minus the standard deviation (Table 2). Overall, the TissueLyser and Dounce techniques had the highest RNA yields from the retinas while the TissueLyser, Dounce, and set screw techniques had the highest yields from the whole eyes. The pellet pestle method had the lowest yields from the retinas, and the pellet pestle and Dounce techniques had the lowest yields from the whole eyes. Statistical analysis was conducted using ANOVA tests with multiple comparisons, and the full list of the results is summarized in Appendix 1 and Appendix 2 for the retinas and the whole eyes, respectively.

To confirm the purity of the RNA obtained via the various homogenization methods, we assessed the A_{260}/A_{280} ratio (Table 3). The ratios are presented in nanometers plus or minus the standard deviation. Overall, the ratios for the retina and the whole eye are very similar across all of the techniques. Statistical analysis was conducted using ANOVA with multiple comparisons, and the full list of the results is summarized in Appendix 3 and Appendix 4 for the retina and the whole eye, respectively.

TABLE 3. MEAN RATIOS OF ABSORBANCE AT 260 NM TO 280 NM ± SD.					
Tissue type	Set screw	TissueLyser	Bullet blender	Pellet pestle	Dounce
Retina	2.054 ± 0.021	2.028 ± 0.013	2.028 ± 0.016	2.006 ± 0.024	2.023 ± 0.028
Whole eye	2.139 ± 0.017	2.149 ± 0.011	2.104 ± 0.048	2.125 ± 0.022	2.146 ± 0.013

Values are mean ratios of absorbance at 260 nm to 280 nm ± SD n=8 for all retina measurements, n=10 for whole eyes except the pellet pestle (n=9). ANOVA multiple comparison analysis compared the mean of each technique within a tissue type (retina or whole eye) with the mean of every other technique for the same tissue. A full list of comparisons with p values is given in Appendix 3 and Appendix 4.

Tissue type	Set screw	TissueLyser	Bullet blender	Pellet pestle	Dounce
Retina	8.33 ± 0.47	7.75 ± 0.64	8.36 ± 0.63	7.91 ± 0.64	8.19 ± 0.81
Whole eye	8.28 ± 0.20	8.18 ± 0.25	8.78 ± 0.18	8.71 ± 0.42	8.65 ± 0.35

TABLE 4. RNA QUALITY BY MEAN RIN SCORE BY DIFFERENT HOMOGENIZATION METHODS ± SD.

Values are mean RIN scores ± SD n=8 for all retina measurements, n=10 for whole eyes except the pellet pestle (n=9). ANOVA multiple comparison analysis compared the mean of each technique within a tissue type (retina or whole eye) with the mean of every other technique for the same tissue. A full list of comparisons with p values is given in Appendix 5 and Appendix 6.

TABLE 5. COSTS AND TIME (ITEMIZED PER 10 SAMPLES) FOR HOMOGENIZATION.

Homogenization method	Capital cost of equipment ($) each	Cost of consumables per 10 samples ($)	Time to prepare 10 whole eye samples (min)
Set Screw	113	5	15
TissueLyser	6497	2.6	15
Bullet Blender	2995	6.2	15
Pellet Pestle	2790	14.7	45
Dounce	131	4	60

Costs were tabulated from recent catalog prices, and summarized for a typical experiment with preparation of 10 independent samples. Column 1 lists the various homogenization techniques, column 2 denotes the cost of any one-time equipment purchase necessary for the technique, column 3 lists the cost of consumables per 10 samples, and column 4 lists the average time necessary to prepare 10 samples.

In addition to quantity and purity, we assessed RNA quality using electropherograms (Figure 2) and RIN scores (Table 4). Representative electropherogram traces from the five different homogenization techniques for both retina and whole eye samples are shown in Figure 2. From these electropherogram traces, a RNA Integrity Number (RIN) score was computed and are presented as a mean plus or minus the standard deviation (SD) and shown in Table 4. We routinely obtained RIN scores for both whole eye and retina for all five techniques in excess of 8.0 provided that RiboLock was included in the initial collection. Thus, all five technique produce comparable quality of RNA. Statistical analysis was conducted using ANOVA tests with multiple comparisons and a full list of results are summarized in Appendix 5 and Appendix 6 for retina and whole eye respectively.

