Evaluation of bead-milling technology for pre-clinical studies involving animal tissues

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1 Introduction

Disease modeling, systems research, and the evaluation of toxicity and safety of potential therapies involve the use of common preclinical animals, including mice. Understanding that there are species and tissue differences, the goal is to help lower the attrition rate of drug candidates. A standardized sample prep process is valuable in multi-omic profiling of animal tissues for pre-clinical stages of research, drug discovery, and drug development. We evaluated the Omni Bead Ruptor Elite bead mill homogenizer for mechanical disruption as a unifying platform for upstream sample homogenization from various relevant tissues for further downstream processes, including protein analysis via immunoassays and genomic applications such as qPCR.

Studies in review

- **Study 1:** High-throughput RNA extraction from mouse kidney, liver, heart, and lung using bead milling vs manual mortar & pestle
- **Study 2:** Automation-enabled DNA extraction from mouse liver
- **Study 3:** Homogenization and protein extraction to assess kidney toxicity in mice treated with antimetabolic agents

2 Materials & Methods

Animals and tissue collection
Mice or tissues were sourced from previous academic/industry collaborations, Bioreclamation, or Charles River Laboratory. Tissues were manually sectioned and flash-frozen, stored on ice, or immediately homogenized after harvest.

Study 1

Sample homogenization
Method 1 – semi-automated bead milling
25 mg of freshly harvested kidney, liver, heart, and lung tissues were placed in pre-chilled re-inforced 2mL tubes containing bead mix with 500 µL of commercially-available RNA lysis buffer containing 2-mercaptoethanol and were homogenized according to settings in Table 1 on the Omni Bead Ruptor 12 bead mill homogenizer.

Sample type	Input (mg)	Tube and bead mix	Speed setting (m/s)	Cycle time (s)
Kidney, liver, heart, lung	25 mg	2 mL, 2.8 mm ceramic beads	2.9	20

Table 1: Sample type, input amount, tube and bead homogenizing mixtures, and instrument settings/cycle times.

Method 2 – manual cryo-milling with mortar & pestle
25 mg of freshly harvested tissue was cryo-milled in prechilled mortar & pestle under liquid nitrogen. Powdered tissue was transferred to a pre-chilled 1.5 mL microcentrifuge tube containing 500 µL of commercially-available RNA lysis buffer containing 2-mercaptoethanol.

RNA extraction, quantification, and integrity analysis
Homogenized samples were taken through a commercially-available universal RNA extraction and purification kit following kit instructions. RNA was eluted in 100 µL DEPC water.

1 µL of purified sample RNA were analyzed on an automated electrophoresis system using an RNA kit chip per kit instruction for yield and RNA integrity number (RIN).

Study 2

Sample homogenization
450 mg of liver tissue were placed in a re-inforced 2mL tubes containing bead mix with 1mL PBS (pH 7.2) and homogenized according to settings in Table 2 on the Omni Bead Ruptor Elite bead mill homogenizer. Lysates were centrifuged at 10,000 x g for 5 mins to pellet cell debris.

Sample type	Input (mg)	Tube and bead mix	Speed setting (m/s)	Cycle time (s)
Liver	450 mg ± 5 mg	2 mL, 2.8 mm ceramic beads	4.5	30

Table 2: Sample type, input amount, tube and bead homogenizing mixtures, and instrument settings/cycle times.

DNA extraction, quantification, and integrity analysis
The following steps were performed on either a JANUS G3 automated workstation or by manual pipette. 100 µL of lysate was transferred to wells of a 96-well plate, and 100 µL of Lysis Buffer (chemagic Tissue DNA Kit) was added to the same wells. 6 µL of Proteinase-K (chemagic Tissue DNA Kit) was transferred to all wells along with 5 µL of RNAse A (Thermo Fisher Scientific). The plate was incubated at 37 °C for 10mins, during which time the Elution Buffer and Magnetic Beads were transferred to respective plates in preparation for automated extraction on the chemagic 360 nucleic acid extractor. The protocol was allowed to run to completion.

After completion, eluted nucleic acid concentration and integrity was determined by A_{260}/A_{280} spectrophotometry.

Quantitative PCR (qPCR)
10 µL of eluted DNA was added to qPCR mix (Bio-Rad) along with 5 µM of forward and reverse 18S primers. The 18S gene was targeted with forward primer 5' - CAG CAG CCG CGG TAA TTC C – 3', reverse primer 5' - CCC GTG TTG AGT CAA ATT AAG C – 3' yielding a product size of 676 bp. An 18S-positive
DNA extract was used as the positive control along with nuclease-free water (negative control). Reactions were run a real time PCR instrument, amplified for 40 cycles and visualized via gel electrophoresis. Sample Cq values were assessed.

Figure 1: Images of table-top units for the Omni BR cryo unit (optional, L) and Omni Bead Ruptor Elite bead mill homogenizer (R).

Study 3

Experimental model
Female nude mice were either untreated or treated with 100 mg/kg of 5-Fluorouracil (5-FU), an antimetabolic and chemoterapeutic agent, three times a week for 2 weeks (n=3, per group). Tissues were harvested after 6 hours of final drug dose in week 2, flash-frozen, and stored in LN_2 until thawed on ice for downstream processing.