Last, we evaluated the costs of using each technique for homogenization (Table 5). The breakdown was by three major categories: disposables, small or major equipment with a long operation lifespan, and the time per manageable group of samples.

The cost of consumables was determined by calculating the cost of a single consumable unit (e.g., one tube) from the cost of a quantity available for purchase. Single unit costs for all components of a given method (e.g., one tube and one bead) were then added together, and the sum was multiplied by ten to give the cost per ten samples. The preparation time for ten samples reflects the approximate time it took the technician to thaw the sample in buffer, homogenize, and store the sample again. The labor time for the Dounce homogenization
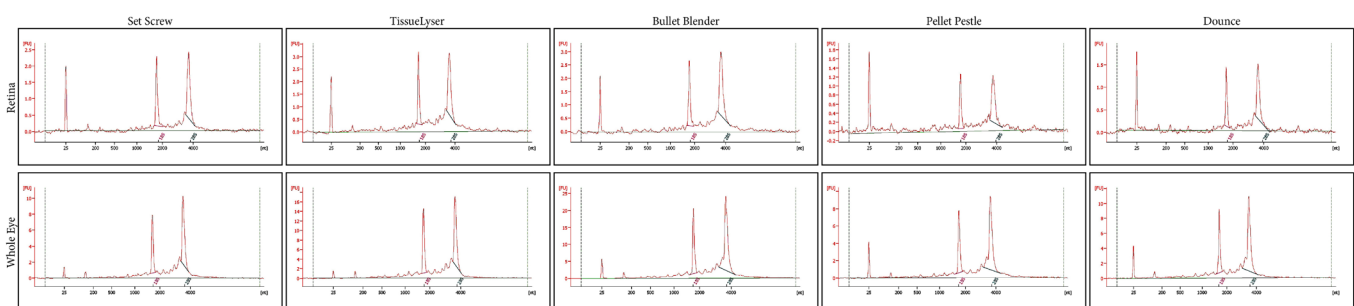

Figure 2. Representative electrophoretic traces from retina and whole eye samples across different homogenization techniques. The first peak in each trace represents a marker, the second peak denotes the 18S rRNA, and the third peak denotes the 28S rRNA.

reflects the use of three homogenizers that had to be washed between uses and includes the washing time.

DISCUSSION

As shown in Table 3 and Table 4, the overall purity and quality of the RNA isolated from these five techniques were roughly the same for the whole eye and the retina. However, when ease of use was compared among the five techniques, the pellet pestle and the Dounce homogenizer were considerably more involved and time-consuming as they require that tissue be sheared by hand, one sample at a time. Additionally, these manual tissue-grinding methods were necessarily subjective in terms of force and speed. This made it virtually impossible to control for the consistency of treatment between different samples and technicians, potentially adding unwanted variability into an experiment.

The pellet pestle and Dounce techniques were also less effective at fully dispersing the whole eye into a uniform homogenate and produced the lowest quantity of RNA from the whole eye. Collecting RNA from the whole eye in some instances is a desirable approach. If the mRNA of interest is expressed in only one tissue or cell type (e.g., the trabecular meshwork or the RPE), then obtaining the mRNA from the whole eye is just as useful and much easier than performing difficult dissections to obtain mRNA from specific cells.

When the remaining three techniques were compared, the bullet blender was more difficult to use because it required aliquoting the small beads without spilling them. The remaining two techniques, the set screw method and TissueLyser, produced comparable amounts of RNA from the whole eye, although the TissueLyser produced more RNA from the retina samples. In addition, the two techniques were virtually identical in ease of use for the technician, the only difference being that the TissueLyser has a built-in timer allowing for an identical homogenization duration for all samples. Although the TissueLyser technique had the highest total RNA yield, the initial $5,383 cost of the machine and the additional $1,114 for the required tube adaptor makes this technique a less favorable option for many laboratories.

Overall, we favor the novel set screw method for homogenizing whole eye and retinal samples. The set screws are very inexpensive and homogenize tissue well. Similar to the bullet blender and TissueLyser, each sample is homogenized by the set screw method in the sample's own individual closed tube. This greatly reduces the chance of cross contamination among samples. In addition, the set screws, similar to the ball bearings used in the TissueLyser, are paramagnetic and can be removed from the sample tube easily before RNA is extracted using a strong magnet. The set screw is less expensive, saves time, reduces errors, and is capable of evenly dispersing the sclera and the cornea. Additionally, the set screw method is an efficient and cost-effective alternative to far more expensive devices that use beads, such as the bullet blender or TissueLyser. We believe that the set screw method is a highly viable option for laboratories that would like the capacity to homogenize numerous whole eye or retinal samples at once without making a large capital purchase.

APPENDIX 1. ANOVA MULTIPLE COMPARISON ANALYSIS COMPARED THE MEAN RNA YIELD FROM RETINAL SAMPLES FOR EACH TECHNIQUE AGAINST THE MEAN RNA YIELD FROM EVERY OTHER TECHNIQUE.

Column 1 states the name of the two techniques that are being directly compared to one another. Column 2 states the p value after being corrected for multiple comparisons. Column 3 indicates whether the p value in Column 1 is statistically significant or not (p value <0.05). Column 4 states a summary of the significant level as indicated by asterisks (n.s.=not significant ; p value <0.05=* ; p value <0.01=** ; p value <0.001=*** ; p value <0.0001=****). If statistical significance is achieved, the first technique listed in Column 1 yielded higher amounts of RNA. To access the data, click or select the words "Appendix 1."

APPENDIX 2. ANOVA MULTIPLE COMPARISON ANALYSIS COMPARED THE MEAN RNA YIELD FROM WHOLE EYE FOR EACH TECHNIQUE AGAINST THE MEAN RNA YIELD FROM EVERY OTHER TECHNIQUE.

Column 1 states the name of the two techniques that are being directly compared to one another. Column 2 states the p value after being corrected for multiple comparisons. Column 3 indicates whether the p value in Column 1 is statistically significant or not (p value <0.05). Column 4 states a summary of the significant level as indicated by asterisks (n.s.=not significant ; p value <0.05=* ; p value <0.01=** ; p value <0.001=*** ; p value <0.0001=****). If statistical significance is achieved, the first technique listed in Column 1 yielded higher amounts of RNA. To access the data, click or select the words "Appendix 2."

APPENDIX 3. ANOVA MULTIPLE COMPARISON ANALYSIS COMPARED THE MEAN 260 NM TO 280 NM ABSORBANCE FROM RETINAL SAMPLES FOR EACH TECHNIQUE AGAINST THE MEAN RNA YIELD FROM EVERY OTHER TECHNIQUE.

Column 1 states the name of the two techniques that are being directly compared to one another. Column 2 states the p value after being corrected for multiple comparisons. Column 3 indicates whether the p value in Column 1 is statistically significant or not (p value <0.05). Column 4 states a summary of the significant level as indicated by asterisks (n.s.=not significant ; p value <0.05=* ; p value <0.01=** ; p value <0.001=*** ; p value <0.0001=****). If statistical significance is achieved, the first technique listed in Column 1 has a higher ratio of absorbance 260 nm to 280 nm. To access the data, click or select the words "Appendix 3."

APPENDIX 4. ANOVA MULTIPLE COMPARISON ANALYSIS COMPARED THE MEAN 260NM TO 280NM ABSORBANCE FROM WHOLE EYE SAMPLES FOR EACH TECHNIQUE AGAINST THE MEAN RNA YIELD FROM EVERY OTHER TECHNIQUE.

Column 1 states the name of the two techniques that are being directly compared to one another. Column 2 states the p value after being corrected for multiple comparisons. Column 3 indicates whether the p value in Column 1 is statistically significant or not (p value <0.05). Column 4 states a summary of the significant level as indicated by asterisks (n.s.=not significant ; p value <0.05=* ; p value <0.01=** ; p value <0.001=*** ; p value <0.0001=****). If statistical significance is achieved, the first technique listed in Column 1 yielded higher ratio of absorbance 260 nm to 280 nm. To access the data, click or select the words "Appendix 4."

APPENDIX 5. ANOVA MULTIPLE COMPARISON ANALYSIS COMPARED THE RIN SCORES FROM RETINAL SAMPLES FOR EACH TECHNIQUE AGAINST THE MEAN RNA YIELD FROM EVERY OTHER TECHNIQUE.