Sample homogenization
150-200 mg of thawed kidneys were weighed and rinsed twice in ice-cold PBS and placed in a pre-chilled re-inforced 2mL tubes containing bead mix with 500 µL/100 mg-of-tissue of ice-cold 1X AlphaLISA SureFire Ultra Lysis Buffer supplemented with protease and phosphatase inhibitors and were homogenized according to settings in Table 3 on the Omni Bead Ruptor Elite bead bill homogenizer. Immediately after homogenization, samples were transferred to pre-chilled Ep-tubes and centrifuged at 14,000 RPM for 10mins at 4°C to separate tissue debris.

Sample type	Input (mg)	Tube and bead mix	Speed setting (m/s)	Cycle time (s)
Kidney	150 - 200 mg	2 mL, 2.8 mm ceramic beads	4.5	30

Table 3: Sample type, input amount, tube and bead homogenizing mixtures, and instrument settings/cycle times.

Protein extraction and quantitation
Soluble protein supernatants were transferred to a new chilled Eppendorf tube to determine total protein concentration using the Pierce Rapid Gold BCA Protein Assay according to kit instructions. Samples were diluted to 5 mg/mL in new Eppendorf tubes and frozen at -80°C until processing.

No-wash AlphaLISA SureFire Ultra immunoassay
Frozen protein samples were thawed on ice prior to use.10 µL of eluted soluble proteins from each kidney were tested in duplicate, at three concentrations of total proteins within the linear range of the assay signal - at 1 mg/mL, 0.5 mg/mL, and 0.25 mg/mL – following kit instructions to measure phosphorylated ERK (thr202/Tyr204)and total ERK. Signals were measured on the Envision 2105 Multimode Plate Reader using pre-programmed Alpha settings.

3 Results

Study 1

A semi-automated bead milling approach produced at least double the RNA yields from liver, heart, and lung compared to a manual cryo-milling mortar & pestle method with comparable average RIN values. Average yield from kidney were also comparable between methods (Table 4). Visual inspection of synthetic gel analysis indicated prominent 28S and 18S bands, as well as comparable electropherograms across both methods. (data not shown).

Tissue – method	Average yields (ng / µL)	Average RIN
Kidney – mortar & pestle	192.3	7.7
Kidney – Omni Bead Ruptor	186.0	8.6
Liver – morter & pestle	167.3	7.5
Liver – Omni Bead Ruptor	733.3	7.1
Heart – morter & pestle	26.7	7.6
Heart – Omni Bead Ruptor	105.7	8.9
Lung – morter & pestle	44.7	7.2
Lung – Omni Bead Ruptor	106.0	7.9

Table 4: Average RNA yield and RIN score for each tissue type processed with mortar & pestle or on the Omni Bead Ruptor unit.

Study 2

Semi-automated bead mill homogenization integrates easily into automation-friendly workflows, which generated an average DNA yield of 2.5 µg from ~ 450 mg of mouse liver tissue. DNA was suitable for downstream qPCR analysis, as indicated in robust Cq values for 18S (Table 5).

Sample ID	Cq	Sample ID cont...	Cq
Liver 1	21.45	Liver 8	21.08
Liver 2	21.21	Liver 9	20.93
Liver 3	21.10	Liver 10	20.75
Liver 4	21.23	Liver 11	20.32
Liver 5	20.56	Liver 12	19.24
Liver 6	20.41	Positive Extraction Ctrl	22.01
Liver 7	20.82	Negative Ctrl	39.07

Table 5: 18S qPCR Cq values from extracted mouse liver DNA.

Study 3

Ample soluble proteins were extracted from homogenized kidneys of untreated or 5-FU treated mice (Table 5). Eluted proteins, without the need for additional cleanup, showed compatibility of method with no-wash immunoassays, though experimental results - confirmed with histological staining (not shown) - preliminarily suggests the implemented two-week regiment was insufficient to elicit acute, observable kidney damage, as measured by normalized phospho-ERK (Figure 4).

Mouse	Group	Concentration (mg/mL)
1	Control	18.94
2	Control	20.78
3	Control	23.61
4	Treated	21.09
5	Treated	21.58
6	Treated	25.57

Table 6: Protein harvest results from each mouse, determined by the BCA protein assay.

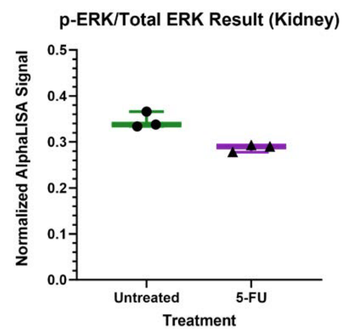

Figure 2: Normalized phospho-ERK/Total ERK results in the kidney of treated vs untreated mice.

6 Summary

As multi-omic studies become more prevalent, the Omni Bead Ruptor Elite bead mill homogenizer leverages mechanical disruption rather than manual methods to unify sample homogenization workflows for downstream extraction of inputs that are compatible with a variety of pre-clinical genomic and protein analysis assays using animal tissues.