Column 1 states the name of the two techniques that are being directly compared to one another. Column 2 states the p value after being corrected for multiple comparisons. Column 3 indicates whether the p value in Column 1 is statistically significant or not (p value <0.05). Column 4 states a summary of the significant level as indicated by asterisks (n.s.=not significant ; p value <0.05=* ; p value <0.01=** ; p value <0.001=*** ; p value <0.0001=****). No statistical significance was achieved. To access the data, click or select the words "Appendix 5."

APPENDIX 6. ANOVA MULTIPLE COMPARISON ANALYSIS COMPARED THE RIN SCORES FROM WHOLE EYE SAMPLES FOR EACH TECHNIQUE AGAINST THE MEAN RNA YIELD FROM EVERY OTHER TECHNIQUE.

Column 1 states the name of the two techniques that are being directly compared to one another. Column 2 states the p value after being corrected for multiple comparisons. Column 3 indicates whether the p value in Column 1 is statistically significant or not (p value <0.05). Column 4 states a summary of the significant level as indicated by asterisks (n.s.=not significant ; p value <0.05=* ; p value <0.01=** ; p value <0.001=*** ; p value <0.0001=****). If statistical significance is achieved, the first technique listed in Column 1 yielded higher RIN scores. To access the data, click or select the words "Appendix 6."

REFERENCES

1. Dounce AL. Enzyme studies on isolated cell nuclei of rat liver. J Biol Chem 1943; 147:685-98. </jrn>.

2. Dounce AL, Witter RF, Monty KJ, Pate S, Cottone MA. A method for isolating intact mitochondria and nuclei from the same homogenate, and the influence of mitochondrial destruction on the properties of cell nuclei. J Biophys Biochem Cytol 1955; 1:139-53. [PMID: 14381436].

3. Potter VR, Elvehjem CA. A modified method for the study of tissue oxidations. J Biol Chem 1936; 114:495-504. .

4. Johnson CJ, Berglin L, Chrenek MA, Redmond TM, Boatright JH, Nickerson JM. Technical brief: subretinal injection and electroporation into adult mouse eyes. Mol Vis 2008; 14:2211-26. [PMID: 19057658].

5. Iuvone PM, Boatright JH, Bloom MM. Dopamine mediates the light-evoked suppression of serotonin N-acetyltransferase

activity in retina. Brain Res 1987; 418:314-24. [PMID: 2445415].

6. Liou GI, Matragoon S. A simple method to homogenize multiple tissue samples in small sizes without cross contamination. Biotechniques 1992; 13:719-[PMID: 1329859].

7. Milner HW, Lawrence NS, French CS. Colloidal Dispersion of Chloroplast Material. Science 1950; 111:633-4. [PMID: 15424657].

8. Sharpe AN, Jackson AK. Stomaching: a new concept in bacteriological sample preparation. Appl Microbiol 1972; 24:175-8. [PMID: 16349927].

9. Smucker RA, Pfister RM. Liquid nitrogen cryo-impacting: a new concept for cell disruption. Appl Microbiol 1975; 30:445-9. [PMID: 810088].

10. von Ziegler LM, Saab BJ, Mansuy IM. A simple and fast method for tissue cryohomogenization enabling multifarious molecular extraction. J Neurosci Methods 2013; 216:137-41. [PMID: 23541735].

11. Hopkins TR. Physical and chemical cell disruption for the recovery of intracellular proteins. Bioprocess Technol 1991; 12:57-83. [PMID: 1367090].

12. Ramanan RN, Ling TC, Ariff AB. The performance of a glass bead shaking technique for the disruption of Escherichia coli cells. – Springer editors. Biotechnol Bioprocess Eng; BBE 2008; •••:613-23. .

13. Ilyin SE, Plata-Salamán CR. An efficient, reliable and inexpensive device for the rapid homogenization of multiple tissue samples by centrifugation. J Neurosci Methods 2000; 95:123-5. [PMID: 10752482].

14. Hunter MJ, Commerford SL. Pressure Homogenization of Mammalian Tissues. Biochim Biophys Acta 1961; 47:580- [PMID: 13716896].

15. Simpson RJ. Disruption of cultured cells by nitrogen cavitation. Cold Spring Harb Protoc 2010; 2010:5513-[PMID: 21041386].